

Supplementary Data

****All supplementary Tables listed below are found in “Supplementary Tables.xlsx”****

Supplementary Data 1. Results of previous and current studies testing anurans for biofluorescence. The excitation light source wavelength, resulting biofluorescence (Y/N), and source for documented tests of frog biofluorescence in the literature, by anuran family, genus, and species. The list includes species we tested for the first time as well as those we re-tested because they were previously examined under only a subset of excitation wavelengths (*Boana cinerascens*, *Boana lanciformis*, *Dendropsophus rhodopeplus*, *Dendropsophus sarayacuensis*, *Phyllomedusa tarsius*, and *Phyllomedusa vaillantii*).

Supplementary Data 2. Species and individuals meeting all criteria for ecological significance of their fluorescent signal. A total of 194 individuals from 86 species across the anuran tree (56.58% of species tested) meet all four criteria. These are the species in which the function of fluorescence for intraspecific communication should be explored, with specific focus on testing for the role of visual communication in those species where this is unknown.

Supplementary Data 3. Pairwise comparison of percent biofluorescent emission by excitation light source. This Pairwise Dunn’s test was performed to determine if fluorescence intensity differed by excitation source. Dunn (1964) multiple comparison p-values were adjusted with the Holm method for each excitation light source wavelength: UV – Ultraviolet (360-380 nm), VI – Violet (400-415 nm), RB – Royal blue (440-460 nm), CY – Cyan (490-515 nm), and GR – Green (510-540 nm). An “*” indicates significance at $\alpha = 0.05$. All pairwise comparisons of percent biofluorescent emission are significantly different except for the biofluorescent emission produced by Ultraviolet and Green light.

Supplementary Data 4. Maximum percent biofluorescent emission by family from each individual under each light source. The excitation light sources are as follows: UV – Ultraviolet (360-380 nm), VI – Violet (400-415 nm), RB – Royal blue (440-460 nm), CY – Cyan (490-515 nm), and GR – Green (510-540 nm). These data are plotted in Fig.1.

Supplementary Data 5. Maximum percent biofluorescent emission by individual. The maximum percent of biofluorescence emission measurements from each individual under each light source. Excitation light sources are as follows: UV – Ultraviolet (360-380 nm), VI – Violet (400-415 nm), RB – Royal blue (440-460 nm), CY – Cyan (490-515 nm), and GR – Green (510-540 nm).

Supplementary Data 6. Maximum biofluorescent emission recording count by body location and excitation light source. The body location from which the maximum biofluorescent emission recording from each individual was taken, separated by excitation light source (the maximum fluorescent emission recording under *any* light source). The excitation

light sources are as follows: UV – Ultraviolet (360-380 nm), VI – Violet (400-415 nm), RB – Royal blue (440-460 nm), CY – Cyan (490-515 nm), and GR – Green (510-540 nm). 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 forelimb, thigh, etc.), throat (including vocal sac), and ventral (including any ventral pattern). The percentage of maximum biofluorescent emission recordings from each of the nine body locations were calculated for each light source.

Supplementary Data 7. Maximum biofluorescent emission recording under each excitation light source by body location. The body location from which the maximum biofluorescent emission recording from each individual, under each excitation light source, was taken (the maximum fluorescent emission recording under *each* light source). The excitation light sources are as follows: UV – Ultraviolet (360-380 nm), VI – Violet (400-415 nm), RB – Royal blue (440-460 nm), CY – Cyan (490-515 nm), and GR – Green (510-540 nm). 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 forelimb, thigh, etc.), throat (including vocal sac), and ventral (including any ventral pattern).

Supplementary Data 8. Assessment of Criteria 1–3 for diurnal individuals in daytime conditions under each light environment and photoreceptor visual sensitivity condition. Bolded values indicate significance at $\alpha=0.05$. None of the fluorescent emissions meet any of the criteria. Sample sizes are extremely small, hence future studies should explore these correlations further with increased sampling.

Supplementary Data 9. Ancestral State Reconstruction of Percent Biofluorescent Emission of Amphibian Basal Node by Excitation Source (estimated via Phytools from data in Fig. S7). Estimates of percent biofluorescent emission at the basal node of the amphibian clade from 1,000 mcmc generations of ancestral state reconstruction are in order of excitation source, left to right: UV – Ultraviolet (360-380 nm), VI – Violet (400-415 nm), RB – Royal blue (440-460 nm), CY – Cyan (490-515 nm), and GR – Green (510-540 nm). Data comparisons are presented in Fig. S8; Blue excitation (440-460nm) produced a greater percent fluorescent emission than any other excitation light source in 85.25% of the percent biofluorescent emission trait estimates for the most recent common ancestor of the amphibian clade.

