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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	All software and code for data collection and analysis is available on Dryad- DOI: 10.5061/dryad.djh9w0w5g
Data analysis	All software and code for data collection and analysis is available on Dryad- DOI: 10.5061/dryad.djh9w0w5g

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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Data availability: All specimen data can be accessed via the museum/institution in which the individual was accessioned (ECM 20001-20528, see Methods section above). All spectrometer recordings and photographs are available by contacting the corresponding author; these raw data files available under restricted access due to vastness (>17,000 spectrometer recordings and >24,000 photographs). All other data are available in the main text or the supplementary materials.

Code availability: All code is available on Dryad (https://datadryad.org, DOI: 10.5061/dryad.djh9w0w5g).

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Ecological, evolutionary & environmental sciences study design

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Study description	This study was designed to discover, document, and assess variation in amphibian biofluorescence. To discover fluorescence across the diversity of amphibians, we conducted field surveys of this trait across eight sites representing much of the amphibian biodiversity of lowland South America, the region with the highest species richness of amphibians in the world. While most specimen acquisition was opportunistic via nightly trail surveys, we focused our efforts on collection of anurans, especially treefrogs from the Hylidae family and the Dendropsophus genus within this family. We made the choice to focus our sampling on Dendropsophus and Hylidae to increase our sample size for future genus- and family-specific studies, balancing the breadth and depth of our data collection. To document biofluorescence, we collected spectrometer recordings and photographs of individual amphibian fluorescence under five excitation sources. To assess variation in the amphibian biofluorescent traits we discovered, we used analysis of variance tests. Specifically, we assessed if biofluorescent emission differed by excitation wavelength to search for evidence of ecological tuning of this novel trait.
Research sample	The research sample was 17,692 spectrometer recordings (biofluorescent measurements) from 528 individuals. This sample included representatives of one salamander family (two species), one caecilian family (one species), and 13 anuran families. Within Anura, there were individuals from 39 genera and 152 species. We quantified biofluorescent emission in response to five different excitation light sources.
Sampling strategy	We determined the peak excitation and emission wavelength and intensity from each spectrometer recording and utilized these intensities to calculate a maximum percent biofluorescence emission: (%BF=(intensity at peak emission λ)/(intensity at peak excitation λ)*100) This provided our focal measure of intensity of fluorescence relative to the amount of reflected excitation light. All characterizations of spectrometer recordings were completed in R version 4.2.3 (R Core Team 2023). An ASCII file, containing intensity in photon counts for wavelengths ranging 200-1000 nm, of each spectrometer recording was saved via the OceanView application at time of acquisition and later loaded into R for analysis. Spectra were smoothed via a fifteen-point moving average filter to reduce noise in the spectrum. Utilizing the photobiology package (Aphalo 2015), smoothed spectra were changed to an object of class spectra and normalized to the highest intensity (corresponding to the excitation light) using the normalize() function. We found peak excitation wavelengths by using the which.max() function and confining the parameters to only look for the maximum intensity within the wavelength range of the excitation light source. For example, finding the wavelength that corresponds to the highest intensity (photon count) within the wavelength range of 360-380 nm for UV excitation light, 400-415 nm for VI light, etc. Some of our excitation peak recordings were saturated (maximum >60,000 photon counts). If this was the case, the maximum intensity value available from the spectrometer recording (at 60,000 photons) was utilized. We expect data affected by this saturation would overestimate the intensity of the fluorescence emission recording, though this should not affect the overall significance of our findings because we estimate that saturation occurred at comparable levels across all excitation light sources. All peak emission wavelengths were found using the get_peaks() function from the photobiology package. As a fluorescent signal can produce multiple peaks, the get_peaks() function found all peaks at a wavelength greater than the longest wavelength of the excitation source (greater than 380 nm for UV, 400 nm for VI, etc.). Because fluorescence absorbs light and re-emits it at a longer wavelength, these peaks are all potential fluorescent signals under the respective excitation light. Within the get_peaks() function, we set the ignore_threshold to 0.01 to only collect peaks with a relative size greater than 1% as compared to the tallest (excitation) peak, and we set the span to 100 to define a peak as a datapoint within the wavelength sequence that had an intensity greater than those within a 50-count window on either side of the point. These parameters were chosen via trials adjusting parameters to find peaks of a subset of spectrometer

