Biological Journal of the Linnean Society, 2023, **139**, 1–17. With 4 figures.

Aposematic coloration of Pacific newts (*Taricha*) provides a qualitatively but not quantitatively honest signal to predators

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Received 3 September 2022; revised 17 January 2023; accepted for publication 22 January 2023

Colourful displays are used by diverse taxa to warn predators of dangerous defences (aposematism). Aposematic coloration is especially widespread among amphibians, which are often protected by harmful toxins. Pacific newts (*Taricha*) are considered a model of aposematism because when threatened, they arch the head and tail upwards to expose a vivid orange ventrum against a dark dorsum. Given that newts are defended by tetrodotoxin (TTX), a lethal neurotoxin, this signal is assumed to warn predators that an attack would be risky. However, colours have not been quantified in *Taricha*, and it remains unknown whether coloration provides qualitatively honest (signalling toxic defence) or quantitatively honest (signalling toxin level) warnings. We used two colour quantification methods (spectrometry and hyperspectral imaging) to measure chromatic (hue) and achromatic (brightness) qualities of ventral and dorsal coloration in two newt species (*Taricha granulosa* and *Taricha sierrae*). We assessed qualitative honesty using visual models of potential predators (snakes, birds and mammals). Next, we evaluated quantitative honesty by measuring TTX in newts and examining the potential correlation between defence level (amount of TTX) and colorimetrics. We found support for qualitative but not quantitative honesty. Selective pressures and evolutionary constraints might impede the evolution of honest quantitative signalling in this system.

ADDITIONAL KEYWORDS: carotenoid – hyperspectral imaging – predator perception – tetrodotoxin – toxic prey – warning coloration.

INTRODUCTION

Colourful displays are used by taxa across the tree of life to communicate both within and between species, from signalling a nectar reward to indicating receptiveness to potential mates or even to ward off would-be predators (Cott, 1940; Hutton *et al.*, 2015; Lim *et al.*, 2019; Hedley & Caro, 2021). One of the most well-known signals is aposematism, the conspicuous coloration used by prey to warn predators of a noxious or toxic defence (Poulton, 1890; Cott, 1940). Aposematic coloration is found across diverse animal phyla (Caro & Ruxton, 2019; White & Umbers, 2021), but is especially well developed in amphibians, which are generally defended by toxins (Rudh & Qvarnström, 2013). In amphibians, warning coloration can be overt, as in showy dendrobatid frogs (Savage, 1968), or revealed only by defensive posturing, when the animal exposes vibrant colours that are otherwise concealed (Hinsche, 1926). A classic example of this behaviour, known as the 'unken reflex', is well characterized in Pacific newts (*Taricha* Gray, 1850), which expose a bright orange ventrum by pulling their heads and tails backwards over a dark dorsal surface (Stebbins, 1951;

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Reimer, 1958; Johnson & Brodie, 1975; Brodie, 1977). Given that newts are well defended by tetrodotoxin (TTX; Brodie, 1968; Hanifin *et al.*, 1999), a highly potent neurotoxin (Lorentz *et al.*, 2016), this behaviour is assumed to provide an honest signal of toxin presence to predators (Stebbins & Cohen, 1997; Petranka, 1998; Stebbins, 2003). However, there have been no tests or analyses on whether the bright ventral colours of Pacific newts are visible to predators. Thus, we lack an understanding of whether newt coloration is a qualitatively honest warning, signalling toxic defence, and whether signal intensity scales with toxin level in a quantitatively honest manner (Számadó, 2011; Blount *et al.*, 2012; Summers *et al.*, 2015).

To meet the criteria for qualitative honesty, conspicuous colour signals must be discernible by primary local predators and be consistently paired with an antipredator defence, meaning that encounters will establish an association between prey coloration and defence, such that future predation attempts are avoided (Sherratt, 2002). These criteria distinguish aposematic signals from deimatic (or 'startle') displays that cause a predator to recoil reflexively in response; that is, deimatism does not require a predator to have a learned or innate aversion (Umbers *et al.*, 2017; Drinkwater *et al.*, 2022). Qualitative honesty in *Taricha* has been investigated using avian predation trials, which showed that some birds can quickly learn that newt coloration signals harmfulness (Johnson & Brodie, 1975; Kuchta *et al.*, 2008), especially when newts are in the unken pose (Johnson & Brodie, 1975). However, there has been no additional work on the effectiveness of this signal to other potential predators. Furthermore, a formal analysis of newt coloration is needed to understand perceptibility to potential predators and to quantify signal variation among individuals and across populations. *Taricha* occupy a broad geographical range and inhabit a variety of aquatic habitats (from permanent creeks to ephemeral pools) and terrestrial communities (from chaparral to coniferous forests; Stebbins, 2003). Thus, differences in the abiotic environment, ecological communities and microhabitats they occupy are likely to influence how predators perceive newt colours. In addition, newts are likely to face geographical differences in dominant local predator types and diversity, in addition to their availability to those predators across the landscape. Differences in local predator communities can influence the evolution of colour (owing to differences in visual systems; Wong, 1989; Hart, 2001; Byosiere *et al.*, 2018) and TTX levels (Hanifin *et al.*, 2008; Stokes *et al.*, 2015; Reimche *et al.*, 2020).

According to a model of quantitative honesty, theory predicts that in populations where all conspecific prey possess toxic antipredator defences, selection should favour the most toxic individuals at the expense of less toxic individuals (Speed & Franks, 2014). In this case, colour signals are expected to be quantitatively honest, indicating the toxin level through enhanced saturation or brightness (Stuckert *et al.*, 2018). A recent meta-analysis found a moderate positive correlation between aposematic colour and chemical defences within and across populations of amphibians, insects and gastropods, but the strength and directionality of this pattern varied among taxa (White & Umbers, 2021). There is strong support for quantitative honesty in some insects (Bezzerides *et al.*, 2007; Blount *et al.*, 2012; Vidal-Cordero *et al.*, 2012; Arenas *et al.*, 2015; White & Umbers, 2021) but mixed support among amphibian studies, with some finding positive (Summers & Clough, 2001; Maan & Cummings, 2012; White & Umbers, 2021), negative (Darst & Cummings, 2006; Wang, 2011; Mochida *et al.*, 2013; White & Umbers, 2021) or no correlation between toxin levels and the aposematic signal (Daly & Myers, 1967; Stuckert *et al.*, 2018; White & Umbers, 2021). Several hypotheses might explain why quantitative honesty does not occur in all systems. Physiological trade-offs might constrain quantitative honesty (Blount *et al.*, 2009; Summers *et al.*, 2015), such that a negative correlation results from resource limitation that prevents the simultaneous increase in both signal strength and toxin level. Alternatively, a negative correlation between signal and defence might occur when prey are so effectively toxic to potential predators that selection might instead reduce the costs incurred by increased conspicuousness of warning coloration (Blount *et al.*, 2009).

Beyond the risk of increased conspicuousness to predators (Sherratt, 2002), both TTX and the pigments contributing to newt coloration might be expensive to produce and store. Actively synthesizing TTX or maintaining endosymbionts that might manufacture the poison (Cardall *et al.*, 2004; Bucciarelli *et al.*, 2017; Vaelli *et al.*, 2020; Gall *et al.*, 2022; Hanifin *et al.*, 2022) could be metabolically costly. Indeed, the reduction or loss of TTX in newt populations allopatric with TTX-resistant garter snake predators (Hanifin *et al.*, 1999; Hague *et al.*, 2016) suggests that maintenance of TTX is costly. Additionally, in many vertebrates, red–orange colours produced by carotenoids must be obtained through the diet (Olson & Owens, 1998; Blount *et al.*, 2009). Given that xanthophores, which typically contain both carotenoids and pteridines (Bagnara *et al.*, 1967), have been found in *Triturus* and *Taricha* (Epperlein, 1982; Tucker & Erickson, 1986), we expect that ventral colour in *Taricha* is also carotenoid based, as in other newts with similar colour patches (Forbes *et al.*, 1973; Matsui *et al.*, 2002). Thus, we expect that newts face energetic demands in obtaining dietary carotenoids in addition to risks

associated with foraging for those pigments. As such, newts might have limited potential to produce both TTX and colour signals in a tightly correlated manner and might experience trade-offs between toxic defence and warning coloration, particularly where high levels of TTX are required to fend off resistant predators.

