101 years of biofluorescent animal studies: trends in literature, novel hypotheses, and best practices moving forward

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ABSTRACT

Biofluorescent animals have become a recent trend in natural history publishing. While the functions and origins of biofluorescence in fauna remains somewhat controversial, trends in biofluorescence throughout published literature may elucidate these concepts. Here we review 101 years of biofluorescent studies encompassing 108 published papers and 977 unique species records. Our results provide insights into areas of improvement that should be made moving forward in biofluorescent studies and hypotheses to be tested. Collated records of biofluorescent indicate that fluorescence is strongly associated with nocturnal and arboreal lifestyles. Our reconstruction of ancestral lineages based on biofluorescent species records indicate a potential origins and trends in evolution of biofluorescence around the Middle Miocene Climate Optimum (MMCO) where fluorescent color diversity proliferates with declining global temperatures and could possibly be indicative of a widespread climate relict among modern taxa; we therefore term this new concept the Biofluorescent Climate Relict Hypothesis (BCRH).

Key words: Biofluorescence, Evolution, Middle Miocene Climate Optimum, Climate Relict Hypothesis, Color

INTRODUCTION

Biofluorescence is a form of photoluminescence which occurs when part of a living creature absorbs and emits radiation in the form of light energy at a longer wavelength. Although unapparent to the naked human eye, biofluorescence can be detected using a UV-A light source with a range of 320-390 nm, the most commonly used being 365 nm. Although biofluorescence was reported as early as the sixteenth century by European observers (Lagorio, et al. 2015), the first biofluorescent animal publications did not occur until the 1920s with the examination of lepidopterans (Mottram and Cockayne 1920; Cockayne 1924). Substantial developments of interest in the study of biofluorescent phenomena did not take hold until the early 1990s with the use of Green Fluorescent Proteins in molecular biology (Reise, et al. 2009) and only recently has it been of international interest across the taxonomic spectrum (Lagorio, et al. 2015; Jeng 2019).

While all major taxonomic groups have been shown to encompass species that exhibit biofluorescence to some extent, there is evidence that the phenomenon is more common in marine life and plants than in terrestrial animals (Lagorio, et al. 2015). However, there are still many data deficient clades that have not been examined, and this may cause a biased interpretation on the true extent of biofluorescence exhibited in species (Lagorio, et al. 2015; Jeng 2019). Filling these data gaps is necessary to determine an accurate origin and evolutionary history of biofluorescence as well as the natural history traits, visual spectrum, and purposes associated with the phenomenon.

Due to the increasing trend in publishing notes and records on biofluorescence in various species, there is a need for standardized format and protocols to ensure future research and reproductions of the examinations can adequately compare results and progress current knowledge. With increasing use of tools to perform web-scraping and systematic reviews, there is also a need to set standards that will allow convenient or automated compilation of data needed to determine evolutionary origins and functions of biofluorescent traits or databases to be established in which they can be retrieved from. Here we compiled an exhaustive list of available literature and records on biofluorescent species to identify current knowledge and data gaps, as well as strengths and weaknesses in reporting. Further, we present a best-practices guide for future research efforts to follow when collecting data, and reporting on biofluorescence in species and discuss highlights of the literature.

METHODS

Data Compilation

In order to compile all relevant literature on biofluorescent animals we performed a systematic Web of Science (webofknowledge.com) search to query publications containing the terms “biofluorescent”, “fluorescent”, “biofluorescence", and “fluorescence” in the title. We further queried Google Scholar (https://scholar.google.com/) to find articles that are relevant to the subject but did not contain these terms in the title. Finally, we explored within-literature references to other papers.

While we wished to compile an exhaustive list of records for our dataset, we did not include several papers on areas of widespread autofluorescence, such as corals, sponges, and some arthropods, instead we added the earliest records of these groups that we could find in the literature and only compiled specimen records when they were already tabulated in later review papers. We gave special attention to older papers published prior to 1960 as they had a higher level of within-text mentions of fluorescent species, rather than reporting them in tables and figures. We did not recorded species from these studies if the biofluorescence was reported as “faint”, “slightly fluorescent”, unclearly reported, or not present when verifying current or updated taxonomy via web search. When a species was reported from compilations of multiple species with reference to prior studies, we recorded the species from both manuscripts. Studies related to the biochemical composition of fluorescent compounds such as Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP) in plants, fungi, and bacteria were removed.

The final dataset consisted of 108 published papers containing both individual biofluorescence natural history notes and compiled species examinations spanning 101 years (1920 - 2021). We then manually examined each study, and compiled a dataset of each reported species in the study based on available photos, and tables both in the manuscript and in the supplementary material (Supplementary Material I). We used our best judgement when manually compiling the data and excluded some species from the dataset when fluorescence reported was ambiguous or unnecessary to record, such as the plants included in Deheyn (2020), random objects, fungi, plants, and eyeshine fluorescence of *Polypedates maculatus* Ramesh (2020), and the entirety of Cottrill (2019), a science fair article which reports 128 freshwater specimens from the great lakes were fluorescent; however, we could not reliable confirm fluorescence in the provided images. Since our study is focused primarily on outward biofluorescence of the organism

itself, when studies presented data on eggs internal tissues and organs that were not visible without dissecting the organism, the data was recorded in our dataset though we do not include them in our analysis.

Literature Analysis and Protocols

In order to determine data reporting quality and potential gaps where future improvements may be warranted for consistency in biofluorescent literature, we created a series of categories under which we examined each paper (Table 1). As a general aspect of data quality and transparency, location data and quality of evidence (images) for each species examined was recorded; all supplementary files were checked for the analysis. Furthermore, we recorded potentially biologically and ecologically relevant features of biofluorescence and/or whether or not they were reported, such as activity periods (nocturnal or diurnal), sex, relative growth stage, lifestyle (e.g. arboreal, terrestrial, marine), type of fluorescence (e.g. skin, bone, organs), general color of fluorescence (e.g. green, blue, red), as well as reported hypothesis suggestions (i.e. reported suspected purpose of fluorescence and reporting predatory behaviors of the organism itself or predators of the organism).

To simplify color categories, we recorded all colors as primary and secondary colors (red, yellow, green, purple, orange, blue) with the addition of "pink". Colors were recorded as green when reported as such, or when green was the first word in a compound (e.g., greenish-yellow), but not with greenish-blue, which was recorded as blue. Colors were classified as yellow when they were reported as yellow or had the word first in a compound (e.g., yellowish-green). Blue was recorded when there was any reference to blue in the record (e.g., blue-green, greenish-blue, pale blue). Colors reported as orange or with orange as the first word in a compound were recorded as orange. Colors between orange and red were considered red (e.g., burnt orange) or when red was the first word in a compound (e.g., reddish-orange); colors between red and purple (e.g., maroon) were considered red. Colors within chromatic range of purple (e.g., violet, lilac, lavender, purple) were considered purple. Finally, reported colors of pink, peach, and magenta were considered pink.