recordings representing the diversity of emission spectra shapes (one emission peak close to the excitation peak, one peak far from excitation, two peaks, no peaks, etc.) and were chosen to reduce the probability that noise in the recording was defined as a peak. Additionally, to further assure that the chosen peaks were significant signals and not noise, any suspected emission peak with an intensity greater than the excitation peak was removed, as this was likely a sign of an inaccurate spectrometer recording. In these cases, and in any cases where no peaks were found, "NA" was input for the peak wavelength and intensity values. For blue-light-induced fluorescence, there were two main emission peaks (Fig. S1), so the second tallest excitation peak was also recorded to account for any individuals with a bi-modal emission spectrum. The intensity at each peak emission wavelength was divided by the intensity at the peak excitation wavelength for the respective spectrometer recording to calculate the average percent intensity of fluorescence relative to the amount of reflected excitation light.

As stated, we took three spectrometer recordings at each location on each individual to have technical replicate measurements of the biofluorescent signal at that body location. The calculated percentages of biofluorescence emission for these three recordings were averaged for each body location. Because our data set contained spectrometer recordings from a different number of body locations, and often different specific body regions, from each individual (due to the variation in physical biofluorescent patterns that we were attempting to capture across a wide range of species), we only used the maximum percent biofluorescence emission (from any body region, under each of the five excitation light sources) for downstream analyses. Hence, for all analyses of variance, the unit of measurement used was the maximum percent of biofluorescence emission recorded from each individual.

We also examined the body location from which the maximum biofluorescent recording from each individual was taken. For this, we used the maximum percent biofluorescence emission (from any body region, under any light source). Utilizing the maximum fluorescent emission recording for each individual insured that only one body location per individual was being considered, although, examining the maximum biofluorescent emission recording for each individual under each excitation light source (as in the analyses above) produced similar percentages of fluorescence by body location. For easier comparison, the body regions were summarized into the following nine groups: cloaca, dorsal (including spectrometer recordings with a body location specified from any dorsal pattern), eye, facial pattern (including lip, spots under the eye, snout, etc.), flank, inguinal region, limb (including forelimbs, thigh, etc.), throat (including vocal sac), and ventral (including any ventral pattern). The percentage of maximum biofluorescent recordings from each of the nine body locations were calculated for each light source.

Statistical Analysis

We evaluated the maximum biofluorescence recordings against the predictions for each criterion proposed by Marshall and Johnsen (2017) for ecological significance for five different light environments irradiance spectra and two relevant photoreceptor visual sensitivity curves. The light environment irradiance spectra we utilized were digitized with permission from Cronin et al. 2014 and consisted of: Daylight, Under Forest Canopy, Twilight, Full Moon, and Starlight. The relevant anuran photoreceptor sensitivity curves we utilized were for the red-sensitive cone and the green-sensitive rod. Photoreceptor sensitivity curves were obtained from Robertson et al. 2022. For assessing ecological significance under environmental conditions relevant to frogs, we paired these light environments to their relevant anuran photoreceptor: the red-sensitive cone with day environments (Daylight and Under Forest Canopy) and the green-sensitive rod with night environments (Twilight, Full Moon, and Starlight) for interpretation of results across criterion.

Evaluate Criterion 1: The fluorescent pigment will absorb the dominant wavelengths of the environment.

To evaluate evidence for Criterion 1, we utilized a randomization test to assess if each range of excitation wavelengths matched the most dominant wavelengths of the environment better than expected by chance. For each set of excitation wavelengths (VI 400 – 415 nm and RB 440 – 460 nm), we determined the average value of irradiance at those excitation wavelengths. This is the test statistic. We then took a sample of the same size (n = 15 and n = 20 respectively) and randomly selected a wavelength between 400 and 700 nm, then calculated a new value for the test statistic for those wavelengths. We repeated this for 10,000 iterations and compared the test statistics in the randomization distribution to the observed test statistic value to determine if the peak fluorescent excitation matched the wavelengths most abundant in the environment better than by chance ($\alpha = 0.05$). We were only able to complete these calculations for the VI and RB excitation sources due to the limited wavelength range of our environment irradiance spectra.

Evaluate Criterion 2: The fluorescence will be viewed against a contrasting background.

To evaluate evidence for Criterion 2, we assessed if the peak emission wavelength of the blue-light-induced fluorescence matched the least dominant wavelengths of the twilight environment better than expected by chance. Fluorescent emission wavelengths that match the least dominant wavelengths of light at twilight will produce the most contrast with the background environment.