The broad variation in TTX levels within and across populations of *Taricha* (Hanifin *et al.*, 2008; Stokes *et al.*, 2015; Reimche *et al.*, 2020) provides an opportunity to test hypotheses of qualitative and quantitative honesty in a natural amphibian system. Here, we used two spectral imaging methods to characterize intra- and interpopulation variation in dorsal and ventral coloration in two *Taricha* species. We assessed whether sympatric predators can visualize the contrasting colours displayed in the unken pose to test the model of qualitative honesty in these newts. We then investigated the potential correlation between newt toxin levels and both chromatic and achromatic measures to test the model of quantitative honesty. For qualitative honesty, we predicted that ventral–dorsal contrast displayed during the unken reflex forms a visible aposematic signal to primary newt predators (avian, mammalian and reptilian). For quantitative honesty, we expected a positive correlation between aspects of warning coloration (i.e. hue, brightness and contrast) and levels of TTX. Alternatively, if newt coloration is largely influenced by abiotic and biotic factors other than predation (e.g. sexual selection) or if trade-offs limit their ability to produce both the dramatic signal and a high toxin load, then we expect that warning colours will be visible to local predators but will not be correlated with individual TTX levels.

MATERIAL AND METHODS

Animal collection and housing

We collected 63 newts from nine sites in California, USA in 2015 (Supporting Information, Table S1) and 75 newts from four sites in California in 2019 (Fig. 1; Supporting Information, Table S2). In both cases, newts were transported to a temperature-controlled room averaging 15–20 °C. Newts were housed communally (three to five individuals per tank) in 38 liter-gallon glass tanks with a depth of 5 cm of dechlorinated water and a substrate of pea gravel with two PVC pipe halves used as both hides and haul-outs. We kept newts on a 12 h light–12 h dark cycle and fed them a variety of frozen (brine shrimp or blood worms) and live food (earthworms) two or three times weekly.

Tetrodotoxin assay

We quantified TTX in the skin of newts using a competitive inhibition enzymatic immunoassay (Stokes *et al.*, 2012). After anaesthetizing the newts by immersion in 1% Finquel MS-222 (tricaine methanesulfonate), we took 3-mm-diameter skin biopsies (Acu-punch; Acu-derm, Fort Lauderdale, FL, USA) from the dorsal surface (the midback) between the pectoral girdle and the pelvic girdle (Hanifin *et al.*, 2002; Lehman, 2007) and extracted TTX following Hanifin *et al.* (2002). We used standards from the linear range of the curve in concentrations of 10–500 ng/mL and diluted all samples between 1:1 and 1:20 (depending on TTX levels) in 1% bovine serum albumin in phosphate-buffered saline. We considered samples with < 10 ng/mL of TTX to have no TTX (N = 1). We extrapolated measurements of TTX in our skin samples to the whole animal using the calculation from the study by Hanifin *et al.* (2004) to yield estimates of whole-newt TTX levels for each individual (in milligrams of TTX per newt; Supporting Information, Tables S1 and S2).

Spectrometry

We measured the spectral reflectance of three regions of interest (ROIs; the dorsum, ventrum and gula) on the 75 newts collected in 2019 representing two species (37 *Taricha granulosa* and 38 *Taricha sierrae*), with between five and 33 individuals per population (Fig. 1; Table 1). We took all measurements in a laboratory using an Ocean Optics spectrometer (QE *Pro*, Largo, FL, USA) with a fibre-optic reflectance probe and a pulsed xenon light source (Ocean Optics PX-2; continuous single strobe with 5 microseconds pulse width). Using the Ocean Optics reflectance protocol (OceanView v.2.0.8), we set the integration time to 30 ms, averaging 30 scans across a boxcar width of 5, with electric dark and non-linearity correction operating. Probably owing to reduced reflectance of the granular skin, we did not encounter differences in peak reflectance with different probe angles (typically cited as caused by glare in other amphibian studies; Hantak & Kuchta, 2018). Therefore, we gently blotted each newt with sterile laboratory paper towel before measurement and positioned the probe in the holder at 90° to eliminate the need for correction of elliptical inclusion of areas outside the ROI. We standardized the location of probe placement and took three repeat measurements of each ROI. To account for lamp drift (Kraemer *et al.*, 2012), we used a Spectralon white reflectance standard (Ocean Optics) and remeasured this standard between each individual.

Hyperspectral imaging

We photographed 63 newts (15 *T. granulosa* and 48 *T. sierrae*) in 2015, with four to ten individuals

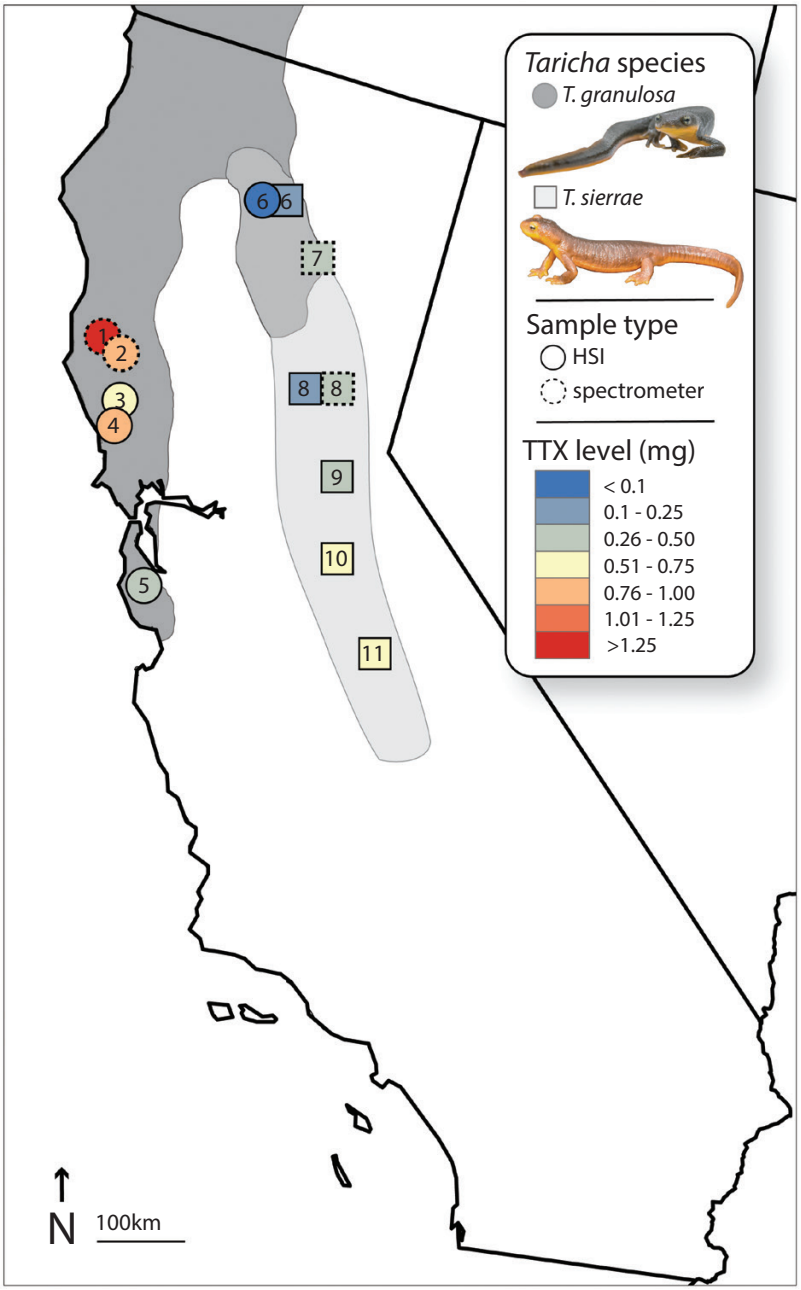

Figure 1. Map of *Taricha* range in California, USA overlaid by sampling method and locality information. Differences in symbol shape represent the two species (circle, *Taricha granulosa*; square, *Taricha sierrae*), and line type represents the colour measurement method (continuous, hyperspectral imaging; dotted, spectrometry). Icon colour is associated with mean population toxin level, which can vary across years (hyperspectral imaging for newts sampled in 2015; spectrometry for newts sampled in 2019).Newt images courtesty of Devon Picklum.

