Naturally occurring fluorescence in frogs

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Fluorescence, the absorption of short-wavelength electromagnetic radiation reemitted at longer wavelengths, has been suggested to play several biological roles in metazoans. This phenomenon is uncommon in tetrapods, being restricted mostly to parrots and marine turtles. We report fluorescence in amphibians, in the tree frog *Hypsiboas punctatus*, showing that fluorescence in living frogs is produced by a combination of lymph and glandular emission, with pigmentary cell filtering in the skin. The chemical origin of fluorescence was traced to a class of fluorescent compounds derived from dihydroisoquinolinone, here named hyloins. We show that fluorescence contributes 18–29% of the total emerging light under twilight and nocturnal scenarios, largely enhancing brightness of the individuals and matching the sensitivity of night vision in amphibians. These results introduce an unprecedented source of pigmentation in amphibians and highlight the potential relevance of fluorescence in visual perception in terrestrial environments.

Amphibia | Anura | Hylidae | visual ecology | fluorophore

Fluorescence occurs when short-wavelength electromagnetic radiation is absorbed and then reemitted at longer wavelength. This phenomenon is broadly distributed in marine and terrestrial environments and is found in distantly related organisms (1). Among aquatic vertebrates, fluorescence is widespread phylogenetically within cartilaginous and ray-finned fishes (2) and has been documented in sea turtles (3), whereas among terrestrial vertebrates, it is only known to occur in parrots (4). With few exceptions (5–7), the molecular basis of most of those reports remains unstudied. Many roles have been suggested for fluorescence in animals, such as photoprotection (8), antioxidation (9), and visual communication (10–14).

Amphibians (frogs, toads, salamanders, newts, and caecilians) have a wide range of skin coloration (15) caused by an integumental pigmentary system in which the combination of different types of chromatophore cells create coloration through the integration of chemical and structural features (16). Although the chemical nature and distribution of chromophores has been studied (16), fluorescence has not been reported in any of the 7,600 species of amphibians (17). Here we report a case of fluorescence in this highly diverse group, introduce a class of fluorescent compounds, and assess its importance by quantifying its contribution to overall coloration under natural light conditions.

Results and Discussion

Fluorescence in *Hypsiboas punctatus*. The South American tree frog *H. punctatus* (Family Hylidae) is unusual among amphibians in possessing a translucent skin, a crystal-containing layer in the peritonea and bladder, and a high concentration of biliverdin in lymph and tissues. We observed that living adults and juveniles illuminated with UV-A blue light produced a bright blue/green fluorescent emission (Fig. 1 *A*–*C*) that was clearly discernible from the body surface of the specimens. To characterize the fluorescence of frogs, we recorded excitation-emission matrices from the dorsum of living adults of both sexes (Fig. 1*D*). Both spectral profiles presented excitation maxima of 390–430 nm and emission maxima at 450–470 nm (blue), with a shoulder at 505–515 nm (green) giving an overall cyan coloration and showing no evident sexual dichromatism.

Anatomical Origin of Fluorescence. Because the skin is seemingly translucent in this species (*SI Appendix*, Fig. S1*A*), we evaluated the contribution of different tissues to fluorescence emission. For this purpose, we recorded fluorescence spectra of isolated skin with absorbed lymph (S+L), yellow glandular secretions obtained through mild electrical stimulation (gl), and three subcutaneous structures. These included the blue green lymph present in lymph sacs (L), muscles (M), and a white underlying connective tissue containing guanine crystals in the dorsal musculature (crystal-containing layer; Fig. 2*A*, *Upper Left* and *SI Appendix*, Fig. S1*B*). All structures showed fluorescence maxima that matched the blue emission peak of living specimens, but only skin (S+L) samples showed the characteristic green shoulder of intact animals (Fig. 2*A*, *Upper Right*). Despite the apparent translucency of the skin, light transmittance at 390–430 nm was less than 10% (Fig. 2*A*, *Lower Left*). A quantitative estimation of the contribution of the different layers to the fluorescent emission (*SI Appendix*, Fig. S1*B*) revealed that muscles and crystal-containing layer accounted for less than 1% of total emission at 400 nm, indicating that the observed fluorescence is mainly a result of the emission of the skin with absorbed lymph (Fig. 2*A*, *Lower Right*).