We utilized a non-parametric Kruskal-Wallis one-way analysis of variance test and Pairwise Dunn's tests with Holm adjustment to determine if the wavelength of biofluorescent emission differed by excitation light source. As above we utilized a randomization test to assess if the emission wavelengths of each excitation source matched the least dominant wavelengths of the environment better than expected by chance. For each set of emission wavelengths within the wavelength range of our irradiance spectra (UV, VI, RB green, and RB orange), we determined the average value of irradiance at those emission wavelengths. This is the test statistic. We then took a sample of the same size (n = 486, 493, 194, and 363 for each emission type respectively, where there are 49 overlapping individuals between blue induced green and orange emission when accounting for multiple peaks) and randomly selected a wavelength between 400 and 700 nm, then calculated a new value for the test statistic for those wavelengths. We repeated this for 10,000 iterations and compared the test statistics in the randomization distribution to the observed test statistic value to determine if the peak fluorescent emission matched the wavelengths least abundant in the environment better than by chance ($\alpha = 0.05$).

Additionally, to correct for unequal samples sizes across phylogenetic groups, we repeated this analysis for 10,000 subsamples of the individuals. For each subsample, we choose one individual from each species at random and repeated the test statistic and null calculations as above, with samples sizes of n =152, 151, 86, and 130 for each emission type respectively. There are 27 overlapping species between blue induced green and orange emission when accounting for multiple peaks. We compared the test statistics in the randomization distribution to the observed test statistic value to determine if the peak fluorescent emission matched the wavelengths least abundant in the environment better than by chance ($\alpha = 0.05$).

Evaluate Criterion 3: Organisms viewing the fluorescence will have spectral sensitivity in the fluorescent emission range.

To evaluate evidence for Criterion 3, we assessed if the peak emission wavelength of the blue-light-induced fluorescence matched the peak sensitivity of the green sensitive anuran rod better than expected by chance. As above, we utilized randomization tests with 10,000 iterations each, for both individuals and species, to determine if the fluorescent emission wavelengths under each light source match the anuran photoreceptor sensitivity curve better than expected by chance ($\alpha = 0.05$). We repeated with for both the

red-sensitive cone and green-sensitive rod sensitivity curves (obtained from Robertson et al. 2022).

Considering that both Criterion 2 and Criterion 3 need to be met for ecological significance, we also compared the blue-light-induced green emission peak to the tradeoff spectrum of twilight irradiance and anuran rod sensitivity, as follows. We divided the green-sensitive rod spectrum (28) by the twilight irradiance spectrum (27) to obtain the tradeoff spectrum. This is the spectrum of wavelengths that would maximize both receiver sensitivity and background contrast. Both the twilight and rod spectra were standardized before this calculation. As above, we then utilized a randomization test to assess if the green emission peak matched the tradeoff spectrum better than expected by chance. We calculated the average tradeoff value at the emission wavelengths of the green peak. This is the test statistic. We then took a sample of the same size (194 individuals) and randomly selected a wavelength between 400 and 700 nm, then calculated the value of the test statistic for those wavelengths. We repeated this for 10,000 iterations and compared the test statistic values in the randomization distribution to the observed test statistic to determine if the green peak fluorescent emission maximized the wavelengths to meet both Criterion 2 and Criterion 3 better than by chance.

Assessing Phylogenetic Structure

To assess whether phylogenetic non-independence between measurements affected our results, we performed several additional tests of the criteria above after accounting for phylogenetic structure. For all phylogenetic analyses we utilized a time calibrated phylogeny obtained from Timetree.org. A list of all species in the analysis was uploaded to Timetree.org, using the "Load a List of Species" option under the "Build a Timetree" function. Three species tips were not available, but inferred from Timetree, based on the same positioning on the tree. These species were Dendropsophus hadaddi inferred from D. brevipollicatus, Physalaemus atlantics inferred from P. ephippifer, and Diasporus gularis inferred from D. diastema. We removed species tips for which we did not have data for a given analysis because the programs we used could not handle missing data for continuous trait evolution models.

To evaluate Criterion 1 within a phylogenetic context, we completed an ancestral state reconstruction for the trait of percent biofluorescent emission under each excitation light source. The percent biofluorescent emission was averaged for each species. We utilized the anc.Bayes function in phytools (implemented in R v4.2.3.) to estimate the percent biofluorescent emission at the basal node of the amphibian tree for each excitation source. This value was estimated in 1,000 mcmc generations and compared across excitation sources.