We recorded whether photos were available, and whether or not both natural and UV light photo comparisons were available. When a study only had a natural light photo available and no UV light, it was considered to be "not available", as there was no direct evidence of fluorescence under UV. We also recorded whether the color of

fluorescence was reported. Color of fluorescence is important to report in the text alongside figures, especially green, orange, and red because those who have red-blind (protanopia), green-blind (deuteranopia), monochromacy (achromatopsia) color-blindness may not be able to distinguish between them. When photos were not included under UV light and color was not reported, we recorded the value as "NA".

We recorded the sample size for each species in each study and whether or not they were biofluorescent if the paper included a comparative survey over taxonomic groups. However, when the sample size was not reported we set the value at n=1 for species with photos. Sample sizes which were not reported and did not have corresponding images were recorded as "NA" and treated as "not reported" in our analysis.

Evidence suggests that fluorescence may diminish between live and preserved specimens (Evtukh 2019; Eipper, et al. 2020; Pine, et al. 1985), and also that fluorescence may become present in dead specimens but not in live specimens. To account for this, we noted whether or not the species examined was a live or preserved specimen as a binary (y/n) variable. If both live and preserved specimens were sampled, we recorded the specimen as preserved; however, if there was a reported difference in any of the variables we were examining, a second row was created to accommodate both the preserved and live specimen. This way, if there is a reported difference in preserved specimens, future efforts can account for them and if not, there is still a record for awareness.

When activity periods (nocturnal/diurnal) were not reported, we noted the absence of the data and explored available literature to fill in the data where necessary. When we could not find data on daily activity periods for a particular species, we recorded the data with what was known about the family in a general sense. Crepuscular activity was considered to be nocturnal, as the purpose of exploring this category was to determine potential evolutionary indicators given low-light or diurnal lifestyles. If both diurnal and nocturnal behavior were reported for a given species, two rows were made for the species denoting each category.

When lifestyle (i.e., marine, terrestrial, aquatic, arboreal, semi-arboreal, semi-aquatic) was not reported, we used available literature and information on the species in question for the record. If there was no data available on lifestyle for a particular species, we recorded what was known about the family. We did not record whether or not lifestyle was reported in the text as a category of reporting quality.

In regards to the type of biofluorescence, we recorded fluorescence shown in photos and reported in the text; however, we compartmentalized categories where possible in order to better visualize potential trends that may arise. The category "skin" was used to describe body fluorescence and included skin (dermal and glandular), fins, and all related bodily structures; the category "coral structure" was used to encompass all fluorescent parts of coral; "cuticle" was used to describe biofluorescent body parts of arthropods and crustaceans (not including eyes); "membrane" was used to describe fluorescence in parts of developed cnidarians (not including polyps), "hair" was used for all animal pelage/fur; "feather" and "beak" were used to describe fluorescence in bird plumage and beak/bills; "bone" was used for all reported bone related fluorescence; "shell" was used for fluorescence in gastropod shells; and "organ" was used to describe reported fluorescence in organs (visible through the skin), though "eyes" were given their own category and only recorded for cephalopods and fish as their fluorescence was reported to have potential ecological significance (De Brauwer, et al. 2018). Fluorescent guanine crystal structures in the eyes of land vertebrates and invertebrates were considered irrelevant to this study and were therefore excluded. More specific categories were recorded for some outlying taxa, such as scales and scutes for one snake (Ramesh 2020), turtles (Gruber and Sparks 2015), and pangolins (Jeng 2019), as well as mucus and radiole fluorescence reported for a marine worm (Deheyn 2020; Michiels, et al. 2008), and Cnidarian polyps (Haddock and Dunn 2015). There were also reported growth stages such as eggs (Willis and Roth 1956; Jeng 2019; Ramesh 2020), and cocoons (Jeng 2019), which did not fall within any of the established categories and were thus treated as their own.

Finally, we recorded whether the purpose of biofluorescence was discussed or not in each paper, as the hypothesis behind the type of fluorescence on potential biological or ecological significance should typically be the purpose of reporting fluorescence. We also created a separate category to analyze whether predator/predation theory was reported, that is to say whether the biofluorescence would make it easier for predators to locate them, or the possibility they use their biofluorescence to attract prey in order to further explore potential dynamics of fluorescence as physiologically interactive feature.

Biofluorescence analysis and ancestral characteristic reconstruction

To identify potential trends throughout ancestral lineages, we created a list of genera from our compiled dataset to match with existing trees from the literature using the rotl package (Michonneau, et al. 2016) our final tree included 375 unique genera from the dataset. We then performed a simulated stochastic character map, and created ancestral

character estimates based on dominant fluorescent coloration of each genera using the phylotools package (Zhang, et al. 2017). We used the plotrix (Lemon, et al. 2015) and phytools (Revell 2012) packages for estimated timeline inferences based on our phylogram. We explored historic climate change data using the timemachine package (Schobben 2020) which compiles and transforms data from the Westerhold, et al., (2020) with corrections from Hansen et al. (2013) for a dataset spanning 66 Million Years Before Present (mybp). References for all packages used for analysis, visualizations, and data manipulation can be found in the supplementary materials. All analyses were conducted in R statistical software v. 4.0 (R Core Team 2020).

RESULTS

Literature Analysis

Published records of biofluorescent animals (**Figure 1**) first began with interest in Lepidopterans (Mottram and Cockayne 1920), followed shortly after by a natural history observation of biofluorescent anemones (Phillips 1927). These publications led to decades more of short notes and biofluorescence records in arthropods and marine life until the first records of various fluorescent colorations in marsupial mammals (Pine, et al. 1985), and years later the first record of fluorescence in birds (Pearn et al. 2001). After the turn of the millennium, biofluorescent species publications gained new attention, with massive new records of arthropods and marine animals. When a group of researchers documented and published a note on biofluorescent coloration in sea turtles (Gruber and Sparks 2015), an exponential increase thereafter in exploration and published records of fluorescence in herpetofauna began.