per site (Fig. 1; Table 1). We used a hyperspectral imaging (HSI) camera (SOC-716 Imager; Surface Optics Corporation, San Diego, CA, USA; e.g. Zhang *et al.*, 2017) which captures radiance across 16 colour channels in wavelength bands ranging from 360 to 660 nm. We photographed each newt in an upright posture and in a venter-up posture. We placed each newt on the same dirt background and took all photographs in the same laboratory as above, but under broad-spectrum studio lights, with a Spectralon white standard and a Macbeth ColorChecker Chart (Edmund Optics, Barrington, NJ, USA) in each series of photographs to confirm calibration and perform any necessary white balance. Note that we did not

Table 1. Summary of newt samples obtained for the two data collection methods, spectrometry and hyperspectral imaging, including newt species (*Taricha*), sample location, latitude and longitude, sample size, estimated whole-newt tetrodotoxin levels (in milligrams) and newt mass (in grams)

Collection method	Newt species	Newt locality	Latitude, longitude	*N*	Total TTX [mg; $\bar{x}$ (SD)]	Mass [g; $\bar{x}$ (SD)]
Spectrometry	*T. granulosa*	Livingston Creek, Mendocino Co., CA, USA	38.8682, –123.2526	19	1.60 (1.12)	15.42 (4.97)
		Rancheria Creek, Mendocino Co., CA, USA	38.8459, –123.2419	18	0.89 (0.58)	15.28 (3.39)
	T. sierrae	Canyon Creek, Nevada Co., CA, USA	39.4429, –120.6581	33	0.46 (0.26)	9.93 (1.69)
		Seneca, Plumas Co, CA	40.1115, –121.0836	5	0.32 (0.18)	15.6 (3.13)
Hyperspectral imaging	*T. granulosa*	Battle Creek, Shasta Co., CA, USA	40.4473, –121.8681	1	0.07 (N/A)	7.4 (N/A)
		Little Strawberry Creek, Sonoma Co., CA, USA	38.7008, –123.0932	5	0.89 (0.53)	15.56 (7.68)
		Tunitas Creek, San Mateo Co., CA, USA	37.2339, –122.2202	4	0.49 (0.26)	11.7 (1.84)
		Warm Springs Creek, Sonoma Co., CA, USA	38.6916, –123.1092	5	0.69 (0.46)	16.24 (3.89)
	T. sierrae	Battle Creek, Shasta Co., CA, USA	40.4473, –121.8681	10	0.17 (0.08)	13.85 (1.70)
		Canyon Creek, Nevada Co., CA, USA	39.4429, –120.6581	10	0.23 (0.17)	13.87 (2.58)
		Deep Creek, Fresno Co., CA, USA	36.9344, –119.2473	9	0.66 (0.57)	13.92 (2.81)
		Ogilby Creek, El Dorado Co., CA, USA	38.7600, –120.4650	9	0.27 (0.14)	14.25 (1.83)
		Yosemite Lakes Campground, Tuolumne Co., CA, USA	37.8103, –119.9474	10	0.69 (0.26)	9.21 (1.15)

Abbreviations: Co., county; TTX, tetrodotoxin.

quantify the background or any contrast between newts and background in the present study; the dirt substrate was simply used as a standard neutral background for each image.

The HSI camera captured the reflectance of each pixel within the photograph, from which we selected specific ROIs and calculated average colorimetrics across each region. We first white-balanced each image using the values from the Spectralon standard. We confirmed good colour calibration by computing the mean value in each colour channel for each square in the 4 × 6 Macbeth colour chart, using the known reflectance spectra of the standard colour squares. No images showed any significant deviation from the standards. Using code written in MATLAB (MathWorks, Natick, MA, USA), we created a polygon around the entire surface of each ROI, referred to as a 'mask', for three ROIs on each newt: dorsum, ventrum and gula. Within each ROI, we took the average, white-balanced value in each of the 16 channels, in addition to the variance in this mean across pixels in the ROI.

Analyses

We processed spectrometer reflectance spectra using the R package pavo 2 (Maia *et al.*, 2019). We used the built-in loess smoothing function, then averaged repeated measures to obtain one data point per ROI per individual (Fig. S1). We then obtained relevant colorimetric variables representing achromatic (brightness) and chromatic (hue) qualities for each ROI. Brightness as calculated in pavo 2 ('B2') is the mean relative reflectance over the entire spectral range, and hue ('H1') is defined as the peak wavelength of maximum reflectance (in nanometres) (Maia *et al.*, 2019; Table 2). We did not find significant interpopulation differences for brightness or hue for any ROI in either species (Fig. 2).

Given that the unken pose typically exposes more of the gula than of the ventrum, we first used a *t*-test to assess whether viewer-independent (not considering predator visual system) colorimetrics of these two ROIs differed, and upon finding that both were significantly different across *T. granulosa* populations

Table 2. Viewer-independent colorimetrics obtained from spectrometry measurements

Newt species	Newt locality	Brightness, relative reflectance [x ± (SD)]			Hue, peak wavelength [nm; x + (SD)]		
		Gula	Ventrum	Dorsum	Gula	Ventrum	Dorsum
T. granulosa	Livingston Creek	16.64 (2.04)	27.50 (3.71)	4.23 (1.90)	635.11 (7.26)	653.47 (46.04)	949.05 (14.79)
T. granulosa	Rancheria Creek	16.96 (2.50)	26.41 (4.20)	4.20 (2.32)	639.50 (9.82)	652.06 (20.99)	952.33 (18.68)
T. sierrae	Canyon Creek	14.82 (1.66)	14.31 (3.14)	6.44 (1.58)	645.33 (28.91)	646.10 (24.81)	923.58 (10.87)
T. sierrae	Seneca	19.67 (4.62)	21.86 (8.20)	10.28 (4.57)	640.8 (1.64)	650.4 (22.30)	930.20 (26.94)

For both newt species (*Taricha granulosa* and *Taricha sierrae*), the mean and SD are provided for achromatic (brightness) and chromatic (hue) measures of each region of interest: gula, ventrum and dorsum.