To evaluate Criteria 2 and 3 within a phylogenetic context, we examined the evolution of the average blue light-induced emission wavelength for each species within the context of each of the five ecological and photoreceptor conditions. The wavelength of biofluorescent emission was averaged for each species. Averages were calculated on the lists of individuals with peak blue light-induced green fluorescence (any peak, first or second) and blue light-induced orange fluorescence (any peak, first or second) from the previous criteria analyses, separately. We found the wavelength of maximum vision/environment tradeoff under each of the five vision/environment conditions. Utilizing RevBayes constrained optima Ornstein-Uhlenbeck evolution models, we ran five analyses for each fluorescent emission dataset where the constrained optimum correlated to the peak wavelength of fluorescence from the tradeoff spectrum of each environment/vision condition. This was done by constraining the moveAppend(theta) parameter weight to 0.01 and constraining the theta prior parameter to 0.01 nm on either side of the peak tradeoff wavelength for each condition as follows. The bounds of the uniform prior for the Daylight/RSCone condition were set at (575.99, 576.01); (591.99, 592.01) for the UnderCanopy/RSCone condition; (512.99, 513.01) for the Twilight/GSRod condition; (496.99, 497.01) for the FullMoon/GSRod condition; and (503.99, 504.01) for the Starlight/GSRod condition (Fig. S9). We ran each model for 100,000 iterations (burnin generations=20000, tuningInterval=100). The average likelihood value from all 100,000 generations of each model was compared using Akaike's information criterion (AIC). Models with a delta AIC value less than two were considered to fit the data equally well.

The analyses were run as above for the blue light-induces oranges fluorescence with a few additions. Three additional conditions were tested in the constrained optima modelling analyses as defined previously. The bounds of the uniform theta prior for these three conditions were defined as (617.99, 618.01) for the Chlorophyll/GSRod condition; (699.99, 700.01) for the Chlorophyll/RSCone condition; and (616.99, 617.01) for the AnuraGreenFluorescence/GSRod condition. Note that unlike all other condition tradeoff spectra, the maximum wavelength for both chlorophyll conditions were calculated by taking the fluorescent chlorophyll b fluorescent spectrum divided by the photoreceptor visual sensitivity curve. This is because these conditions were chosen to test whether the orange fluorescence of frogs could be utilized as a cryptic signal for camouflage. Hence, we wanted to find the wavelengths that maximize matching both the peak emission of the fluorescent chlorophyll background environment and the peak Red-sensitive cone visual sensitivity (in contrast to finding the wavelengths that maximize peak visual sensitivity while also maximizing contrast with the background environment, as defined in Marshall and Johnsen's original Criterion 2 and 3). Finally, the emission spectra utilized for the green anuran fluorescent signal in the final condition tested, was taken from an individual of the species Osteocephalues buckleyi (Field number ECM 20505). For this indiviudal Osteocephalus buckleyi we have both an orange fluorescent throat and a green fluorescent flank spectrometer recording under Blue (440-460nm) excitation light. As both the flank and ventral surface of this species is mottled, we believe that the fluorescent recording from the flank is a good approximation for the fluorescence of the ventral skin surface.

Data collection

Study sites

We collected data at eight sites spanning four countries across South America. We chose study sites to maximize the diversity of hylid frogs, as preliminary work has shown that this group has the highest presence and diversity of biofluorescence found to date (13–18). We focused efforts on collecting individuals from the genus Dendropsophus; our collection sites include the geographic ranges of 77 of the 108 recognized Dendropsophus species. These sites include field stations in four South American countries: Colombia, Ecuador, Peru, and Brazil, including four states within Brazil (SP, ES, BA, AM). Data collection occurred from March to May of 2022, within the breeding season of most anurans at these sites. Our field collection schedule was as follows: March 2nd-10th at Yasuní Scientific Station, Ecuador; March 13th-21st at El Amargal Nature Reserve, Nuquí, Chocó, Colombia; March 24th-April 2nd at Los Amigos Conservation Hub (CIRCA), Peru; April 8th-13th sampling in Fazenda Michelin, Ubatuba, São Paulo (SP); April 17th-20th sampling in Estação Biológica Augusto Ruschi, Aracruz, Espirito Santo (ES); April 23rd-27th sampling in Reserva Michelin, Igrapiúna, Bahia (BA); May 2nd-5th sampling in Manaus, along Rio Negro via boat, State of Amazonas (AM); May 6th-13th sampling in Presidente Figueiredo, AM.