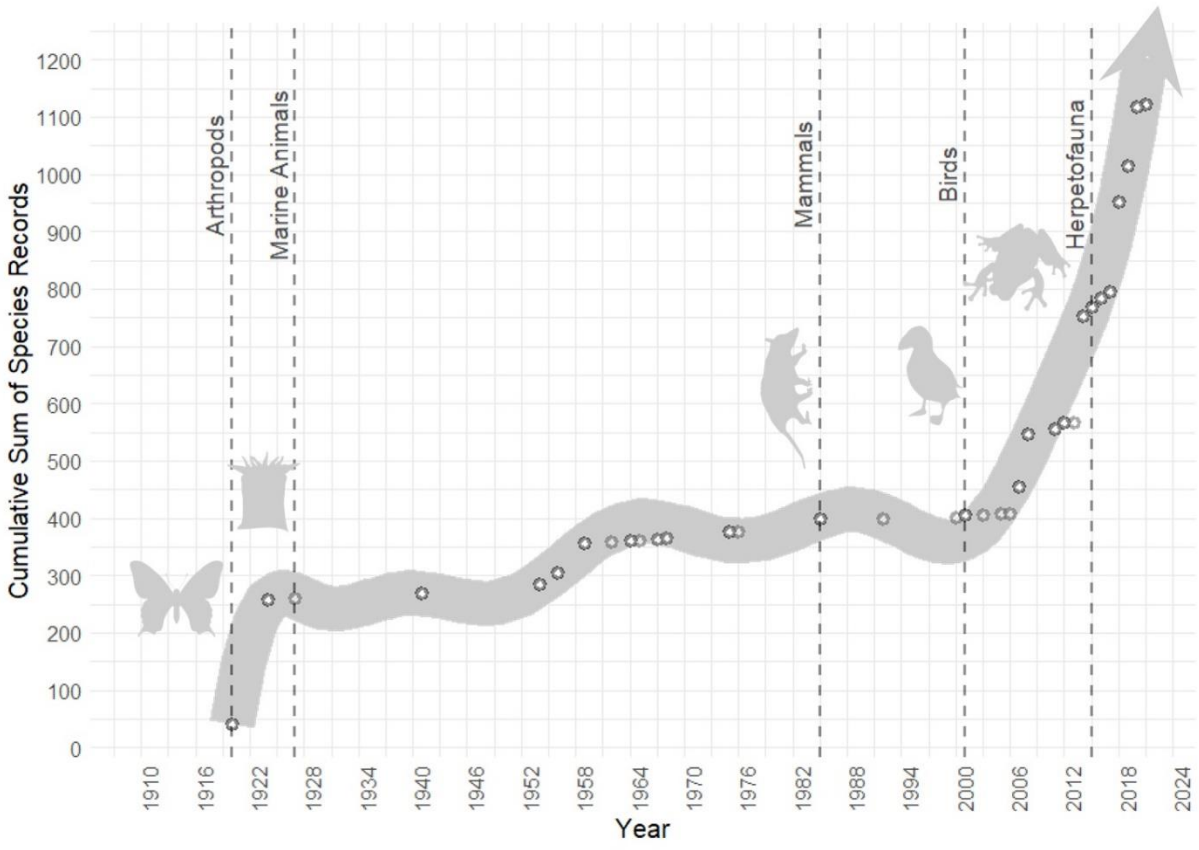

Figure 1. Published records of biofluorescent species ranging from 1920 - 2021 (n=1121); vertical lines indicate the first records of a given group, points indicate individual studies (n=108); transparency of points was set low to allow darkening of overlapping records to show for a given year; A LOESS regression with increased size shows the local regression trend of the data.

Literature on biofluorescent animals had varying levels of reporting quality (**Table 1**). There were some noticeable areas in need of improvement (<50%), including the reporting of both fluorescent and non-fluorescent species examined, reporting the sex of examined individuals, reporting growth stage information, activity periods, location data, providing photos for each animal examined including shots under both natural and UV light, and predator-prey relationships which may be associated with the species.

Table 1. Reporting quality of literature on biofluorescent species (R = Reported; NR = Not Reported)

Category	**R**	**NR**
Reported sample size or provided evidence of sampled species	90%	10%
Examined a range of species and reported both fluorescent and non-fluorescent specimens	23%	77%
Reported sex of the specimens examined	38%	72%
General information about growth stage reported	32%	68%
Reported nocturnal or diurnal activity periods	16%	84%
Reported location data for the specimens examined	48%	52%
Photo provided for each specimen examined	40%	60%
Photos with both natural and UV light provided for each specimen	31%	69%
Reported wavelength of UV used to examine specimens	89%	11%
Reported tool used for UV examination	87%	13%
Color of fluorescence reported for each biofluorescent specimen	61%	39%
Theorized purpose of biofluorescence discussed	92%	8%
Predator/predation theory discussed	54%	46%

Trends in biofluorescent animal records

Our data compilation efforts yielded 1524 records in total (including 401 non-fluorescent records), which is comprised of 977 total unique species records examined (245 non-fluorescent species), which include 24 fluorescent species

which were only classified to the family level (Supplementary Material). Biofluorescent species were comprised of 328 fish, 327 lepidoptera, 86 Arachnids, 77 reptiles, 64 coral, 44 amphibians, 43 coleoptera, 34 mammals, 22 blattodea, 19 cnidaria, 18 birds, 17 mollusks (including two cephalopods), 12 myriapods, six decapods, five porifera, four annelids, three orthoptera (*Liogryllus bimaculata*, *Melanoplus herbaceus,* and one Tettigoniid), two diptera (*Drosophila melanogaster* and *Acinia picturata*), two echinoderms (*Ophiopsila californica* and *Temnopleurus toreumaticus*), two hemiptera (*Brochymena sulcata,* and eggs of *Pentatomidae*), two odonata (*Cordulegaster* sp. and *Pachydiplax longipennis*), one hymenoptera (*Dieunomia nevadensis*), one mantidae (*Mantis religiosa* subsp. *inornata*), one neuroptera (*Chrysoperla* sp.), and one phasmid (*Necroscia punctata*).

The majority of biofluorescent species overall (69%) were nocturnal, arthropods had the least amount of biofluorescent nocturnal species (64%), followed by birds (64%), while the vast majority of biofluorescent herpetofauna (97%), marine animals (98%) and 100% of mammals were nocturnal. Arboreal lifestyles were the most dominant throughout the recorded species (46%), followed by marine (39%), and semi-arboreal (8%), while very few terrestrial (3%), semi aquatic (2%), and aquatic (1%) species were recorded to be fluorescent.

In regards to overall biofluorescent coloration, green (33%), blue (25%), and red (15%), and yellow (8%) were among the most common, while pink, purple, orange, and mixtures of each color comprised <5% of the total records. Of the 953 unique species (excluding family-only classification records), there were distinct trends throughout fluorescent colors, type of animal, and type of biofluorescence (**Figure 2**). Green and red coloration was often associated with skin and coral structures of marine animals as well as cuticles of arthropods. Blues and yellows were more often found in arthropod cuticles and butterfly wings. Terrestrial vertebrates exhibited green biofluorescence

more often, while mammals exhibited almost exclusively pink and purple fluorescence.