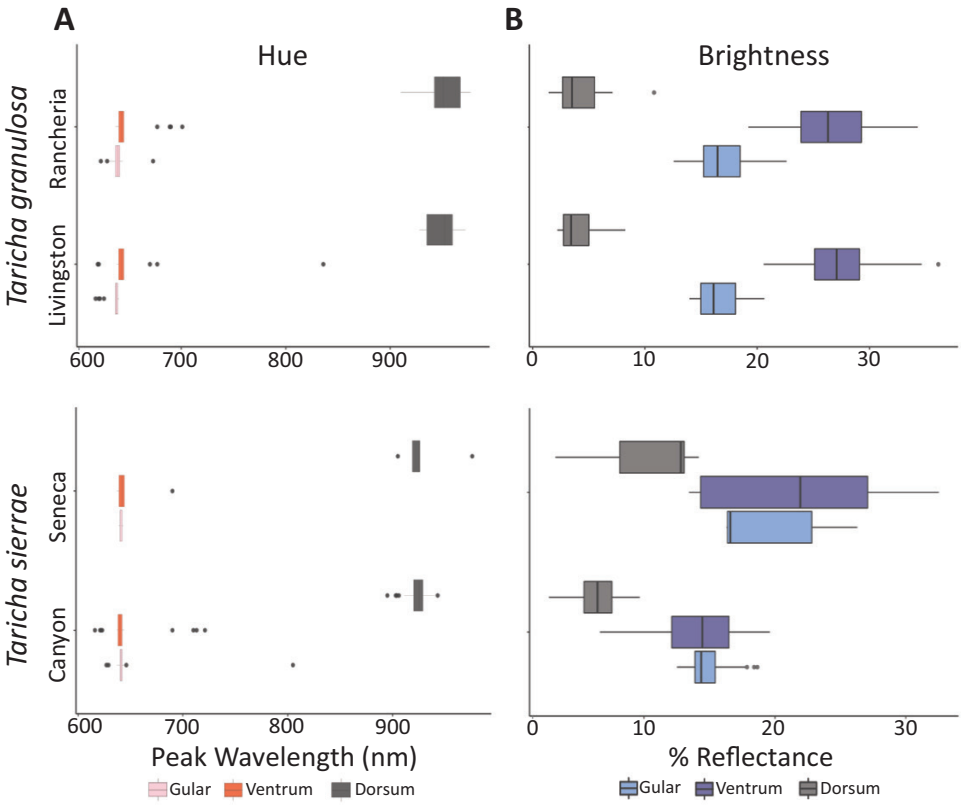

Figure 2. Average peak wavelength (in nanometres) and mean brightness (percentage reflectance) by population and region of interest in two Pacific newt species. *Taricha granulosa* (top panels; N = 37) did not show significant population influence on hue (A) or brightness (B) for the gula (hue $t_{31.26} = -1.54$, $P = 0.13$; brightness $t_{32.93} = -0.42$, $P = 0.68$), ventrum (hue $t_{25.47} = 0.12$, $P = 0.90$; brightness $t_{33.93} = 0.84$, $P = 0.41$) or dorsum (hue $t_{32.39} = -0.59$, $P = 0.56$; brightness $t_{32.92} = 0.04$, $P = 0.97$). The same was true for *Taricha sierrae* (bottom panels; N = 38) for the gula (hue $t_{33.26} = 0.89$, $P = 0.38$; brightness $t_{4.16} = -2.33$, $P = 0.08$), ventrum (hue $t_{5.62} = -0.39$, $P = 0.71$; brightness $t_{4.18} = -2.04$, $P = 0.11$) and dorsum (hue $t_{4.20} = -0.54$, $P = 0.61$; brightness $t_{4.15} = -1.86$, $P = 0.13$).

(B2 $t_{57.27} = -13.65$, $P < 0.001$; H1 $t_{40.35} = -2.58$, $P = 0.01$; Fig. 2), proceeded by analysing the contrast between the ventrum and dorsum and between the gula and dorsum.

The HSI camera outputs an average intensity in each channel for each mask, representing a measure of brightness in that colour band. We plotted all 16 and subset our analyses to include the five brightest (580, 600, 620, 640 and 660 nm), which match how newt ventrums are perceived by human observers, spanning the 'yellow' to 'orange/red' range. For all populations of both species, the 640 nm channel was the brightest of our ROIs (dashed boxes, Supporting Information, Fig.

S2). To contextualize the colour intensity of the regions while a newt is in the unken pose, we evaluated the contrast between the ventrum and dorsum of each individual as a response variable.

Qualitative honesty

After processing the spectrometer data, we used PAVO 2 to create visual models (Maia *et al.*, 2019) for predators representing three major groups: violet-sensitive tetrachromat birds (*Pavo cristatus*, Indian peafowl), dichromat mammals (*Canis familiaris*, domestic dog) and trichromat colubrid snakes (*Thamnophis sirtalis*, common garter snake). We used snake visual parameters drawn from the paper by Sillman *et al.* (1997), which listed cone absorptions of 360, 482 and 554 nm. Peafowl have been used as a violet-sensitive representative for corvids and raptors, which are potential predators at our sites (Håstad *et al.*, 2005; Calderon-Chalco & Putnam, 2018). We used standard daylight illumination ('D65') values for all models. From these models, we collected noise-weighted Euclidian colour distances, which were calculated in-package using the receptor-noise model of Vorobyev & Osario (1998).

We used the colour distance tools in PAVO 2 to calculate just noticeable difference (JND) values for chromatic and achromatic contrasts between the ventrum and dorsum and between the gula and dorsum to represent conspicuousness to potential predators of the unken signal (Table 3). As described by Vorobyev & Osorio (1998), a JND value above one suggests that regions are visually distinct to the viewer, and higher values indicate increasing distinguishability. We used the graphical tools provided in PAVO 2 and *ggplot2* (Wickham, 2016) for all figures.

Quantitative honesty

We used linear regression models to investigate potential relationships between whole-animal TTX levels (strength of prey defence) and both viewer-independent and viewer-contextualized brightness

Table 3. Just noticeable differences calculated from predator visual models for birds (*Pavo cristatus*), mammals (*Canis familiaris*) and snakes (*Thamnophis sirtalis*)

Newt species	**Newt locality**	**Predator group**	**Body contrast**	**Chromatic JND**	Achromatic JND
				dS [*x* + (SD)]	**dL [*x* + (SD)]**
T. granulosa	Livingston Creek	Bird	Ventrum	16.02 (2.59)	27.29 (6.33)
			Gula	8.85 (3.59)	23.77 (6.96)
		Mammal	Ventrum	14.17 (2.44)	20.00 (6.96)
			Gula	7.49 (3.94)	19.52 (7.52)
		Snake	Ventrum	5.69 (1.25)	23.74 (6.49)
			Gula	3.18 (1.23)	21.46 (7.18)
	Rancheria Creek	Bird	Ventrum	16.31 (2.41)	28.71 (9.80)
			Gula	10.22 (3.14)	25.49 (8.96)
		Mammal	Ventrum	14.18 (2.28)	20.94 (10.14)
			Gula	8.60 (3.37)	20.46 (9.27)
		Snake	Ventrum	5.78 (1.02)	24.84 (9.67)
			Gula	3.56 (1.33)	22.72 (8.91)
T. sierrae	Canyon Creek	Bird	Ventrum	6.24 (3.69)	18.26 (4.81)
			Gula	4.71 (1.41)	19.90 (4.39)
		Mammal	Ventrum	5.29 (3.50)	15.71 (5.46)
			Gula	3.52 (2.19)	22.04 (4.93)
		Snake	Ventrum	2.72 (1.60)	17.31 (4.93)
			Gula	2.14 (0.93)	20.37 (4.56)
	Seneca	Bird	Ventrum	9.76 (7.19)	20.10 (6.11)
			Gula	6.62 (3.58)	20.95 (4.69)
		Mammal	Ventrum	7.97 (6.84)	15.96 (5.36)
			Gula	5.34 (3.31)	21.62 (5.97)
		Snake	Ventrum	4.16 (2.68)	18.15 (5.03)
			Gula	2.87 (1.98)	20.75 (4.81)

The mean and SD of chromatic (dS) and achromatic (dL) just noticeable differences (JNDs) are listed for both contrasts: ventrum–dorsum and gula–dorsum.

and hue (strength of prey signal). We regressed brightness or hue for each ROI separately by species against ln-transformed whole-newt TTX levels. For spectrometer data, we examined the relationship between TTX levels and brightness or hue as single values across the entire visual spectrum, split by species for an intrapopulation measure of quantitative honesty.

For HSI data, we used linear regression to assess the relationship between ventrum–dorsum contrast for the five focal channels and ln-transformed estimated whole-animal TTX levels (Table 4). We split analyses by species for an interpopulation measure of quantitative honesty. We plotted results using *ggplot2*.