Field capture and specimen preparation

We collected individuals during night surveys on the trails surrounding each research station we visited. We captured individual

amphibians by hand and placed them in a labelled Ziploc plastic bag with air, substrate, and water for transport back to the field station. Each individual was given a field number, and GPS coordinates were taken at the point of capture using a handheld Garmin GPS system. At the field station, all biofluorescent measurements were taken on the same night of capture (see methodology below). The following morning, we euthanized each individual, determined species and sex via dissection, collected tissues, and prepared samples for museum accession. Individual species IDs were determined via region-specific field guides and knowledge of local collaborators. All specimen capture and sample acquisition followed appropriate permit requirements for the specific site (see Acknowledgements).

Collecting biofluorescence measurements

At the field station, and on the same night of capture, we tested each individual for fluorescence under five different excitation sources spanning a nearly 200nm wavelength range (365-460nm) using the Xite Fluorescent Flashlight System (NightSea). The five excitation wavelength ranges were as follows: UV – Ultraviolet (360 – 380nm), VI – Violet (400 – 415nm), RB – Royal blue (440 – 460nm), CY – Cyan (490 – 515nm), and GR – Green (510 – 540nm). We focused on this range of wavelengths to encompass and expand upon wavelengths of biofluorescent excitation used in previous studies (13–18) and to ensure that we did not miss any novel biofluorescent traits. The individual was held by hand beneath the light source, which was suspended above the organism via a YSLIWC Gooseneck Tripod stand. We obtained biofluorescent emission spectra for each individual by utilizing a Maya2000 Pro Series UV-VIS Portable Spectrometer with its attached fiber optic cable held above the surface of the amphibian's skin while beneath the excitation light. This instrument provided information on the emission spectra and intensity of any biofluorescent signals from the frog (200nm-1000nm) in response to each of the five excitation wavelengths. NightSea barrier filter glasses matching the respective excitation source were utilized to view and identify potential locations of biofluorescence to test on the body of the amphibian. We utilized the live spectrometer recording acquisition feature of the OceanView computer application to determine if a specific location had a fluorescent signal (as defined by the presence of a visible peak at a longer wavelength than the excitation source). If any intensity peak was visible, or if it was questionable if a peak was visible, a spectrometer recording was taken with the probe held approximately two millimeters above that area of skin. We used a 1,000 ms integration time for each spectrometer recording and took three recordings at each location to have technical replicate measurements of the biofluorescent signal at that body location. In addition, we photographed patterns of biofluorescence under each light source, as well as under a full-spectrum headlamp light source, using a Canon digital camera and Tiffen camera filters matching the respective excitation source (as specified by NightSea). The camera was a Canon EOS REBEL T7i with a 55mm lens and settings for fluorescent photographs are as follows: f/6.3, 1/8sec, ISO 6400, EXP 0, no flash. Along with these quantitative measurements, we recorded qualitative descriptions of the color of skin and the color of fluorescence. Fluorescent measurements of vocal sac fluorescent were most often taken when vocal sac was not inflated.

All biofluorescent recordings were captured via spectrometer by CEW and RMB. CEW held the frog under the excitation source and positioned the fiber optic probe, while RMB examined the live acquisition and clicked the collection button on the interface to save the spectra data. All dissections and sample preparation were performed by CEW, RMB, and various field assistants as mentioned in the Acknowledgments section. All statistical analyses were performed by CEW with guidance from AL.

Timing and spatial scale

See "Study Sites" information in Data Collection section above. Data collection occurred every night at specific field station. Our field collection schedule was as follows: March 2nd-10th at Yasuní Scientific Station, Ecuador; March 13th-21st at El Amargal Nature Reserve, Nuquí, Chocó, Colombia; March 24th-April 2nd at Los Amigos Conservation Hub (CIRCA), Peru; April 8th-13th sampling in Fazenda Michelin, Ubatuba, São Paulo (SP); April 17th-20th sampling in Estação Biológica Augusto Ruschi, Aracruz, Espirito Santo (ES); April 23rd-27th sampling in Reserva Michelin, Igrapiúna, Bahia (BA); May 2nd-5th sampling in Manaus, along Rio Negro via boat, State of Amazonas (AM); May 6th-13th sampling in Presidente Figueiredo, AM. Gaps in between collection periods were due to travel time between sites. The data cover a spatial scale of over 6,000 miles. Sites include field stations spanning four South American countries: Colombia, Ecuador, Peru, and Brazil, including four states within Brazil (SP, ES, BA, AM).