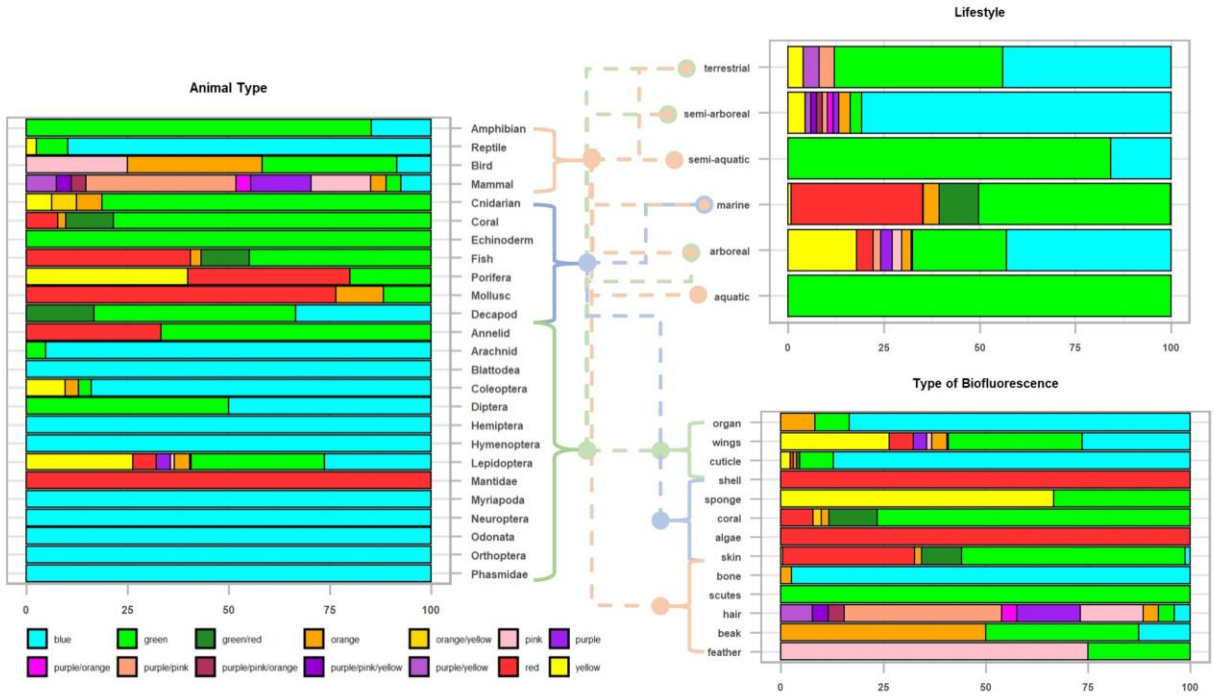

Figure 2. Trends in biofluorescent coloration in relation to animal type, lifestyles, and type of biofluorescence. Values were calculated as 100% proportions of their respective categories using a dataset of only unique species records (n=.931). Colored lines represent terrestrial vertebrates (orange), marine animals (blue) and arthropods (green) with the inclusion of decapods and annelids within both marine and arthropod groups. Types of biofluorescence with <5 records were omitted. Note that "wings" refers only to butterfly wings.

Ancestral Reconstruction and trends in biofluorescence

Ancestral reconstruction (**Figure 3**) provided insights into phylogenetic history and patterns of fluorescent colors. Ancient root nodes (> 20 mybp) were composed mostly of green fluorescence, while diversifying in later fish lineages to red biofluorescence between 15-20 mybp. Blue biofluorescence, commonly associated with autofluorescence in arthropods, was also present in ancient root nodes which underwent a marked transition in color diversity within other arthropod clades around 10 mybp onward. In terrestrial vertebrate groups, green fluorescence was dominant, with pink, and purple fluorescence occurring only in mammalian lineages, while pink and orange occurred in a few bird

lineages. The time of biofluorescent color diversification appears to correspond to decreasing average global temperatures following the Middle Miocene Climate Optimum (MMCO).

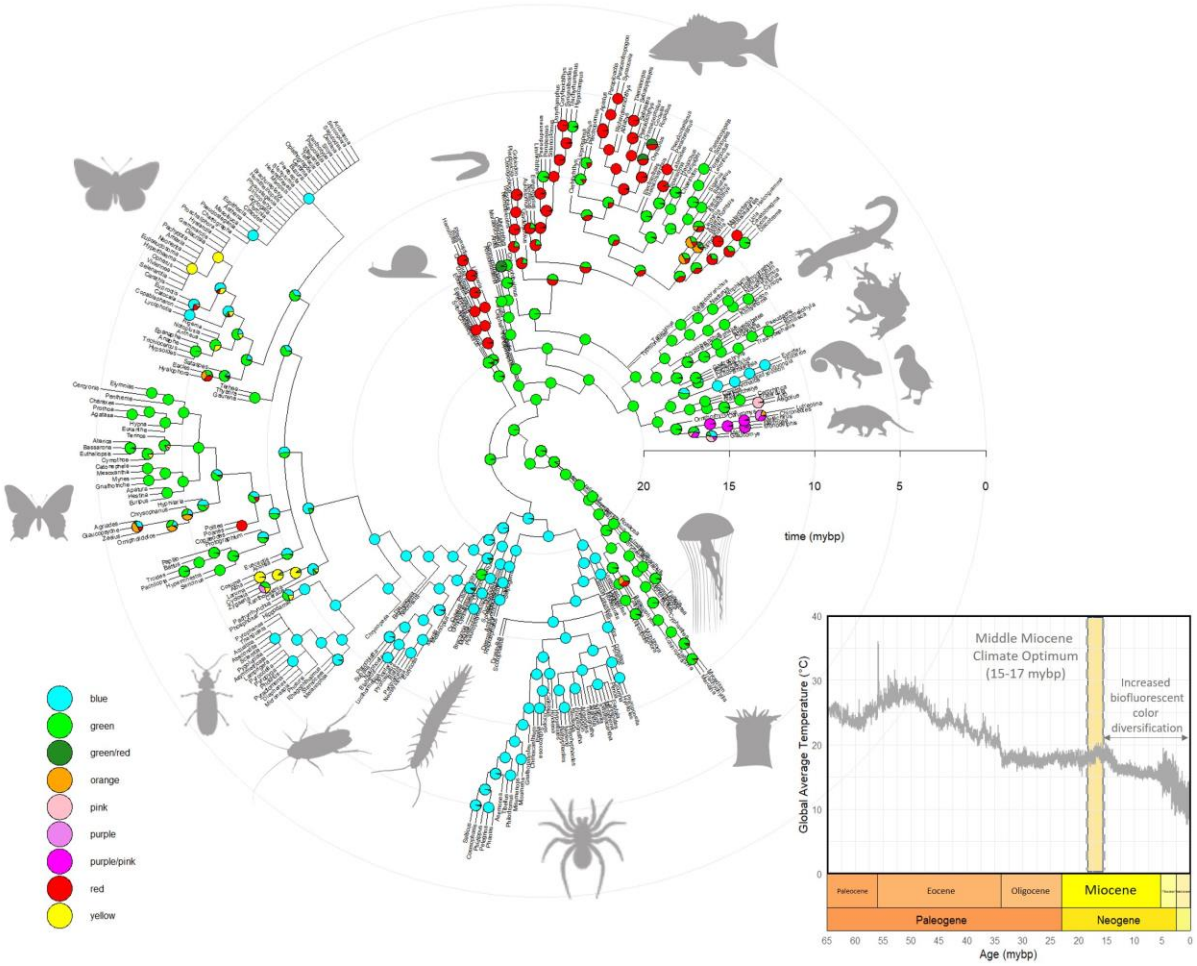

Figure 3. Ancestral reconstruction of dominant fluorescent colorations of taxonomic groups of unique genera (n=375); pie charts represent nodular proportions of dominant genera colorations per tip. Lineages are represented through a timeline of 20 million years before present (mybp) based on their respective branch lengths; compounding colors (e.g., Green/Red) indicates there were two colors present in a single species of a given genera. Animal silhouette markers are general representatives of major taxonomic groups; however, not all groups are represented by markers. Climate reconstruction represents 65 million years of mean global temperatures and highlights the Middle Miocene Climate Optimum (MMCO).