Detailed images of skin using confocal microscopy of transverse sections revealed that excitation at 405 nm caused emission in the

Significance

In this interdisciplinary study, we report naturally occurring fluorescence in amphibians; specifically, in a common South American tree frog. We show that fluorescence is traceable to a class of compound that occurs in lymph and skin glands. Our study indicates that in our model species, in low-light conditions, fluorescence accounts for an important fraction of the total emerging light, largely enhancing brightness of the individuals and matching the sensitivity of night vision in amphibians. These findings open an exciting perspective into frog visual physiology and ecology and into the role of fluorescence in terrestrial environments, where classically it has been considered irrelevant.

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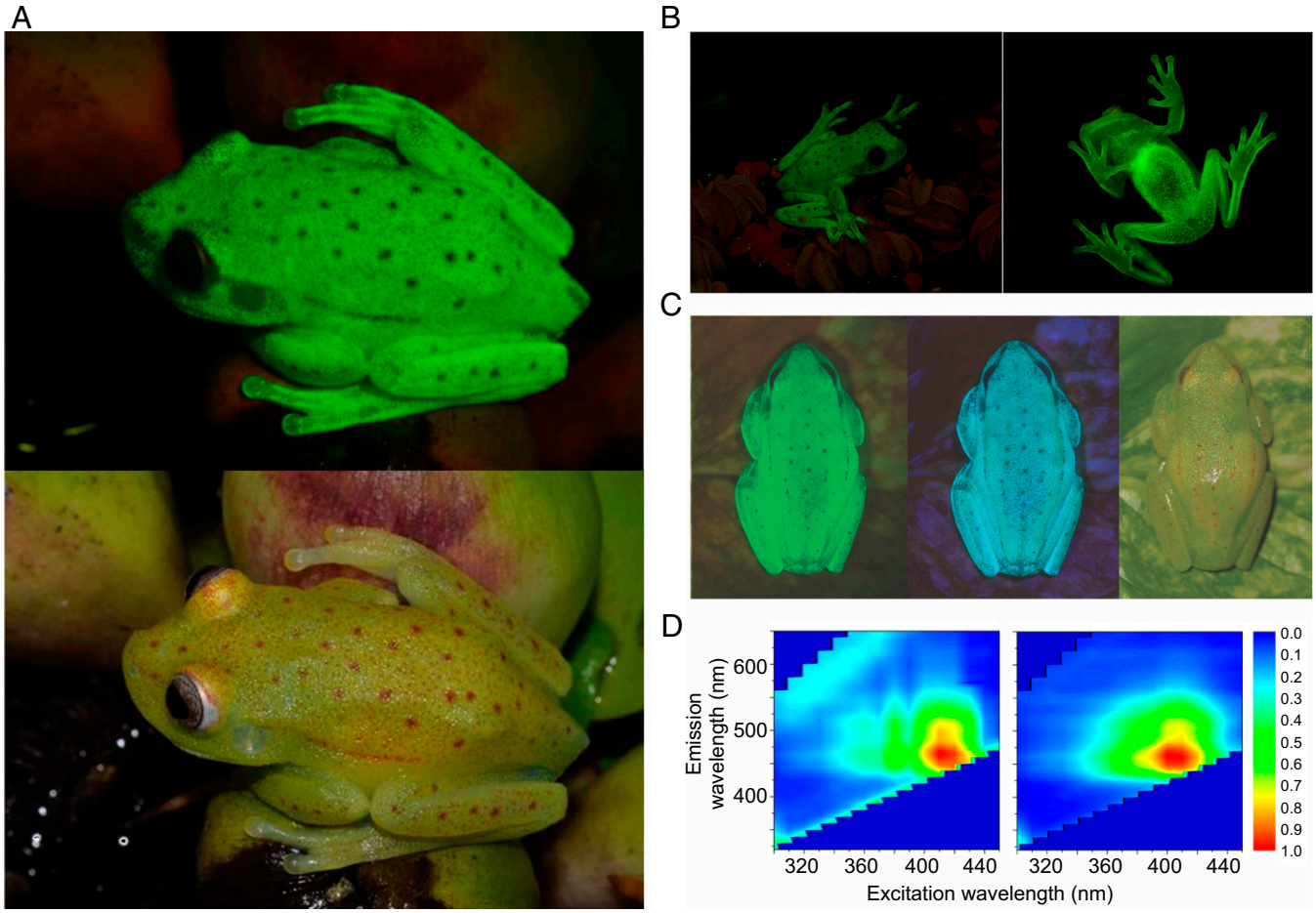