RESULTS

Species and population differences in TTX levels

The TTX levels among newts measured with a spectrometer varied between species (ln-transformed whole-animal TTX: $t_{65.09} = -5.68$, $P < 0.001$), with *T. granulosa* having a mean estimated whole-animal TTX level of 1.26 mg, nearly triple the mean TTX level in *T. sierrae* (0.44 mg; Table 1). Within species, we found a moderate difference between populations of *T. granulosa* ($t_{34.99} = 2.11$, $P = 0.04$) but not *T. sierrae* ($t_{4.97} = 1.173$, $P = 0.29$; Table 1). Levels of TTX among newts measured with the HSI camera also varied between species ($t_{79.19} = 4.60$, $P < 0.001$), with *T. granulosa* having the lower mean whole-animal TTX level (0.39 mg) compared with *T. sierrae* (0.66 mg). Within species, toxin levels varied significantly across populations in *T. granulosa* ($F_{3,41} = 4.699$, $P = 0.007$) and *T. sierrae* ($F_{4,138} = 22.29$, $P < 0.001$; Table 1).

Qualitative honesty

For all three predator groups, both ventral–dorsal and gular–dorsal contrast had mean values above the JND threshold. Achromatic contrast was consistently higher than chromatic contrast for both species, both masks and all predators (Table 3).

Within *T. granulosa*, chromatic ventral–dorsal contrast was differentially discriminable ($F_{2,108} = 265.40$, $P < 0.001$; Fig. 3) for all predators, with snakes predicted to have a reduced ability to discriminate these regions in comparison to both birds (Tukey's HSD $P < 0.001$) and mammals (Tukey's HSD $P < 0.001$). Avian predator models outperformed mammal models in discriminability of the two regions (Tukey's HSD $P = 0.0002$). This pattern held for gular–dorsal chromatic contrast ($F_{2,108} = 42.73$, $P < 0.001$; snake–mammal Tukey's HSD $P < 0.001$, snake–bird Tukey's HSD $P < 0.001$), but mammals and birds were not predicted to view the chromatic contrast differently (Tukey's HSD $P = 0.09$). Predator models

Table 4. Population averages of colour intensity in the five focal hyperspectral imaging channels (580, 600, 620, 640 and 660 nm), with values listed for the two measured regions of interest, ventrum and dorsum

Newt species	Newt locality	Region of interest	Hyperspectral imaging channel intensity (x)				
			580 nm	600 nm	620 nm	640 nm	660 nm
Taricha granulosa	Battle Creek	Ventrum	0.00105	0.00165	0.00247	0.00835	0.00273
		Dorsum	0.00016	0.00023	0.00028	0.00089	0.00032
	Little Strawberry Creek	Ventrum	0.00091	0.00141	0.00208	0.00659	0.00233
		Dorsum	0.00021	0.00029	0.00038	0.00124	0.00045
	Tunitas Creek	Ventrum	0.00095	0.00143	0.00206	0.00645	0.00231
		Dorsum	0.00020	0.00029	0.00038	0.00129	0.00047
	Warm Springs Creek	Ventrum	0.00097	0.00153	0.0023	0.00748	0.00261
		Dorsum	0.00022	0.00030	0.00038	0.00126	0.00045
Taricha sierrae	Battle Creek	Ventrum	0.00086	0.00134	0.00203	0.00681	0.00230
		Dorsum	0.00020	0.00027	0.00035	0.00120	0.00042
	Canyon Creek	Ventrum	0.00073	0.00119	0.00194	0.00701	0.00228
		Dorsum	0.00021	0.00028	0.00037	0.00133	0.00046
	Deep Creek	Ventrum	0.00082	0.00127	0.00190	0.00640	0.00220
		Dorsum	0.00024	0.00034	0.00048	0.00172	0.00062
	Ogilby Creek	Ventrum	0.00091	0.00143	0.00215	0.00718	0.00243
		Dorsum	0.00020	0.00003	0.00036	0.00126	0.00044
	Yosemite Lakes Campground	Ventrum	0.00082	0.00137	0.00214	0.00724	0.00243
		Dorsum	0.00022	0.00005	0.00042	0.00146	0.00052

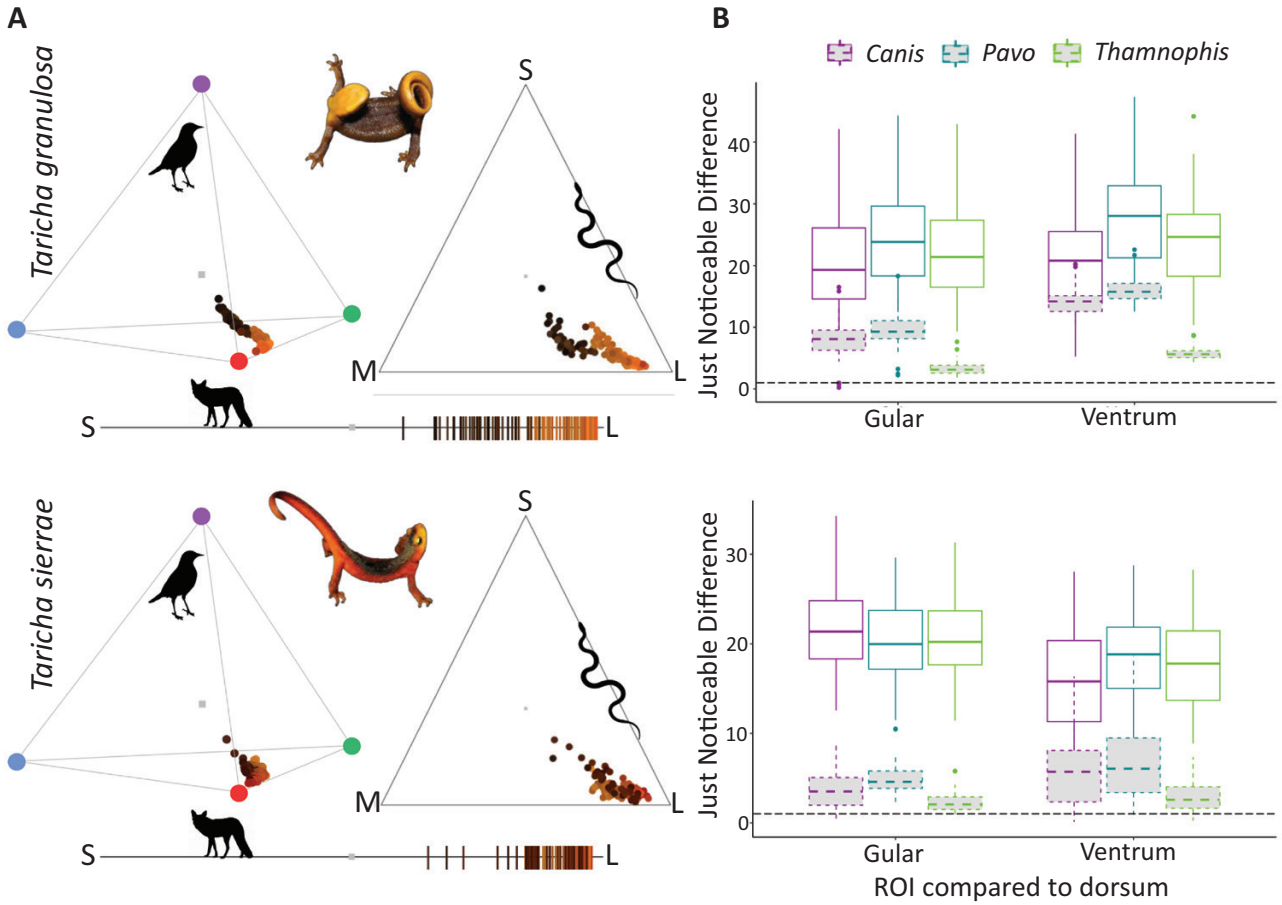

Figure 3. Predator visual models and contrasts for all regions of interest (gula, ventrum and dorsum) for *Taricha granulosa* (top panels; $N = 37$) and *Taricha sierrae* (bottom panels; $N = 38$). A, we modelled newt measurements in colour space for expected primary predators: tetrachromat birds (*Pavo cristatus*; four photoreceptors represented by coloured dots), dichromat mammals (*Canis familiaris*; S and L denote short- and long-wavelength photoreceptors) and trichromat garter snakes (*Thamnophis sirtalis*; in addition to the S and L photoreceptors of mammals, garter snakes have a medium-wavelength photoreceptor). Data points are coloured as they would be visualized by a human, with darker dorsal measures skewing towards the midpoint of each graph, and orange–red ventral measures grouping towards long wavelengths. B, from these models, we pulled relative colour distances to calculate the just noticeable difference (JND), a representation of whether the contrasting signal of the unken reflex is qualitatively honest. For both achromatic (open boxes) and chromatic (grey boxes) measures, the average JND was above the threshold of being discriminable to all predators (black horizontal line; JND = 1), supporting a qualitatively honest aposematic signal. Abbreviation: ROI, region of interest. Newt images Images courtesy of Gary Nafis.

also differed in their ability to differentiate achromatic ventral–dorsal contrast ($F_{2,108} = 7.68$, $P < 0.001$), with the same prediction that birds visualize this cue most distinctly and with both bird and mammal models outperforming snakes (bird–mammal Tukey's HSD $P < 0.001$). All predator models performed similarly when perceiving gular–dorsal contrast ($F_{2,108} = 3.045$, $P = 0.05$), again with the bird expected to discriminate best (bird–mammal Tukey's HSD $P = 0.04$).