Data exclusions

Some spectrometer recordings were excluded as defined in the methodology hereafter. We determined the peak excitation and emission wavelength and intensity from each spectrometer recording and utilized these intensities to calculate a maximum percent biofluorescence emission:

(%BF=(intensity at peak emission λ)/(intensity at peak excitation λ)*100)

This provided our focal measure of proportion of excitation light re-emitted as fluorescence. All characterizations of spectrometer recordings were completed in R version 4.2.3 (R Core Team 2023). An ASCII file, containing intensity in photon counts for wavelengths ranging 200-1000nm, of each spectrometer recording was saved via the OceanView application at time of acquisition and later loaded into R for analysis. Spectra were smoothed via a fifteen-point moving average filter to reduce noise in the spectrum. Utilizing the photobiology package (Aphalo 2015), smoothed spectra were changed to an object of class spectra and normalized to the highest intensity (corresponding to the excitation light) using the normalize () function. We found peak excitation wavelengths by using the which.max() function and confining the parameters to only look for the maximum intensity within the wavelength range of the excitation light source. For example, finding the wavelength that corresponds to the highest intensity (photon count) within the wavelength range of 360-380 nm for UV excitation light, 400-415 nm for VI light, etc. Some of our excitation peak recordings were saturated (maximum >60,000 photon counts). If this was the case, the maximum intensity value available from the spectrometer recording (at 60,000 photons) was utilized. We expect data affected by this saturation would overestimate the intensity of the fluorescence emission recording, though this should not affect the overall significance of our findings because we estimate that saturation occurred at comparable levels across all excitation light sources. All peak emission wavelengths were found using the get_peaks() function from the photobiology package. As a fluorescent signal can produce multiple peaks, the get_peaks() function found all peaks at a wavelength greater than the longest wavelength of the excitation source (greater than 380 nm for UV, 400 nm for VI, etc.). Because fluorescence absorbs light and re-emits it at a longer wavelength, these peaks are all potential fluorescent signals under the respective excitation light. Within the get_peaks() function, we set the ignore_threshold to 0.01 to only collect peaks with a relative size greater than 1% as compared to the tallest (excitation) peak, and we set the span to 100 to define a peak as a datapoint within the wavelength sequence that had an intensity greater than those within a 50-count window on either side of the point. These parameters were chosen via trials adjusting parameters to find peaks of a subset of spectrometer recordings representing the diversity of emission spectra shapes (one emission peak close to the excitation peak, one peak far from excitation, two peaks, no peaks, etc.) and were chosen to reduce the probability that noise in the recording was defined as a peak. Additionally, to further assure that the chosen peaks were significant signals and not noise, any suspected emission peak with an intensity greater than the excitation peak was removed, as this was likely a sign of an inaccurate spectrometer recording. In these cases, and in any cases where no peaks were found, "NA" was input for the peak wavelength and intensity values. The intensity at each peak emission

wavelength was divided by the intensity at the peak excitation wavelength for the respective spectrometer recording to calculate the average percent of the excitation light that is re-emitted as fluorescence.

As stated, we took three spectrometer recordings at each location on each individual to have technical replicate measurements of the biofluorescent signal at that body location. The calculated percentages of biofluorescence emission for these three recordings were averaged for each body location. Because our data set contained spectrometer recordings from a different number of body locations, and often different specific body regions, from each individual (due to the variation in physical biofluorescent patterns that we were attempting to capture across a wide range of species), we only used the maximum percent biofluorescence emission (from any body region, under each of the five excitation light sources) for downstream analyses. Hence, for all analyses of variance, the unit of measurement used was the maximum percent of biofluorescence emission recorded from each individual.

We also examined the body location from which the maximum biofluorescent recording from each individual was taken. For this, we used the maximum percent biofluorescence emission (from any body region, under any light source). Utilizing the maximum fluorescent emission recording for each individual insured that only one body location per individual was being considered, although, examining the maximum biofluorescent emission recording for each individual under each excitation light source (as in the analyses above) produced similar percentages of fluorescence by body location. For easier comparison, the body regions were summarized into the following nine groups: cloaca, dorsal (including spectrometer recordings with a body location specified from any dorsal pattern), eye, facial pattern (including lip, spots under the eye, snout, etc.), flank, inguinal region, limb (including forelimbs, thigh, etc.), throat (including vocal sac), and ventral (including any ventral pattern). The percentage of maximum biofluorescent recordings from each of the nine body locations were calculated for each light source.