DISCUSSION

Improving biofluorescent literature

Based on our analysis of the literature, we propose a series of best practices and information that could be included in natural history notes regarding biofluorescence (**Figure 4**) in order to optimize scientific value and use in future research. These guidelines have been identified by the strengths and weaknesses of data quality, reporting and evidence of biofluorescent fauna and provides a framework for future natural history notes on the phenomenon to follow. While we understand spectrometer readings are a valuable data source for fluorescent range, we also recognize that spectrometers are expensive, and otherwise may not be realistically accessible at the time of observation, as many notes on biofluorescence come from field-derived data where novel fluorescence was not the primary study target. Therefore, these best-practice guidelines should not be seen as protocols, but rather information and inferences that could be made where available to optimize research output.

That said, in addition to guidelines for the more common natural history notes and additional spectrometer readings of biofluorescent species, we recommend that more elaborate future studies follow account for certain factors and follow an additional set of protocols when making inferences about biofluorescence. While pertaining to design of fluorescent material, Shieber (2001) introduces modelling frameworks such as the MacAdam limit to quantify suprathreshold appearance of fluorescent colors and varying relative luminance, which may allow future studies to avoid using subjective terms such as “dull” or “bright” to describe biofluorescent emission. Photos under UV light should use a yellow filter to absorb residual light emitted under blue wavelengths for clarity of the fluorescent colors. Finally, when exposure and white balance settings are altered to elucidate fluorescent coloration, photos should be accompanied by non-ambiguous reference scale or object such as those evident in Figure 2 of Kohler et al. (2019).

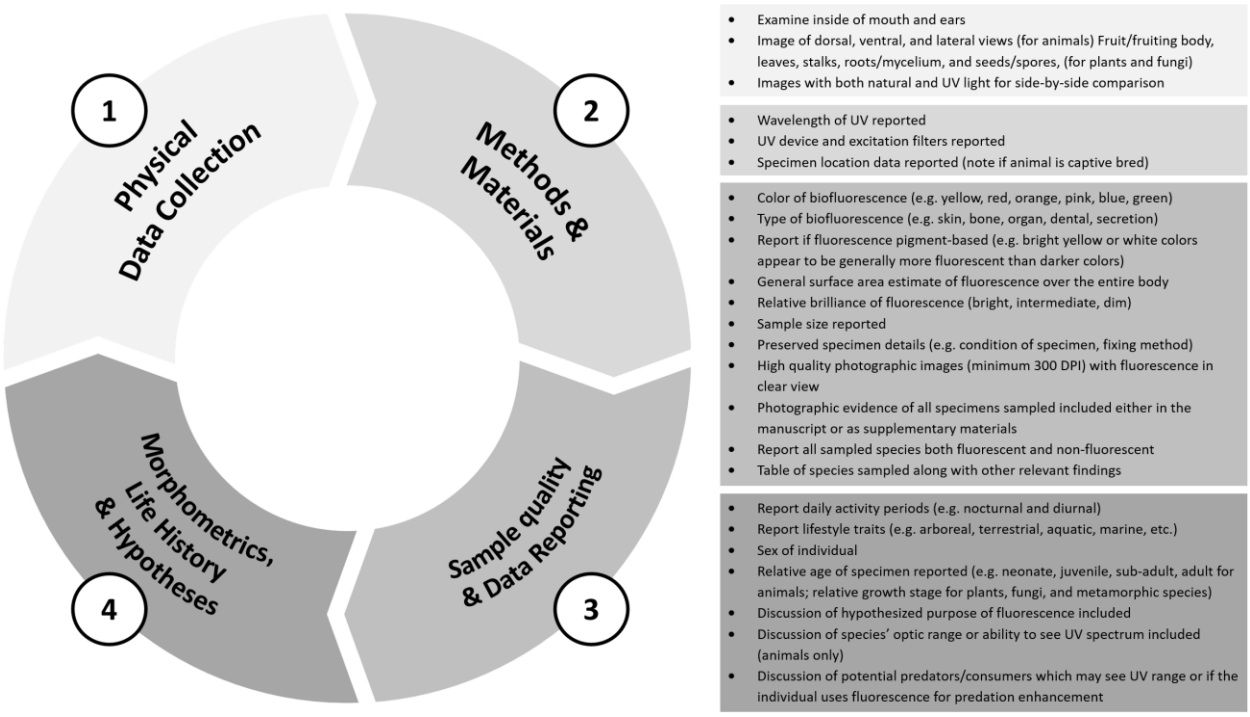

Figure 4. Four divisions of data collection, quality and reporting to optimize scientific value of biofluorescent species publications.

While most published records satisfied our selected categories for analysis. There were marked weak spots in the literature that we feel should be recognized. In terms of supporting evidence, many published studies were missing images of their reported species from both the manuscript itself and available supplementary files. This was primarily an issue for older papers which were published prior to 1960, where cameras were not as widely used for photo documentation. Old studies which encompass numerous biofluorescent species records, such as that of Cockayne (1924) and Mottram and Cockayne (1920) should be verified with images for archival purposes. Several more recent papers also failed to provide photographic evidence as well. For example, the species table from Welch, et al., (2012), provided graphs of fluorescent range from spectrometer readings, but provided no evidence of fluorescence under UV light in any of the provided images, while also citing several other papers which also did not provide photographic evidence. Other than missing images, image quality was also an issue. The report on blind snake fluorescence by Eipper, et al., (2020) is one example where there were both missing images for the reported species, but the image quality was also <300 dpi in the published study, which becomes pixelated and unclear when expanded.