Fig. 1. Fluorescence in the tree frog *H. punctatus*. (*A*) Adult male under UV-blue light (400 nm; *Upper*) and white light (*Lower*). (*B*) Fluorescence of dorsum (*Left*) and venter (*Right*) of a male. (*C*) Female under UV blue light excitation (400 nm) and long-pass emission filters (*Left*: 435 nm; *Middle*: 516 nm), or under white light and no emission filter (*Right*). (*D*) Normalized representative excitation-emission matrices of the dorsal surfaces of female (*Left*) and male (*Right*) specimens. Maximum emission signal was detected at 460–470 nm with a shoulder at 510 nm and corresponded to an excitation maximum of 390–430 nm. Photos in *B* were taken with a band-pass excitation filter attached to the flash and a long-pass emission filter (516 nm) attached to the lens.

430–490-nm bandwidth in the epidermis, dermal connective tissue, and glands (Fig. 2*B*, *Upper* and *SI Appendix*, Fig. S1*C*). This is in stark contrast to other hylid species examined, in which no fluorescence was detected under the same experimental conditions, and faint fluorescence was only observed from the interface of the strata spongiosa and compacta of the dermis (pteridine layer) (18) when laser power was enhanced 15-fold (Fig. 2*B*, *Lower* and *SI Appendix*, Fig. S1*D* and *Discussion*). Skin chromatophores located immediately below the epidermis in *H. punctatus* did not show fluorescence in the blue bandwidth analyzed (Fig. 2*B*, *Upper* and *SI Appendix*, Fig. S1 *C* and *E* and *Discussion*). As this layer includes yellow pigments mainly found in xanthophores (16) (*SI Appendix*, Fig. S1 *F* and *G*), we recorded transmittance spectra from both intact and lymph-washed skin and assessed its effect on fluorescence emission. The resulting filtered glandular fluorescence spectrum (Fig. 2*C*, *Right* and *SI Appendix*, Figs. S1 *I* and *H*) revealed a clear attenuation and showed a shape that matches that of living specimens and skin with lymph, with its characteristic green shoulder (Fig. 2*A*, *Upper Right*). Accordingly, glandular ducts, which are not attenuated by chromatophores, fluoresced more brightly than adjacent tissue (Fig. 2*C*, *Left*).

Chemical Source of Fluorescence. To elucidate the chemical nature of fluorescence, we extracted and purified water-soluble fluorophores from lymph and skin interstitial tissue, epidermis, and multicellular exocrine skin glands and analyzed them by HPLC-Ion Trap-MS, assisted by TOF-MS, MS/MS-based fragmentation, and 1D-2D NMR spectroscopies (*SI Appendix*, Fig. S2 *A* and *B*). We isolated a major fluorescent compound from lymph with molecular formula $C_{22}H_{31}NO_4$, as determined by TOF-MS data (molecular weight accuracy <5 ppm; Fig. 3*A*, peak H-L1, and *SI Appendix*, Fig. S2*B*). We designated this molecule as Hyloin-L1. The Hyloin-L1 fluorescence profile (Fig. 3*B*) closely matched the blue emission maximum of lymph, skin, and living specimens (Fig. 2*A*, *Upper Right* and *SI Appendix*, Fig. S2*C*) and exhibited solvatochromicity (Fig. 3*B*). Combined 1D and 2D NMR experiments allowed a full structural elucidation, revealing an *N*-methyl-dihydroisoquinolinone core, with an unsaturated C_{11} fatty acid and a methoxy group as substituents of the aromatic ring (Fig. 3*C* and *SI Appendix*, Figs. S3 and S4). A minor, related compound with molecular formula $C_{22}H_{29}NO_4$, Hyloin-L2, was also isolated (Figs. 3*A*, peak H-L2, and 3*C* and *SI Appendix*, Figs. S2 *A* and *B* and S5) and exhibits the same fluorescence profile (*SI Appendix*, Fig. S5*D*) as Hyloin-L1. The identification of additional hyloin analogs was assisted with molecular networking (19), which grouped related parent ions in different clusters based on similarities in MS/MS fragmentation patterns. The resulting clusters allowed the identification of compounds of the homologous series based on systematic fragmentation studies considering the MS/MS spectra of the isolated hyloins (Fig. 3*D* and *SI Appendix*, Figs. S5*D*, S6*A*, S7, and S8). Several variants involving