Within *T. sierrae*, chromatic ventral–dorsal contrast was estimated by models as perceptible to all predators ($F_{2,111} = 11.33$, $P < 0.001$; Fig. 3) and, similar to *T. granulosa*, was less discriminable in the garter snake model than in either the bird model (Tukey's HSD $P < 0.001$) or the mammal model (Tukey's HSD $P = 0.003$), which performed very similarly (Tukey's HSD $P = 0.41$). This pattern held for chromatic gular–dorsal contrast ($F_{2,111} = 20.17$, $P < 0.001$; snake–mammal Tukey's HSD $P = 0.002$, snake–bird Tukey's HSD $P < 0.001$), although birds were predicted to have a greater ability to discriminate the two regions than mammals (Tukey's HSD $P = 0.017$). Achromatic contrast was equally visible in all predator models for both ventral ($F_{2,111} = 2.86$, $P = 0.06$) and gular ($F_{2,111} = 1.89$, $P = 0.156$) contrasts.

Quantitative honesty

Viewer-independent analyses of the spectrometer data revealed no significant relationship between ln-transformed whole-newt TTX levels and ROI for either *T. granulosa* brightness (gula: $r = 0.01$, $r^2 = 0.0002$, $P = 0.92$; ventrum: $r = 0.02$, $r^2 = 0.0004$, $P = 0.90$) or hue (gula: $r = 0.25$, $r^2 = 0.06$, $P = 0.14$; ventrum: $r = 0.14$, $r^2 = 0.02$, $P = 0.36$; Supporting Information, Fig. S3), and the same was found for *T. sierrae* brightness (gula: r –0.17 =, $r^2 = 0.03$, $P = 0.29$; ventrum: $r = -0.26$, $r^2 = 0.07$, $P = 0.10$) and gula hue ($r = 0.22$, $r^2 = 0.05$, $P = 0.18$). However, there was a significant decrease in ventral peak wavelength in more toxic individuals ($r = -0.36$, $r^2 = 0.13$, $P = 0.03$), although this relationship might be inflated by the presence of a few individuals with peaks closer to the red and infrared spectrum (700 nm) with low toxin levels (see Supporting Information, Fig. S3).

Viewer-dependent analyses of spectrometer data from *T. granulosa* did not reveal a relationship between chromatic (dS) or achromatic (dL) JNDs and newt toxin levels for either ventral–dorsal or gular–dorsal contrast as viewed by most predators (Fig. 4), with the exception of a relationship between chromatic ventral–dorsal contrast as predicted to be viewed by bird predators ($F_{1,35} = 4.86$, $P = 0.03$, $r = 0.35$, $r^2 = 0.12$) and snake predators ($F_{1,35} = 6.68$, $P = 0.01$, $r = 0.4$, $r^2 = 0.14$; Fig. 4). Analyses of *T. sierrae* spectrometer data did not reveal significant relationships between any colorimetric trait and toxin levels for any predator model (Fig. 4).

Across populations and for both newt species, we found few relationships between ln-transformed toxin levels and dorsal–ventral contrast as recorded in our five focal HSI camera channels. Colour contrast for both species peaked in the 640 nm channel for intensity, yet there was no significant correlation between toxin level and contrast in this channel across populations of *T. sierrae* ($F_{1,45} = 3.68$, $r = -0.28$, $r^2 = 0.08$, $P = 0.06$) or *T. granulosa* ($F_{1,13} = 1.96$, $r = -0.36$, $r^2 = 0.13$, $P = 0.19$; Fig. 4). In fact, toxin level and contrast were not correlated for any HSI camera channels across *T. granulosa* populations. However, we found moderate negative correlations in three channels in *T. sierrae*: 580 nm ($F_{1,45} = 4.38$, $r = -0.3$, $r^2 = 0.09$, $P = 0.04$), 600 nm ($F_{1,45} = 4.22$, $r = -0.3$, $r^2 = 0.09$, $P = 0.05$) and 620 nm ($F_{1,45} = 4.02$, $r = -0.28$, $r^2 = 0.08$, $P = 0.051$).

DISCUSSION

Pacific newts (*Taricha*) perform the unken reflex when threatened, displaying a vivid ventrum against a starkly contrasting dorsum (Stebbins, 1951; Reimer, 1958; Johnson & Brodie, 1975; Brodie, 1977). We predicted that both bright ventral colour and, especially, its contrast against the darker dorsum represent a qualitatively honest aposematic signal to primary predator groups. Our results demonstrate qualitative honesty within populations for the two newt species we sampled (*T. granulosa* and *T. sierrae*).

Our support for qualitatively honest signalling confirms previous interpretations from avian predation trials using live newts (Johnson & Brodie, 1975) and newt models (Kuchta *et al.*, 2008). To varying degrees and depending on the colour trait (chromatic or achromatic contrast), all three predator visual models (bird, mammal and snake) clearly distinguish contrasting regions in both newt species. Bird and mammal visual models had consistently higher chromatic JND values than snakes, particularly for *T. granulosa*. All three predators are predicted to distinguish achromatic contrast of both newt species similarly. Although all predators are expected to visualize both chromatic and achromatic contrasts that a newt advertises during the unken reflex, our results suggest that brightness is a more perceivable signal. Although newts are active at any time of day (especially in water), they generally wait for nightfall or rainy and overcast conditions for overland movements (Stebbins, 1951, 2003; Stebbins and Cohen, 1997). In such conditions, brightness rather than specific hue might be more easily perceived by predators, especially crepuscular or nocturnal mammalian carnivores. This finding also aligns with the literature (Prudic, 2007; Sandre *et al.*, 2010), particularly on birds, which are thought to rely more heavily on achromatic cues when detecting small objects (Osorio *et al.*, 1999; Schaefer *et al.*, 2006; Kraemer & Adams, 2014).

We also tested the hypothesis that newts convey a quantitatively honest signal, in which there is a positive correlation between colour or brightness and the toxin level (Speed & Ruxton, 2007; Számadó, 2011). We measured quantitative honesty at two scales, using spectrometry to investigate colorimetrics against toxin level across individuals within a population and using hyperspectral imaging to measure this relationship across multiple populations per species. Regardless of the scale or method, our results showed no support or mixed support for a model of quantitative honesty.