Reproducibility

In order to assess reproducibility within our own sample collection, we did the following during data collection. We utilized the live spectrometer recording acquisition feature of the OceanView computer application to determine if a specific location had a fluorescent signal (as defined by the presence of a visible peak at a longer wavelength than the excitation source). If any intensity peak was visible, or if it was questionable if a peak was visible, a spectrometer recording was taken with the probe held approximately two millimeters above that area of skin. We used a 1,000 ms integration time for each spectrometer recording and took three recordings at each location to have technical replicate measurements of the biofluorescent signal at that body location. We also attempted to capture and test multiple individuals of each species, when possible.

However, we did not attempt to fully repeat the data collection and analyses to verify reproducibility of the entire study.

Randomization

For all randomization tests, we ran a sensitivity analysis to determine at which effective sample size our statistical interpretations held. To do so, we changed the sample size of the randomization distribution and determined at which sample size, n, the null distribution of test statistic values was not significantly different than the observed test statistic, as defined above at $\alpha = 0.05$. The effective sample size for all significant randomization tests was n = 2, except for that test comparing the “green” peak fluorescent emission and twilight irradiance spectrum whose effective sample size was n = 13. Hence, our statistical interpretations stated in the Results section above hold as long as our data contains at least two and thirteen independent groups respectively. As we had representatives from 82 species within our sample size of 192 “green” peak individuals, this effective sample size is very likely, despite the unknown influence of phylogeny on anuran biofluorescence.

Blinding

As stated, all biofluorescent recordings were captured via spectrometer by CEW and RMB, with the setup as follows. CEW (wearing filer glasses corresponding to the specific excitation source) held the frog under the excitation source and positioned the fiber optic probe, while RMB examined the live acquisition and clicked the collection button on the interface to save the spectra data. CEW could not see the computer screen/live aquisition of the OceanView program during this process, so was blind to the biofluorescent information so as it did not affect the position of the probe to preferentially choose a location to record. Additionally, during data analysis Field number was the only individual identifier so as to be blind to the specific species, genus, family, location, etc.; this more specific specimen information was added to the dataset after the maximum percent biofluorescence emission values were calculated.

Did the study involve field work? ☒ Yes ☐ No

Field work, collection and transport

Field conditions

We collected individuals during night surveys on the trails surrounding each research station we visited.

Location

Our sampling sites include field stations in four South American countries: Colombia, Ecuador, Peru, and Brazil, including four states within Brazil (SP, ES, BA, AM). Data collection occurred from March to May of 2022, within the breeding season of most anurans at these sites. Our field collection schedule was as follows: March 2nd-10th at Yasuní Scientific Station, Ecuador; March 13th-21st at El Amargal Nature Reserve, Nuquí, Chocó, Colombia; March 24th-April 2nd at Los Amigos Conservation Hub (CIRCA), Peru; April 8th-13th sampling in Fazenda Michelin, Ubatuba, São Paulo (SP); April 17th-20th sampling in Estação Biológica Augusto Ruschi, Aracruz, Espirito Santo (ES); April 23rd-27th sampling in Reserva Michelin, Igrapiúna, Bahia (BA); May 2nd-5th sampling in Manaus, along Rio Negro via boat, State of Amazonas (AM); May 6th-13th sampling in Presidente Figueiredo, AM.

Access & import/export

We made every effort to access habitats, collect data, and export samples in a responsible manner and in compliance with local, national, and international laws.

Collections in Ecuador were made under the authority of the Ministerio de Ambiente, Agua y Transición Ecológica No. 2034. Vouchers were deposited in The Zoology Museum at the Pontifical Catholic University of Ecuador (QCAZ). Collections in Peru were made under the authority of the Ministerio de Agricultura y Riego No. 2022-0000809. Vouchers were deposited in the Museo de Historia Natural San Marcos (UNMSM). Collections in Colombia were made under the authority of the Permiso marco No. 1177 of 09 October 2014 granted by the Autoridad Nacional de Licencias Ambientales (ANLA) to the Universidad de los Andes (UniAndes). Relevant animal care and use protocols for amphibians were approved by the CICUA of UniAndes under POE 22-001 and vouchers were deposited in the Museo de Historia Natural C.J. Marinkelle at UniAndes. Collections in Brazil were made under the authority of

	the Brazil Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) No. 66597 to FGG via collaboration with the Instituto Butantan. Vouchers were deposited at the Instituto Butantan and registered in the SisGen system by time of publication. All data collection methods were approved by the IACUC under protocol IPROTO202100000007.
Disturbance	We minimized disturbance to the local flora and fauna by utilizing established trails around each research station for local collections when available. At times when mitigation of effects was not possible, disturbances caused by the study include capturing of local amphibian fauna and the effect of persons within the environment (potentially causing light disturbance from head lamps at night, noise disturbance, and/or disturbance to local flora when capturing specimen).