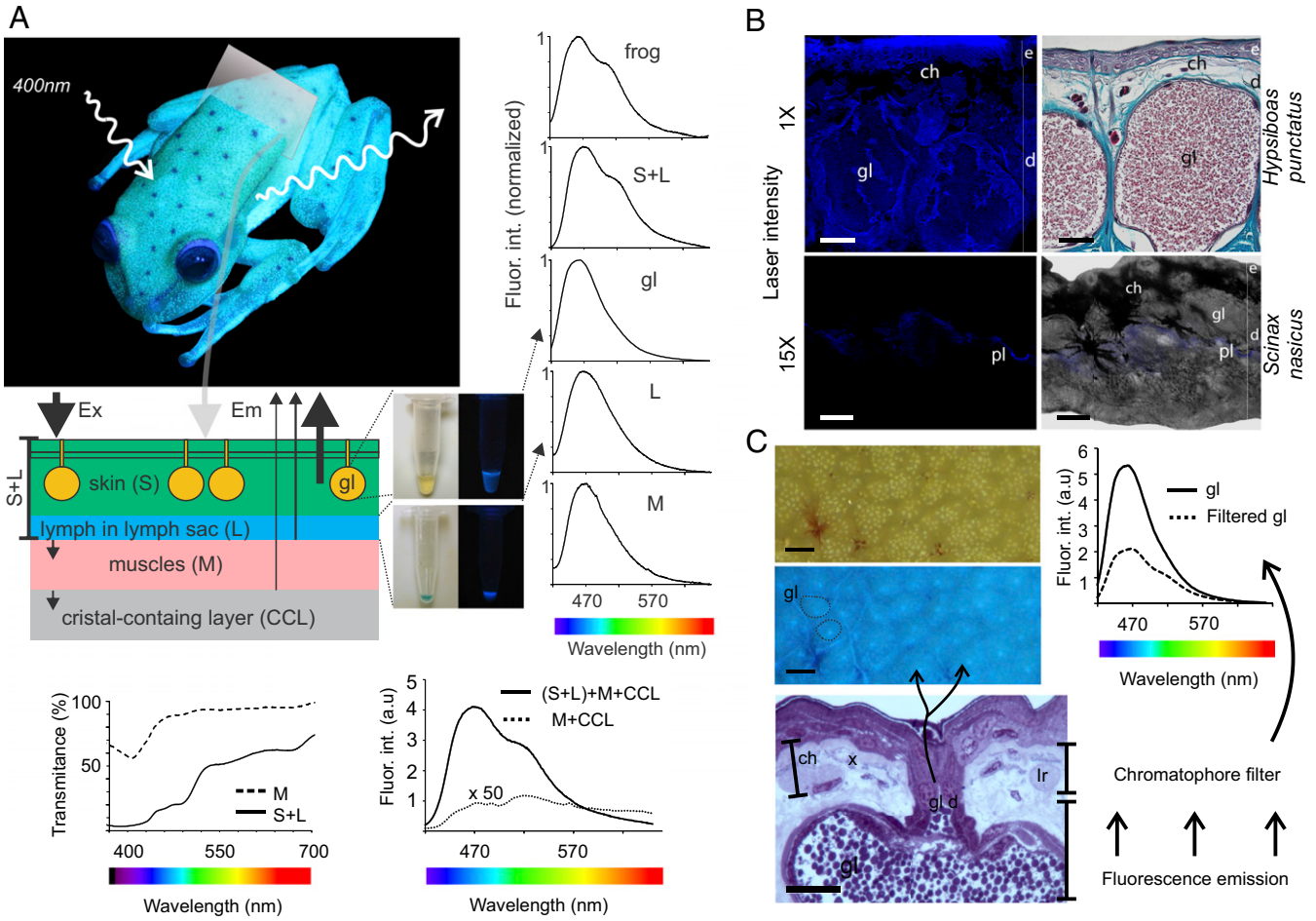

Fig. 2. Anatomy of fluorescence in *H. punctatus*. (*A*) Fluorescence is observed in the skin and isolated subcutaneous structures. Incident excitation light and fluorescence emission from each tissue layer are attenuated by the structures above and depend on the transmittance of each layer (*Lower Left*). Fluorescence from subcutaneous structures is almost completely filtered by skin with lymph (*Lower Right*). (*B*) Transverse sections of dorsal skin of *H. punctatus* and *Scinax nasicus*. (*Left*) Confocal images of fresh samples using a 405-nm laser line. (*Right*) Stained histological section of *H. punctatus* and unstained sections of *S. nasicus* superimposed to confocal image. Fluorescence emission in *H. punctatus* is observed from epidermis (e), dermis (d), and glands (gl), whereas in *S. nasicus*, it is restricted to the pteridine layer (pl) of the dermis. No fluorescence is detected from chromatophores (ch). (Scale bar, 50 μm.) (*C*) Chromatophores lie immediately beneath epidermis, as seen in the semithin skin section (*Lower*). (Scale bar, 20 μm.) They impart coloration to skin (*Upper*, stereomicroscope image of living specimen) (Scale bar, 150 μm.) Fluorescence emission from glands and dermis is filtered by the chromatophores, and hence fluorescence intensity is attenuated mainly in the blue region. A green shoulder is observed in the filtered emission, as in living frogs. Chromatophore attenuation is evident, as unattenuated glandular ducts (gl d) fluoresce more brightly than the rest of the gland (gl; *Middle*) (Scale bar, 150 μm.)

different lengths (10–12) and degrees of unsaturation (0–2) of the side chain (Fig. 3*D*) were detected in crude lymph and epidermal samples (*SI Appendix*, Fig. S2 *A* and *B*). Analyses of glandular secretions revealed the presence of the compounds extracted from lymph, but as amide derivatives with the amine agmatine (Hyloin-G1, for glandular; Fig. 3*D* and *SI Appendix*, Figs. S2*B*, S6*A*, and S9). Glandular secretions from other hylid species did not include the novel compounds described