Another limitation of images was the lighting of the photo, and also the surface area of fluorescence on the animal examined. In Figure 2 by Whitcher (2020), frogs photographed in the figure are reported to show biofluorescent, however there appears to be nothing other than purple specs on each, which may be due to the natural light setting of the photograph, the small surface area coverage of the fluorescence, or no fluorescence. Unclear photos such as these should be revised before publication. Since images are the primary source of evidence for biofluorescence, they are one of the most important forms of documentation. To improve future studies, a general percentage estimate of fluorescent coverage on each individual examined should also be reported. Finally, as a recommendation for metadata quality for future systematic reviews and data collation efforts, we suggest that titles of biofluorescent literature should include the species name and family or order of the animal as well as the term "*biofluorescen(t/ce)*" or "*fluorescen(t/ce)*". If multiple animals were examined, we suggest that the highest taxonomic rank (common denominator) and number of species it encompasses should be mentioned. A table of all species examined, and all relevant information should be tabulated and added as a spreadsheet in the supplementary materials.

Apart from photo documentation and general reporting quality, there were several areas of improvement in the reporting of natural history traits and morphometrics. Of the 108 studies we analyzed and the 1524 total records, many did not report location data for the examined individuals (n=449), location is important in terms of wild documented, captive bred, or preserved specimens records due to potential geographic variability in coloration, and as a result, possibility of geographic variation in fluorescence. In regards to natural history features, 1191 individuals examined had no reported sex of the species they examined, which prevents any further inference regarding the potential presence or role of sexually dimorphic biofluorescence. Another 77 studies did not include information on the growth stage of the animals they examined (we omitted papers specifically covering larvae and butterflies, as they were obvious and unnecessary to report). Reporting size and growth stage of species examined for biofluorescence is important in determining whether or not there are any ontogenetic shifts in a given species from non-fluorescent to fluorescent or vice versa. Ontogenetic shifts in the coloration of species are known to occur for various reasons, some of which are related to predation behaviors and corresponding shifts in diet as the animal grows. Light-colored caudal lures in some viper species (Neill 1960; Rabatsky and Farrell 1996) are present in juveniles to lure in specific, smaller prey, while the color fades in adulthood when the animal can take larger prey which is unattracted to the luring behavior. Biofluorescent lures have already been documented in some species of frogfish (De Brouwer and Hobbs 2016), and ontogenetic shifts in fluorescence have been documented in birds (Weidensaul, et al. 2011) Other than

predatory functions, ontogenetic shifts in biofluorescence may occur due to presence or lack of biochemical secretions when the organism ages or metamorphosizes. An observation by Taboada, et al (2017), fluorescent secretions were documented as a result of contact from the treefrog *Boana atlantica*, while this observation provided no evidence of ontogenetic shift, it does raise the question of whether or not tadpoles of the species, or any other fluorescent frog species for that matter, also exhibit natural fluorescence. Overall, natural history traits and predator-prey relationships are important to account for and report when publishing literature on coloration and fluorescence, by exploring these variables, we will be able to make more effective inferences on the potential evolutionary functions of biofluorescence.

Hypotheses testing and future research in biofluorescent studies

Fluorescent signaling hypotheses

Evidence of utilizing biofluorescent features as a behavioral mechanism is prominent throughout the literature in regards to potential sexual signaling interactions, intimidation or defensive behaviors, as a form of mimicry, and as a predatory function. Fluorescent patterns in the skin and bone structures of cryptic and well camouflaged species such as the mossy bushfrog (*Philautus macroscelis*) and several chameleon species are thought to signal conspecific mates while remaining hidden from predators (Gray 2019; Protzel, et al. 2018). In arthropods, sex-specific fluorescent signaling has also been documented in spiders (Brandt and Masta 2017; Jiang, et al. 2009; Lim, et al. 2007) and tick species (Shade 2016). Use of fluorescence as an enhancement in defensive signaling was documented in mantis shrimp (Mazel, et al. 2003), and as a predatory lure in frogfish (De Brauwer and Hobbs 2016). However, one of the more interesting finds was a case of fluorescent structures evolving as a potential defensive function. A study by Reboucas, et al., (2019) determined that the conspicuous bone fluorescence in pumpkin toadlets was aposematic and may have evolved in cryptic colored species as a means of predator avoidance. Each of these behavioral concepts merits further study and may reveal currently unforeseen trends in biofluorescent adaptations and utilization by species of plants, animals, and fungi.

Intraspecific variation hypotheses

Similar to color morphology, intraspecific biofluorescence may be more likely to occur in some populations and not others due to morphological selection. Fluorescent prey may stand out against vegetation and shelter features making them more visible to predators. Therefore, intraspecific variation of fluorescence could be a predictor of the dominant predators in a given population and whether or not they can perceive the UV spectrum, while in other populations where predators cannot see UV ranges, species may select for biofluorescent patterning due to enhanced mate signaling capabilities or intimidation without the threats of predation. On a longer evolutionary timeline, fluorescence may also be used by predators to lure in prey, such as anemones (Haddock and Dunn 2015) or frogfish (De Brauwer and Hobbs 2016) which means that variation in populations, or taxa could also be attributed to an evolutionary specialization for prey that can perceive the UV spectrum. Future research should first identify species which exhibit inter-population variations of fluorescent patterning while accounting for dominant predators in each population to determine whether or not they are accurate predictors of microevolutionary trends.

Solar UV photoprotective hypotheses

While the origins of biofluorescence remain controversial, two leading hypotheses suggest they are functions of visual (Kloock, et al. 2010; Meadows, et al. 2014) and photoprotective attributes (Salih, et al. 2000; Hsiung, et al. 2014). While there are certainly compelling arguments for both, this the photoprotective hypothesis requires much more data to construct a large enough phylogeny with confirmed fluorescent and non-fluorescent species. Future studies should also account for anomalous lineages such as Chaerilid scorpions (Lourenco 2012)**,** which do not exhibit autofluorescence as all other scorpions do, as they may be the key to realizing the first adaptations of cuticular autofluorescence. Future studies should focus on both micro- and macro- evolutionary processes in UV protection. For an example of microevolutionary mechanisms of photo protection, studies may document patterns of fluorescent species abundances and intraspecific variation in closed canopy forests or caves, and compare them to open canopy forests or ecosystems with direct sunlight exposure. For macroevolutionary studies, future research may focus on latitudinal and altitudinal variation of biofluorescence.

Biochemical composition hypotheses

Origins of biofluorescence may also be a compositional side-effect or artifact of some physiological structures, secretions, or compounds which cause autofluorescence. For example, fluorescence in species which contain guanine

crystals in their eyes (Ramesh 2020, Sparks, et al. 2014), and fluorescence in the bioluminescent organs in fireflies (Jeng 2019). Another study by Weisenborn (2011) revealed that biofluorescence in riparian insects was associated with nitrogen content, while autofluorescence in roaches was found to increase in brilliance with diet (Beckert, et al. 2017). Many fluorescent chemical compounds have already been widely studied in biological systems (Lagorio, et al. 2015). Biochemical processes as a driver of biofluorescent evolution may be difficult to study, but compositional analysis of each known biofluorescent species will provide future insights to potentially meaningful compositional shifts in taxonomic lineages.