Supplementary Data 10. Species and individuals with blue light-induced orange fluorescence. These are the species in which the function of should be explored, with specific focus on testing for the role of fluorescence in both intraspecific communication and crypsis signaling.

Supplementary Data 11. Average percent biofluorescent emission for each species in the tree, by excitation source.

Supplementary Code 1.

Supporting material for publication submitted to Nature Communications. We conducted tests to assess the variability of amphibian biofluorescence and provide insight into its potential ecological significance. This dataset includes R Code and relevant raw data for all statistical analyses conducted. The order of the scripts in this file, follows the order of analyses run and their presentation in the manuscript. All scripts were run utilizing R v4.2.3.

Description of the Data and file structure

MainManuscriptScript.R: code for (1) spectrometer analysis, (2) Kruskal-Wallis one-way analysis of variance test and Pairwise Dunn's tests for assessing differences in excitation and emission of biofluorescence by excitation light source (graphs for Fig. 1, Fig. 3, and Supp. Fig. 1 in manuscript), (3) visualization of criterion 4 of ecological significance, and (4) visualization of all blue-light-induced spectrometer recordings for graph in Fig. 2 of manuscript

ALLindivmax_dat.csv: input for MainManuscriptScript.R, the maximum fluorescent emission recording for each individual under each light source

MAXindivmax_dat.csv: input for MainManuscriptScript.R, the maximum fluorescent emission recording for each individual under any light source

RandTest.R: code for randomization tests for criteria 2 and 3 of ecological significance of biofluorescence

Rod and Spectra.csv: input for RandTest.R, rod spectra from Yovanovich et al. 2017 and twilight spectra digitized with permission from Cronin et al. 2014

Individual_Data.csv: input for RandTest.R, individual metadata (species, genera, family, etc. by field number)

Individ_em_wv_round_less550.csv: input for RandTest.R, "green" emission peak data

Individ_em_wv_round_greater550.csv: input for RandTest.R, "orange" emission peak data

Additions Post Revisions

RandTest_UpdatedFromRevisions.R: code for randomization tests for criteria 1, 2, and 3 of ecological significance of biofluorescence updated post revisions. Plots for Figures 4 and 5.

Individ_em_wv_round_green_eitherpeak.csv: input for RandTest_UpdatedFromRevisions.R, "green" emission peak data including first and second green peaks from emission data (to account for bi-modal emission spectra)

Individ_em_wv_round_orange_eitherpeak.csv: input for RandTest_UpdatedFromRevisions.R, "orange" emission peak data including first and second orange peaks from emission data (to account for bi-modal emission spectra)

ALLindividmax2_dat_RB.csv: input for MainManuscriptScript.R taking into account bi-modal peaks under RB excitation. The maximum fluorescent emission recording of the first two peaks for each individual under RB (440-460 nm) light source

Phylogenetic Correction Analyses

AncStateRec.R: code for Ancestral State Estimation of biofluorescent emission intensity by excitation wavelength

BF_species_NEXUS.nex: input for AncStateRec.R, time calibrated phylogeny for all species in the analysis available on and downloaded from Timetree.org

AvgInt_UV.csv, AvgInt_VI.csv, AvgInt_RB.csv, AvgInt_CY.csv, AvgInt_GR.csv: input files for AncStateRec.R, average biofluorescent emission intensity by species under each excitation wavelength (UV, VI, RB, CY, GR).

##RevBayes scripts and data files: constrained optima under Ornstein-Uhlenbeck evolution analyses

mcmc_OU_MODIFIED.REV: code for constrained optima under Ornstein-Uhlenbeck evolution analyses

pruned_nexustree_greens.nex, pruned_nexustree_oranges.nex: input files for mcmc_OU_MODIFIED.REV, trees pruned from BF_species_NEXUS.nex to included species for which we have blue light-induced green and orange fluorescent emission data respectively.

BF_speciesTrimmed_MaxEmissionGreenSpecies.nex,
BF_speciesTrimmed_MaxEmissionOrangeSpecies.nex: input files for mcmc_OU_MODIFIED.REV, character files of fluorescent emission wavelength of blue light-induced green and orange fluorescent emission data respectively, averaged by species from Individ_em_wv_round_green_eitherpeak.csv and Individ_em_wv_round_orange_eitherpeak.csv datafiles respectively.

AIC.R: code for comparison of average likelihood from RevBayes output files for the constrained optima Ornstein-Uhlenbeck evolution models under each condition