as indicated from molecular network results (*SI Appendix*, Fig. S6*B*), and no UV-A absorbing molecules were detected under the same HPLC-diode array detector conditions used to analyze *H. punctatus* secretions (Fig. 3*E* and *SI Appendix*, Fig. S6*C*). Electronic structure calculations suggest that an intramolecular interaction accounts for a very polar structure, consistent with the observed high aqueous solubility of these new molecules (*SI Appendix*, Fig. S10 and *Discussion*).

Fluorescence and Total Emerging Light. The biological relevance of fluorescence in *H. punctatus* from a visual ecology perspective depends on the quantitative contribution of fluoresced photons to the total emerging light (fluoresced + reflected photons). Two factors would increase this contribution: a large fluoresced photon flux that depends on the quantum yield (Φ_f) of the frog, and a low reflected photon flux in the spectral range that matches fluorescence emission, which depends on reflectance properties of the skin. Furthermore, these two factors also depend on the environmental light availability, which increases the fluorescence when there is a sufficiently large ratio between ambient light irradiance in the spectral excitation and emission ranges of the fluorophores present in the tree frog. We empirically determined a Φ_f value of 0.12 ± 0.03 at 400 nm and developed a methodology to quantify fluorescence emission at other excitation wavelengths (*SI Appendix*, Fig. S11 *A* and *B*). The reflectance of *H. punctatus* for the 420–550-nm range (where most fluorescent photons are emitted; Fig. 1*D*) is relatively low (less than 8% in the blue part of the spectrum; Fig. 4*A*). As *H. punctatus* is a crepuscular and nocturnal species (20), we estimated the contribution of fluorescence to the total emerging light under twilight, moonlight, and moonless night irradiances (21). Given our results and the ambient irradiance distribution, our calculations show that fluorescence contributes from 18.5 ± 2.6% in a full moon night to 29.6 ± 3.2% during twilight (Fig. 4*B* and *SI Appendix*, Fig. S11*C*).