We did not find a viewer-independent correlation between the toxin level and ventral hue in *T. granulosa*, whereas we did find a negative correlation between TTX and the ventrum in *T. sierrae* ($r = -0.36$, $r^2 = 0.13$, $P = 0.03$). Conversely, when colorimetrics were contextualized by predator visual models, *T. granulosa* displayed positive correlations between TTX and the chromatic dorsal–ventral contrast, as predicted to be viewed by both birds ($r = 0.35$, $r^2 = 0.12$, $P = 0.03$) and snakes ($r = 0.4$, $r^2 = 0.16$, $P = 0.01$). However, after accounting for visual parameters of potential

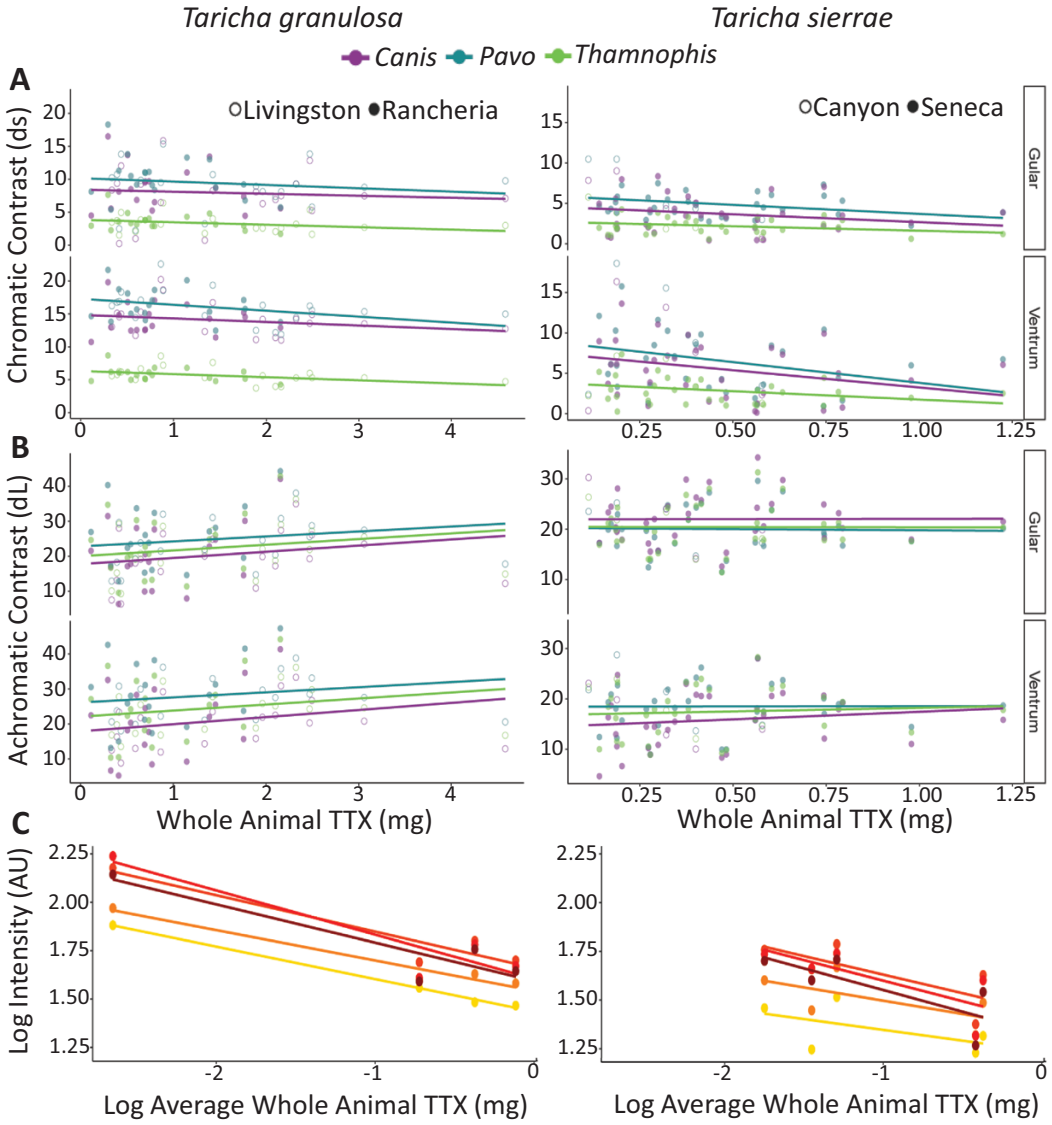

Figure 4. Intrapopulation assessment of quantitative honesty via correlations between predator-contextualized just noticeable difference (JND) and newt tetrodotoxin (TTX) levels. A, correlations between JNDs for chromatic (hue) contrasts. B, correlations between JNDs for achromatic (brightness) contrasts. Populations of *Taricha sierrae* (top; $N = 38$) and *Taricha granulosa* (bottom; $N = 37$) are indicated by open versus filled circles, whereas predators are indicated by colour. The TTX levels in *T. sierrae* (right panels) individuals were not correlated with chromatic or achromatic ventrum contrasts for any predator. We found similar results for *T. granulosa* (left panels), except for the chromatic contrast of the ventrum as viewed by bird predators ($F_{1,35} = 4.86$, $r = 0.35$, $r^2 = 0.12$, $P = 0.03$) and snake predators ($F_{1,35} = 6.68$, $r = 0.4$, $r^2 = 0.14$, $P = 0.01$). These results suggest mixed support for intrapopulation quantitative honesty, dependent on newt species and predator visual system. C, interpopulation assessment of quantitative honesty via correlations between colour intensity and newt TTX levels (ln-transformed). Each vertical line of five points is representative of the five focal channels (580, 600, 620, 640 and 660 nm) in one population. All species peaked in the 640 nm channel (red; Supporting Information, Fig. S3). We calculated contrast within each channel by dividing the intensity of the lighter ventrum by the intensity of the darker dorsum for each newt. We did not find strong evidence of quantitative honesty for either species (*T. granulosa*, $N = 15$; *T. sierrae*, $N = 48$) but did find a moderate negative correlation between TTX and colour intensity [in arbitrary units (AU)] in channels 580, 600 and 620 nm in *T. sierrae*, the opposite of our predictions.

predators, we found no such correlations between TTX level and ventrum colorimetrics in *T. sierrae*.

At a broader spatial scale (across several populations), our HSI data revealed only three weak, marginally significant correlations between toxin level and dorsal–ventral contrast in *T. sierrae* [580 nm ($r = -0.3, r^2 = 0.09, P = 0.04$), 600 nm ($r = -0.28, r^2 = 0.09, P = 0.05$) and 620 nm ($r = -0.28, r^2 = 0.08, P = 0.05$)]. In all cases, these correlations were negative with contrast, decreasing as the TTX level increased. These findings do not support the presence of quantitative honesty at either the intra- or interpopulation scale in *Taricha*.

Negative correlations between toxin level and colour can be expected when the two traits compete for resources (Blount *et al.*, 2009). This might be the case when colour is based on carotenoid pigments, which cannot be synthesized by vertebrates and must instead be obtained through foraging (Goodwin, 1984). Carotenoid availability might be limited further for coloration, because these pigments are also used as antioxidants to protect tissues against autotoxicity (Blount *et al.*, 2009; Summers *et al.*, 2015). Thus, a greater understanding of the availability of carotenoids and their physiological roles in newts is needed to understand patterns of colour variation in *Taricha*. Likewise, knowledge of the costs of toxin production and maintenance is needed to understand potential correlations with warning coloration. Although it has yet to be identified definitively whether TTX is synthesized, sequestered or produced by symbionts (Cardall *et al.*, 2004; Bucciarelli *et al.*, 2017; Kudo *et al.*, 2020; Vaelli *et al.*, 2020; Gall *et al.*, 2022), several lines of evidence suggest that TTX is costly to acquire or produce. First, we see the reduction and even loss of TTX in populations allopatric with resistant predators (Brodie & Brodie, 1991; Hanifin *et al.*, 1999; Hague *et al.*, 2016). Second, it can take > 9 months for newts to regenerate TTX in laboratory experiments (Cardall *et al.*, 2004). If precursors to TTX are foraged from the environment, then variation in these resources might decouple the signal (Kodric-Brown, 1989; Mochida *et al.*, 2013; Crothers *et al.*, 2016; Casas-Cardonas *et al.*, 2018). However, previous experiments have established that newts are able to maintain and regenerate TTX in laboratory conditions (Hanifin *et al.*, 2002; Gall *et al.*, 2022), suggesting that TTX is not a resource obtained from the diet. Given that we did not find strong or consistent evidence of trade-offs in the form of negative correlations, resource-based limitations might not be (entirely) responsible for the lack of quantitative honesty within and across populations of *Taricha*.