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	N/A
Wild animals	We collected individuals during night surveys on the trails surrounding each research station we visited. We captured individual amphibians by hand and placed them in a labeled Ziploc plastic bag with air, substrate, and water for transport back to the field station. Each individual was given a field number, and GPS coordinates were taken at the point of capture using a handheld Garmin GPS system. At the field station, all biofluorescent measurements were taken on the same night of capture. We tested each individual for fluorescence under five different excitation sources spanning a nearly 200nm wavelength range (365-460nm) using the Xite Fluorescent Flashlight System (NightSea). The five excitation wavelength ranges were as follows: UV – Ultraviolet (360 – 380nm), VI – Violet (400 – 415nm), RB – Royal blue (440 – 460nm), CY – Cyan (490 – 515nm), and GR – Green (510 – 540nm). We focused on this range of wavelengths to encompass and expand upon wavelengths of biofluorescent excitation used in previous studies (13–18) and to ensure that we did not miss any novel biofluorescent traits. The individual was held by hand beneath the light source, which was suspended above the organism via a YSLIWC Gooseneck Tripod stand. We obtained biofluorescent emission spectra for each individual by utilizing a Maya2000 Pro Series UV-VIS Portable Spectrometer with its attached fiber optic cable held above the surface of the amphibian's skin while beneath the excitation light. This instrument provided information on the emission spectra and intensity of any biofluorescent signals from the frog (200nm-1000nm) in response to each of the five excitation wavelengths. NightSea barrier filter glasses matching the respective excitation source were utilized to view and identify potential locations of biofluorescence to test on the body of the amphibian. We utilized the live spectrometer recording acquisition feature of the OceanView computer application to determine if a specific location had a fluorescent signal (as defined by the presence of a visible peak at a longer wavelength than the excitation source). If any intensity peak was visible, or if it was questionable if a peak was visible, a spectrometer recording was taken with the probe held approximately two millimeters above that area of skin. We used a 1,000 ms integration time for each spectrometer recording and took three recordings at each location to have technical replicate measurements of the biofluorescent signal at that body location. In addition, we photographed patterns of biofluorescence under each light source, as well as under a full-spectrum headlamp light source, using a Nikon digital camera and Tiffen camera filters matching the respective excitation source (as specified by NightSea). Along with these quantitative measurements, we recorded qualitative descriptions of the color of skin and the color of fluorescence. The following morning, we euthanized each individual, determined species and sex via dissection, collected tissues, and prepared samples for museum accession. Individual species IDs were determined via region-specific field guides and knowledge of local collaborators. All specimen capture and sample acquisition followed appropriate permit requirements for the specific site (see collection section above).
Reporting on sex	Data on sex was collected, though sex was not considered in the study design or data analysis. Sex-based analysis was not performed due to limited confidence of sex determination of most individuals after dissection. Further genetic work is needed to confirm sex of individuals before that trait could be included in the analysis.
Field-collected samples	We captured individual amphibians by hand and placed them in a labeled Ziploc plastic bag with air, substrate, and water for transport back to the field station. At the field station, and on the same night of capture, we tested each individual for fluorescence. After data collection, specimen were returned to their Ziploc bag, water was refilled if necessary, and then the individual was euthanized the next morning. For a few individuals in Colombia, as specified by the local permitting, individuals were toeclipped and

	then released the following day to the exact field location of collection (by GPS data point).
Ethics oversight	Ethical approval and guidance was completed by the permitting authorities. Collections in Ecuador were made under the authority of the Ministerio de Ambiente, Agua y Transición Ecológica No. 2034. Collections in Peru were made under the authority of the Ministerio de Agricultura y Riego No. 2022-0000809. Collections in Colombia were made under the authority of the Permiso marco No. 1177 of 09 October 2014 granted by the Autoridad Nacional de Licencias Ambientales (ANLA) to the Universidad de los Andes (UniAndes). Relevant animal care and use protocols for amphibians were approved by the CICUA of UniAndes under POE 22-001. Collections in Brazil were made under the authority of the Brazil Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) No. 66597 to FGG via collaboration with the Instituto Butantan. All data collection methods were approved by the IACUC under protocol IPROTO202100000007.

Note that full information on the approval of the study protocol must also be provided in the manuscript.