Ontogenetic shifts in biofluorescence

Ontogenetic variation in biofluorescence was found to be dependent on growth stages from younger to older owls with marked variation in the pink fluorescent plumage (Weidensaul et al. 2011). Also, eggs were recorded in some studies which provided evidence of fluorescent color variability throughout the animal kingdom, such as red fluorescence in chicken (*Gallus gallu*) eggs (Ramesh 2020), greenish-yellow fluorescence in cockroach and other insect eggs due to riboflavin (Willis and Roth 1956; Busnel and Drilhon 1942; Bodine and Fitzgerald 1947; DeLerma 1952), and blue fluorescence in Pentatomid eggshells (Jeng 2019). Other metamorphic species show brilliant fluorescence in their larval stages, which may only exhibit minute fluorescence in their adult stages (Messenger and Borzee 2019). Future studies should explore potential biofluorescent shifts in the metamorphic stages of amphibians, and explore ontogenetic shifts across other known (and unknown) biofluorescent taxa.

Biofluorescence as a tool for Ecologists

Biofluorescent species also have value as tools for ecologists including systematic studies, marine surveys, age determination and diet. Back in 1920's Mottram and Cockayne (1920) published a note in the Proceedings of the Transactions of the Royal Entomological Society of London about their findings of biofluorescent lepidoptera with a follow-up by Cockayne (1924) in later years. Those papers appear to have been the start of chemical fluorescent divisions in systematic for butterflies and moths, after the discovery of seemingly identical species with pale yellow wings that had different degrees of fluorescence, or none at all. The authors crushed dried wings in powder and documented whether or not they were soluble in alcohol and water, and then tested the UV color of the resulting liquid

mixture to test for fluorescence. Some of their suggested generic taxonomic revisions are still in place today, and fluorescence in systematic studies also persists among several taxonomic kingdoms.

As a survey tool, De Brauwer et al., (2018) suggested that biofluorescence could be used as a survey technique for cryptic species in marine surveys. Similar surveys could be done to document biofluorescent terrestrial fauna while accounting for survey effort to determine the overall abundance of biofluorescent animal species in a systematic sample.

Regarding diet, other than the biochemical findings associated with fluorescence in arthropods, (Beckert, et al. 2017; Weisenborn 2011), recent field surveys by author R. Gray have revealed that teeth of *Acanthosaura coronata* appear to be brightly autofluorescent, in comparison to other closely related Agamid species such as *Calotes mystaceus* (**Figure 5**). This increase in fluorescent brilliance may be a result of dietary niche, as *Acanthosaura* are typically forest dwelling lizards, whereas *Calotes* are more of a generalist species which occupies a wide range of habitats. While the compositional difference of the fluorescent dentin in both genera could be due to diet, this hypothesis requires further study, as *Acanthosaura* are severely data deficient and have no dietary records in published literature. However, if the hypothesis proves to be true, dental fluorescence may be used in future studies to make inferences about the diet and natural history of rare or preserved agamid specimens.

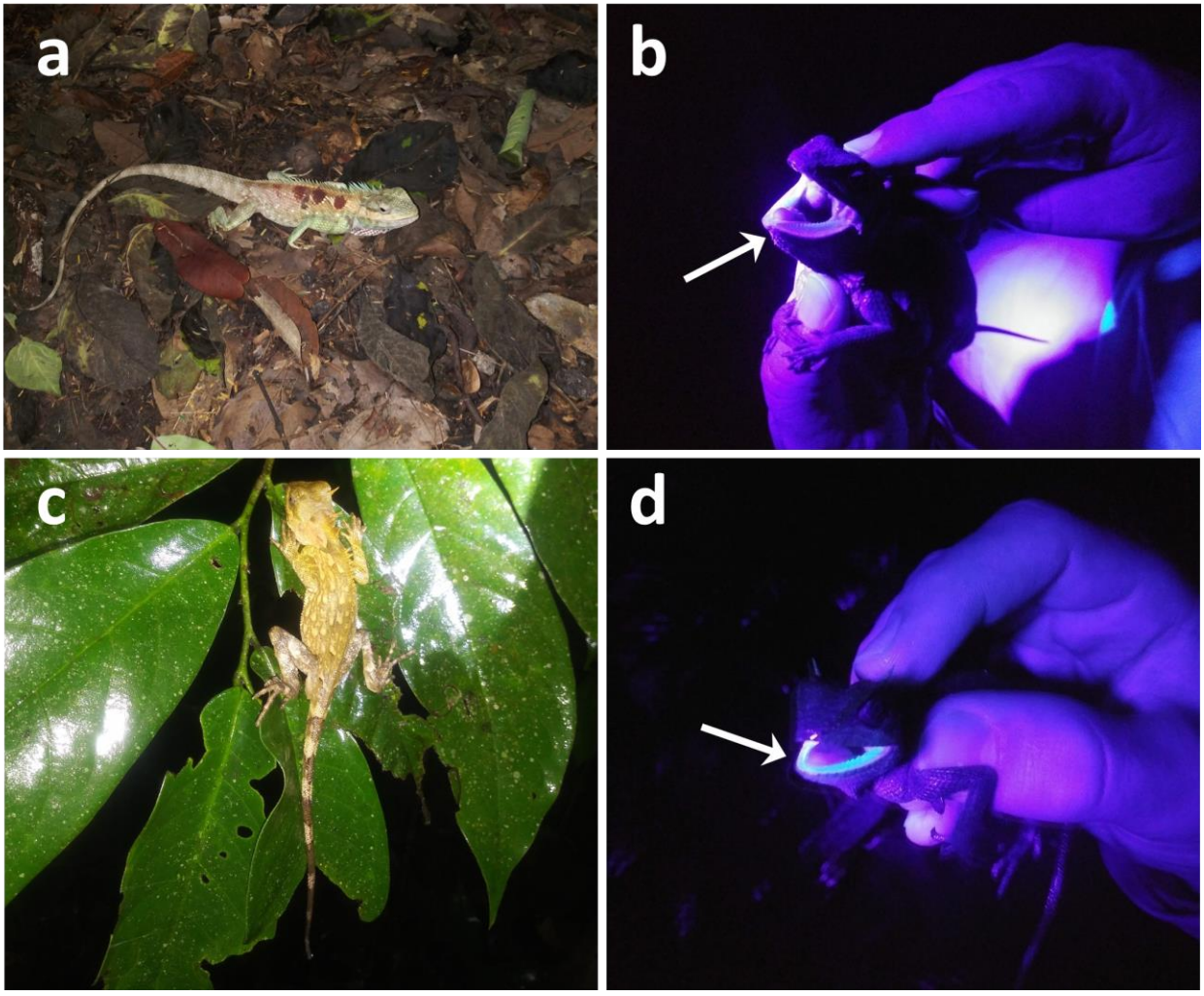

Figure 5. (a) adult male *Calotes mystaceus* from Sakaerat Environmental Research station, Thailand under white light conditions and **(b)** the same individual exhibits little to no dental fluorescence under 365 nm UV light (handheld UV flashlight); **(c)** *Acanthosaura cardamomensis* from the same location (possibly female) under white light conditions; and **(d)** the same individual exhibiting brilliant blue dental fluorescence under UV from the same flashlight.