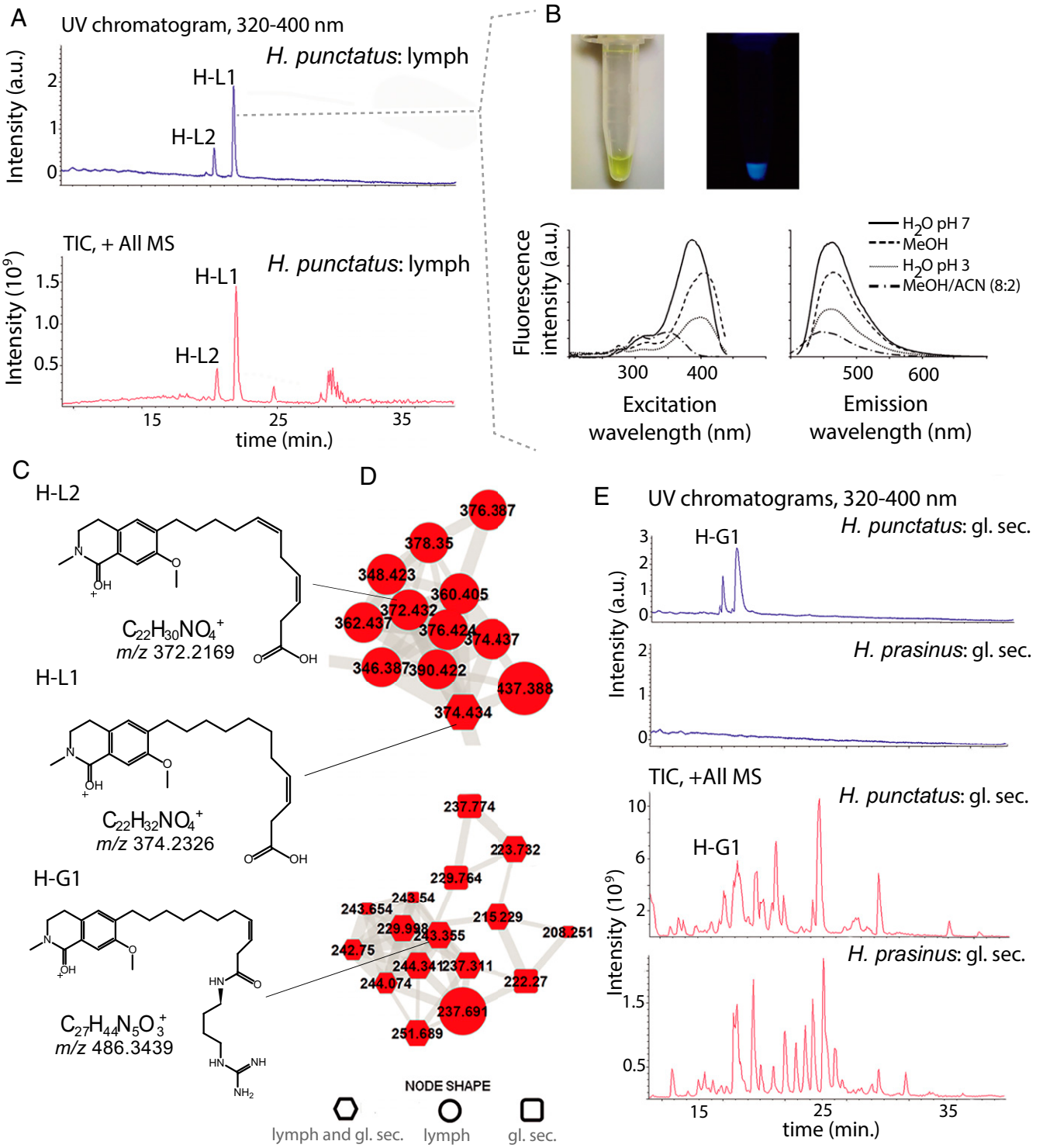

Fig. 3. Novel natural fluorophores present in *H. punctatus*. (*A*) HPLC profile of partially purified subcutaneous lymph showing peaks corresponding to Hyloin-L1 (H-L1) and Hyloin-L2 (H-L2). Monitoring was performed by UV detection in the range 320–400 nm and by total ion current (TIC). (*B*) Purified Hyloin-L1 at neutral pH under white (*Upper Left*) and UV (*Upper Right*) light, showing characteristic fluorescence. The compound is highly solvatochromic (*Lower*) and matches the living animals' fluorescence emission spectra at neutral pH. (*C*) Structures of two hyloins identified from lymph by combination of NMR and MS/MS data. Hyloin-G1 (H-G1; G stands for glandular), found in glandular secretions is an amide derivative of Hyloin-L1. (*D*) Molecular network of hyloin clusters based on LC-MS/MS (ESI+) spectral similarities. Many hyloin variants are detected either in its acidic (*Upper*) or in its amide derivative form (*Lower*). (*E*) HPLC profile of glandular secretions from *H. punctatus* and another hylid, *H. prasinus,* monitored as in *A*. No compounds with detectable absorbance in this region could be traced in the other hylid species studied.

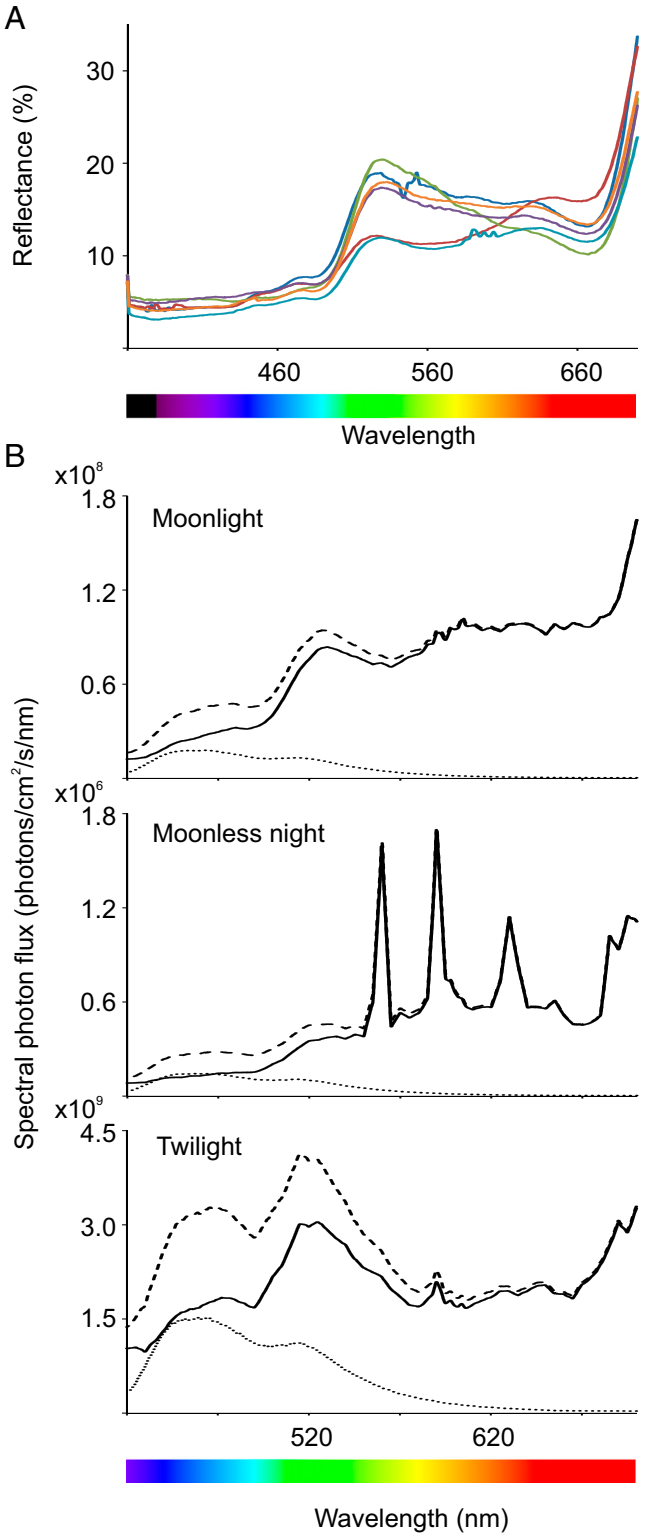

Fig. 4. Contribution of fluoresced photons to total emerging light in *H. punctatus*. (*A*) Reflectance spectra of the dorsal surface of six specimens. (*B*) Spectral photon flux (photons/cm^2/s/nm) emerging from the dorsal surfaces of one of the specimens under three different natural illuminants. Reflected light (solid line; reflectance × irradiance for every λ), fluoresced light (dotted line; calculated with the empirical quantum yield and the methodology described in *SI Appendix*), and the sum of both components (dashed line) show a large contribution of fluorescence to the total amount of photons for all the analyzed scenarios. Maximum contribution corresponds to the blue/green range (420–550 nm).