Newts might also experience differential selection on signal and TTX levels because of local variation in predator density or diversity (Mochida, 2009). Selection might differ owing to variation in predator visual systems (Maan & Cummings, 2012) or, in this case, the degree of TTX resistance of sympatric garter snakes (*Thamnophis*). Certain populations of garter snakes are among the few predators of newts (Brodie, 1968; Brodie *et al.*, 2002, 2005; Feldman *et al.*, 2009; Greene & Feldman, 2009), and these snakes have less-developed colour vision than most avian predators (Sillman *et al.*, 1997; Macedonia *et al.*, 2009) and rely heavily on motion and scent cues (Ford & Burghardt, 1993; Williams *et al.*, 2003). Thus, newt populations depredated by TTX-resistant garter snakes might be under more intense predation pressure. Such pressure might disrupt or decouple selection that would otherwise promote an honest link between coloration and toxin level by predators with more acute vision (i.e. avian predators; Sillman *et al.*, 1997; Macedonia *et al.*, 2009). Likewise, newt eggs and larvae are vulnerable life stages (Stebbins & Cohen, 1997; Petranka, 1998), and mothers provision eggs with TTX (Hanifin *et al.*, 2003; Gall *et al.* 2012a, 2014), which appears to transfer to larvae until metamorphosis (Gall *et al.*, 2011, 2022), affording protection against some aquatic predators (Gall *et al.*, 2011, 2012b). Thus, selection might promote newt mothers with high toxin levels, regardless of coloration, again disrupting a potential link between defence and warning signal. Finally, it might be that most newts in our study contain more than enough TTX to incapacitate local predators (Brodie, 1968; Abal *et al.*, 2017). Consequently, beyond a sufficient toxin threshold, a correlation between signal and defence might no longer be informative or beneficial (Speed *et al.*, 2012; Stuckert *et al.*, 2018). Greater sampling across the range of newt phenotypes, especially from populations with little to no TTX (Hanifin *et al.*, 1999, 2008; Stokes *et al.*, 2015; Hague *et al.*, 2016), might be required to detect a link between variation in the warning signal and the toxin level.

Although we found strong evidence that predators can visualize the contrast between colours presented when newts are in the unken pose, further work is needed to understand all the mechanisms that might impact coloration in newts (Guilford, 1988). For example, although newts appear to possess aposematic coloration, other factors, such as newt density and signalling conditions (Sherratt, 2002; Mappes *et al.*, 2005; Speed & Ruxton, 2007; Speed *et al.*, 2010), abiotic variation (Mochida, 2011), presence of non-toxic mimics (Kuchta, 2005; Kuchta *et al.*, 2008; Maan & Cummings, 2012; Kraemer *et al.*, 2015), differences in genetic background (Hague *et al.*, 2020) and intraspecific communication (Maan & Cummings, 2009), might drive variation in colour and brightness. For example, in other amphibian systems, intraspecific communication and sexual selection influence signal development (Jiggins *et al.*, 2001; Maan & Cummings, 2009), especially when toxin

levels exceed a threshold at which predator learning is no longer the primary selective agent (Cummings & Crothers, 2013). Intraspecific communication in newts (especially courtship) can rely heavily on pheromones (Thompson *et al.*, 1999), and although larval and adult *Taricha torosa* have functional pineal photoreceptors (Hendrickson & Kelly, 1971), their visual pigments are composed entirely of rhodopsin, which is not as sensitive to red light as other pigments (Crim, 1975). More research is needed to understand how colour, brightness and contrast are visualized by *Taricha*.

Understanding the signals used in communication between ecological partners is important for identifying the selective pressures acting on prey and predator populations. When both signal and defence require costly energetic inputs to produce or maintain, variation in the availability of these resources and ability to acquire them might disrupt the expectation that these traits are tightly linked and instead lead to qualitative honesty without concurrent quantitative honesty. In systems where prey might be, on average, dangerous to almost all predators, further subtlety in signal level might not be needed to communicate danger to sympatric predators.

ACKNOWLEDGEMENTS

We dedicate this paper to Judy and Butch Brodie, who conducted pioneering work on aposematism in newts and other salamanders. We thank California Department of Fish & Wildlife and Oregon Department of Fish and Wildlife for scientific collection permits (to C.R.F.) and approval of live animal protocols by University of Nevada, Reno's Institute of Animal Care and Use Committee (UNR IACUC) (to C.R.F.). For access to important sampling sites, we thank Margot Rawlins (Sonoma State University Galbreath Wildlands Preserve). We thank Kelly Robinson, John Gray, Amber Durfee and Taylor Disbrow for assistance with live animal care. We are grateful to Jenny Ouyang for generously providing spectrometry hardware and software. We are indebted to Devon Picklum for invaluable assistance in forming a spectrometer protocol. We thank Andrea Rummel for assistance with HSI data collection. We thank Devon Picklum and Gary Nafis (californiaherps.com) for photographs. We appreciate helpful feedback on this manuscript from Matt Forister, Jack Hayes and the UNR EvolDoers, especially Davide Baldan, Kelly Robinson, Danny Nielsen, Abigail Miller and Jamie Voyles. This manuscript was improved by the generous feedback from three anonymous reviewers. This work was supported by National Science Foundation (NSF) grant IOS1355221 (to C.R.F.), NSF Division of Ocean Sciences grant 1129897 (to R.T.H.) and a Nevada NSF Established Program to Stimulate Competitive Research - Undergraduate Research Opportunity Program (EPSCoR UROP) grant (to S.M.K.). The authors have no conflicts of interests to declare.

DATA AVAILABILITY

The data and code underlying this article are available in the online Supporting Information for the article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article on the publisher's website.

Table S1. Species, locality, mass (grams) and estimated whole newt TTX level (mg) data for two Pacific newt species (Taricha granulosa and T. sierrae) measured by hyper-spectral imaging camera in 2015.
Table S2. Species, morphometric [mass (g) and snout-vent length (cm)], estimated whole newt TTX level (mg), locality, viewer-independent brightness (% relative reflectance) and hue (peak wavelength in nanometers) for all three regions of interest (gula, ventrum, dorsum) in two Pacific newt species (Taricha granulosa, T. sierrae) measured by spectrometry in 2019.
Figure S1. Raw and averaged (mean + SD) spectrographs of newt gula, ventrum and dorsum for the two focal species (*Taricha granulosa* and *Taricha sierrae*). Hue is determined from these data as the wavelength (in nanometres) at which each spectrum peaks, which is then averaged across individuals for a given region of interest.
Figure S2. Population range in colour intensity for the focal five channels (580, 600, 620, 640 and 660 nm) for *Taricha granulosa* (left) and *Taricha sierrae* (right) split by region of interest (ventrum and dorsum). All regions across populations and species peaked in the 640 nm channel (dashed red boxes).
Figure S3. Viewer-independent correlations between chromatic (hue; left) and achromatic (brightness; right) measurements and estimated whole-animal toxin level. Correlations were calculated for both *Taricha granulosa* (top) and *Taricha sierrae* (bottom) gular regions (plus signs, dashed lines) and ventrums (dots, continuous lines). There were no correlations between TTX levels and colorimetrics, with the exception of *T. sierrae* ventral hue ($r = -0.36$, $r^2 = 0.13$, $P = 0.028$).
File S1. Data files associated with Moniz *et al*. 2023 including data collected by both Hyper-Spectral Imaging (HSI) camera and Spectrophotometer. The 7 Excel spreadsheets represent a mix of raw data and analysis outputs that are read into the associated code for replication of findings. Newt collection and individual information can be found in supporting tables S1 and S2. Please see the included README file for further metadata by spreadsheet. Cite as: Moniz HM *et al*. (2023). Aposematic coloration of Pacific newts (*Taricha*) provides a qualitatively but not quantitatively honest signal to predators, Biological Journal of the Linnean Society, Dataset, File S1, DOI: 10.1093/biolinnean/blad007.