Predictions for evolution of biofluorescence and current hypothesis

The evolutionary origins of biofluorescence in animals has been hypothesized to function either as a photoprotective mechanism (Hsiung, et al. 2014; Salih, et al. 2000) or a visual function corresponding with levels of light (Kloock 2010; Meadows, et al. 2014). While the photoprotective hypothesis references by Salih, et al., (2000) and Hsiung, et

al., (2014) may seem compelling at first, given that biofluorescence functionally absorbs and emits radiation similar to cellular melanin; however, this hypothesis falls short as species which fluoresce have been shown to contain melanin already (Hsiung et al. 2015). Furthermore, as our results show, the majority of biofluorescent species appear to be nocturnal and many of them exhibit biofluorescence in locations on the body where solar radiation would not be likely to reach, such as the lateral patterning on frogs which is blocked by their thigh (Gray 2019), the inner-wing plumage of birds (Weidensaul, et al. 2011). Therefore, it seems more likely that biofluorescence develops and evolves in low-light conditions for visual purposes. Our preliminary examination of published records and recent cladistic diversification of marine species support Meadows, et al., (2014) hypothesis that biofluorescent color diversity in marine species is driven by proximity to the ocean's surface and levels of blue light. We expand on these concepts by providing evidence that they are also influenced by the age of phylogenetic lineages and suggest new concepts for land-dwelling species.

In contrast to marine species, terrestrial invertebrates show evidence that ancient lineages, such as scorpions and millipedes, exhibit full-bodied autofluorescence, whereas more recent lineages of arthropods exhibit patches of fluorescence indicative of more complex functions. For terrestrial vertebrates, older lineages, such as sea turtles, exhibit fluorescence due to their marine lifestyles whereas terrestrial testudines do not fluoresce. The difference in marine and terrestrial fluorescence in testudines lineages may be due to terrestrial species' tolerance to extreme prehistoric climates. Non-testudine reptiles are broadly non-fluorescent with few exceptions. Amphibians show full bodied autofluorescence in treefrogs and caudates which may indicate lineages derived from cave-dwellers or some alternative evolutionary mechanism of biofluorescence. Marine birds, such as puffins, may followed a similar trend as other marine species, whereas general depth of marine environments in which they forage for fish could be a niche predictor for color of green or orange beak fluorescence (Evtukh 2019). However, the evolutionary pathways which lead to pink plumage of birds and pink and violet hair of mammals remains to be understood.

According to our ancestral reconstruction of biofluorescent colors, there was a marked diverging pattern in both Marine and land-dwelling speciation from the late Miocene onward. Green and blue fluorescence was present at the root nodes, and various colors diversified millions of years later with more recent lineages. For arthropods, blue autofluorescence began with ancient lineages of arachnids and myriapods, while undergoing an explosive diversification with coloration of lepidopterans approximately 5 million years later. With marine life, fluorescence

started out green, and later diversified to oranges and reds in fish, coral, and cnidarians. For terrestrial vertebrates (birds, reptiles, amphibians, and mammals), fluorescence started out green and later evolved to blue, pink and purple in mammalian fur.

Overall, trends in our data show that common predictors of modern terrestrial biofluorescent species are nocturnal, arboreal, and semi-arboreal lifestyles, though other notable natural history trends are evident throughout different taxonomic groups which correspond to low-light conditions and biofluorescence. The dominance of nocturnal behaviors in biofluorescent species may be indicative that thermal intolerance in species may be a factor in the evolutionary development of biofluorescence. Based on patterns of biofluorescent mammals in our records, future studies should examine the hair of nocturnal and arboreal mammals such as bats, and nocturnal primates. Cryptozoic reptiles such as (more) blind snakes, and worm lizards which evolved in subterranean, low-light conditions, may exhibit biofluorescence as a visual communication feature and should also be examined. Birds, like butterflies and moths appear to exhibit biofluorescence as a feature of their coloration, and our current records are too deficient to make any predictions about them. Small body size in arthropods may be a predictor of biofluorescence; other than scorpions, cuticular fluorescence appears to be most common in smaller species.

Finally, following the trends throughout the years of biofluorescent literature, our results support the hypothesis that biofluorescence may have originally evolved as a visual function. Ancient green and blue autofluorescent and subsequent color diversification appears to correspond with prehistoric temperature records surrounding the decline of the MMCO. While in marine environments the diversification of colors at various depths as reported by Meadows, et al. (2014) is evident as a function of visual evolution, the diversification of terrestrial animals may have undergone a different process. Evidence of a Climatic Relict Hypothesis (CRH) suggests that prolonged climate change extremes may cause surface-dwelling invertebrates to take on subterranean lifestyles, isolating them from hardier surface-dwelling counterparts (Ballarin and Li 2018; Botosaneanu & Holsinger 1991; Holsinger 2000). In fossorial, subterranean, or cave-dwelling conditions, animals may have evolved a visual mechanism as an adaptation to locate conspecifics. This new Biofluorescent Climate Relict Hypothesis (BCRH) concept would explain the irregularities and absences in biofluorescence throughout various lineages, and may also explain why autofluorescent is not present in all arthropods. Following the same hypothesis for terrestrial vertebrate groups may provide insight into why more thermal tolerant species such as reptiles do not have green or blue

autofluorescent scales or scutes unless they are marine dwelling species (Gruber and Sparks 2015) or blind snakes (Eipper et al. 2020). While there is an outlying exception to this rule for reptiles, the ventral coloration of a noturnal desert gecko (Prötzel 2021), recent evidence shows that ventral coloration in ectotherms is influenced by thermal properties of the substrate in which they live (Goldenberg et al. 2021), which may actually provide further evidence of thermal influence on the evolution of biofluorescence. Therefore, the origins of more ancient green and blue autofluorescence may have evolved as a visual function for species which were driven to low-light, thermally regulated environments due to an intolerance of the extreme climate conditions surrounding the MMCO. As global temperatures decreased, the need for these low-light, thermally regulated climate refugia also decreased, and as a result, the requirement of a visual mechanism became less of an evolutionary pressure. Thereafter, ecological drivers became more prominent and thus were more likely to influence evolutionary diversification. Pre-existing biofluorescence may have evolved into more recent taxa as an artifact which was thereby incorporated into evolutionary adaptations for more complex ecological interactions and behavioral functions (e.g., predatory luring, sexual signaling, threat displaying). Although these hypotheses and conclusions are limited to available information on biofluorescent species in literature and in our ancestral reconstruction, we hope that our contribution will fuel future contributions in biofluorescent studies, and studies on the optimal ranges of species to provide better insights, updated theory, and support new concepts or confirm our own.

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