Fluorescence and Visual Perception in Terrestrial Environments. Even though the visual system of *H. punctatus* has not been studied, two retinal rod classes involved in nocturnal scotopic vision (under low-light conditions) have been identified in several amphibian species and studied thoroughly in the Green tree frog *Hyla cinerea* (22). The close match between scotopic spectral sensitivity in this species (maxima at 435 nm and at 503 nm) and fluorescence emission spectra in *H. punctatus* indicates that fluorescence could contribute to the visual perception of individuals, enhancing brightness by converting irradiance from the UV-blue portion of the spectrum, where visual sensitivity is low, to longer wavelength emission, where sensitivity is higher. This information suggests that fluorescence is a component of the pigmentary system in *H. punctatus*, constituting a novel extrachromatophore source of coloration. Quantitative estimations of fluorescence contribution to total emerging light in other fluorescent organisms under natural irradiances are limited to marine organisms (23), in which spectral distribution of the ambient illumination is nearly monochromatic. Although fluorescence has been suggested to be irrelevant in terrestrial environments (24), our quantitative results are striking, in that they show that fluorescence could be an important component of total emerging light in the chromatically more complex terrestrial environments as well.

A survey indicates that seven anuran amphibian families (Arthroleptidae, Centrolenidae, Hemiphractidae, Hylidae, Hyperoliidae, Mantellidae, Rhacophoridae) include at least some species having similar characteristics to *H. punctatus* (translucent skin, a crystal-containing layer in the peritonea and urinary bladder, and a high concentration of biliverdin in lymph and tissues) and should be tested for fluorescence (*SI Appendix*, *Discussion*). Our report of fluorescence in *H. punctatus* and its role in frog coloration raises an exciting perspective on the study of the molecular origin, evolution, and relevance of fluorescence in amphibian visual perception and biology.

Materials and Methods

Adult specimens of the tree frog *H. punctatus* were collected at night in the outskirts of the city of Santa Fe, Santa Fe, Argentina. Other species included in different parts of this study were *Aplastodiscus leucopygius*, *Aplastodiscus perviridis*, *Hypsiboas prasinus*, *Hypsiboas raniceps*, and *Scinax nasicus*. Collection permits were issued by Secretaria de Medio Ambiente, Ministerio de Aguas, Servicios Públicos y Medio Ambiente, Province of Santa Fe, Argentina (021-2011 and 063-2013), Ministerio de Ecología, Province of Misiones, Argentina (010-2015), and Instituto Chico Mendes de Conservação da Biodiversidade/SISBIO (Permit 50071-1). All procedures involving animals were carried out according to the regulations specified by the Institutional Animal Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (Res C/D 140/00), and those specified by Conselho Nacional de Controle de Experimentação Animal, Ministério da Ciência, Tecnologia e Inovação, Brazil.

The fluorescent compounds were isolated and purified from lymph and dermal glands, using published chromatographic methods. Chemical characterization was performed by NMR, mass spectrometry, and electronic structure calculations. Fluorescence measurements were performed using a steady-state spectrofluorometer (QuantaMaster; Photon Technology International) on specimens of *H. punctatus* held immobilized, and on specific dissected tissues and glandular secretions. Diffuse reflectance and transmittance measurements were performed by means of a spectrophotometer (UV3101PC; Shimadzu) equipped with an integrating sphere (ISR-3100; Shimadzu). The fluorescence quantum yield (Φ_f) of intact adult specimens was estimated using a published methodology. Fluorescence influence on total emerging light under two nocturnal and one twilight scenarios were taken into account using published irradiance data (21). See *SI Appendix* for a full description of methods.

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