Chapter

Biofluorescence in Terrestrial Animals, with Emphasis on Fireflies: A Review and Field Observation

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Abstract

The mysterious world of biofluorescence in terrestrial ecosystems is mesmerizing. Though not as ubiquitous as in the ocean, it is not a rare phenomenon on land. Fluorescence occurs in all major phyla of terrestrial animals (Platyhelminthes, Mollusca, Annelida, Nematoda, Onychophora, Arthropoda, and Chordata) and their subgroups, with diverse fluorophores and performance. In this chapter, we make a general review on the fluorescence in terrestrial animals first, including their systematic distribution, research history, fluorophores, and proposed functions for each group among several other aspects. A systematic observation on the fluorescence of fireflies is reported for the first time. The co-occurrence of biofluorescence and bioluminescence in luminescent land snails, earthworms, potworms, millipedes, and fireflies is a fascinating issue. Though the biochemical mechanism of photogenesis is not fully understood in many terrestrial animals except fireflies, it appears that biofluorescence and bioluminescence do not have clear interaction during the light production process. However, fluorophores and luminophores are usually biochemically related and are different from the photogenic mechanism of jellyfish and several marine creatures whose ultimate light emission is made through energy transfer from bioluminescence to biofluorescence by green fluorescent protein (GFP) or its variants. The role of fluorescence is disputative. In general, nocturnal animals or animals having cryptic living styles, e.g., in earth or under shelters like tree bark or rocks, tend to exhibit UV fluorescence more frequently than animals that are diurnal or inhabit open environments. This pattern is evident in fireflies wherein only nocturnal and luminescent species exhibit noticeable UV fluorescence (likely from luciferin), which is dim or absent in diurnal or crepuscular fireflies. It is unlikely that occasionally induced UV fluorescence in natural environments can play a significant role in intra- or interspecific communication in fireflies or other nocturnal animals.

Keywords: biofluorescence, terrestrial animals, review, fireflies, photogenic organ, nocturnal, luciferin, adaptationism

1. Introduction

Fluorescence is a form of photoluminescence that occurs when a substance emits light caused by absorbing light or electromagnetic radiation [1]. Some amount of the absorbed energy is dissipated in the process, and the rest undergoes an internal

energy transition to re-emit light (longer wavelength) almost immediately after the absorption. The substance ceases to fluoresce virtually simultaneously when the stimulating source is removed. A similar but slightly different photoluminescent phenomenon is phosphorescence. It is characterized by slow energy transition, hence a longer emission of light after original excitement. Early scientists tended to use the term phosphorescence to describe all kinds of light emission that did not produce heat (cold light), including bioluminescence (e.g., [2, 3]). The latter is a form of chemiluminescence and should not be mistaken for one another in modern science [4].

European observers reported biological fluorescence (aka biofluorescence) from the sixteenth century [5], but this received significantly less attention when compared to bioluminescence. Although green fluorescent protein (GFP) was discovered and purified in the 1960s [6], it was not until the late 1990s that GFP and its variants suddenly vaulted from obscurity to the limelight, serving as one of the most widely exploited tools in biochemistry and molecular cell biology [7]. Since then, biofluorescence has been cumulatively reported in diverse organisms and microorganisms. It has been found to occur in marine, freshwater, as well as terrestrial ecosystems [5, 8–12]. Interestingly, it appears that biofluorescence is much more common in marine lives and plants than in terrestrial animals [5, 13]. Marine animals fluoresce by absorbing ambient blue or ultraviolet (UV) light in the sea or from their own bioluminescence and glow in cyan, green, orange yellow, or red in human vision. In contrast, terrestrial biofluorescent animals mostly glow under UV illumination, but different light excitations have also been reported [5, 14].

In a broad sense, animals are more or less fluorescent because their cells and tissues contain various endogenous fluorescent substances, such as flavins, reduced NADH and NADPH, lipofuscins, reticulin fibers, collagen, elastin, and chitin, among others [15]. Such autofluorescence in vitro is beyond the scope of the current report; however, it is difficult to separate autofluorescence from externally visible biofluorescence in many circumstances.

In this chapter, we will first review literatures on biofluorescence in terrestrial animals, including systematic distribution, fluorophores, proposed functions, connection with bioluminescence in luminous groups, and applications especially in taxonomic use. We will then move on to the fluorescence in fireflies, giving a historical review of related fluorescence in vitro and introduce a macroscopic observation on externally visible fluorescence in living fireflies.

2. Biofluorescence in terrestrial animals, a review

All major phyla of terrestrial animals, including platyhelminthes, mollusks, annelids, nematodes, onychophorans, arthropods, and chordates, have been shown to have fluorescent species [5, 13], with some taxa better studied than others. The current review covers only truly land-living animals (aka terrestrial), and freshwater and intertidal dwellers have been excluded.

2.1 Platyhelminthes

The epithelium and mucus trail of some land planarians (Tricladida: Terricola) exhibit fluorescence under UV torch or fluorescence microscopy. Yellow and pale brown pigments in integument, likely pheomelanins, give off a dim yellowish fluorescence. It has been suggested that secreted compounds within mucus fluorescence may have repugnatorial or toxic functions [16]. UV-induced crimson fluorescence from rhabdoids was recorded in deparaffinized sections of *Platydemus manokwari* which is indicative of the existence of uroporphyrins. Living specimens, however, did not fluoresce in their rhabdoids [17].

Figure 1.
The glowing land snail Quantula striata *(from Singapore) from ventral aspect under white light (left) and 365 nm UVA torch (right), notice the fluorescent spot of the photogenic organ on suprapedal gland. By Tsan-Rong Chen (CTR).*

Parasitic flatworms have also been reported to be fluorescent in vitro. For instance, *Schistosoma japonicum* (Trematoda: Strigeidida), a blood fluke causing schistosomiasis in humans, can emit a broad spectrum of fluorescence between 500 and 600 nm under different excitation light sources in confocal microscopy. Green (514 nm) and blue (488 nm) lights yielded strong fluorescence of yellowish green (550–580 nm) and are best for microscopic observation [18].

2.2 Mollusca

The land snail *Quantula striata* has been well-known for its bioluminescence [19]. Its eggs and newly hatched snails glow, while immature individuals give off rhythmic flashes in green from a photogenic organ located in the anterior part of the supra pedal gland. A fluorescent substance has been extracted and partially purified from its photogenic organs. The compound exuded green fluorescence (λmax 515 nm) under UV light (365 nm), similar to the snail's bioluminescent spectrum. Dim fluorescence of the photogenic organ in vivo can be excited by UVA light and is visible externally to the naked eye (**Figure 1**). The compound is not water-soluble and shows similar properties to flavins [20, 21].

Fluorescence in land snails may occur either on the shells or the soft body or both. Different fluorescent color patterns have been observed under near UV and near infrared light across different snail families. The color patterns under different illuminations are thought to be environment adaptive [22]. Fluorescent substances from the snails' soft body have been evaluated for systematic and taxonomic application. Using paper chromatography, Kirk et al. compared the characteristic fluorescence and absorption patterns of the extracted substances from seven species of European land snails. The results indicated it is species-specific but were ineffective to differentiate age and geographic or dietary variations within the species [23]. Fluorescent pigments of a sibling species pair of *Bradybaena* snails from Japan were confirmed to be useful in species diagnosis [24].

2.3 Annelida

More than 30 species from five families of earthworms and potworms (subphylum Clitellata: Oligochaeta) are known to be luminescent. Some of them, such as the cosmopolitan *Microscolex phosphoreus*, North American *Diplocardia longa* (both in Haplotaxida and Acanthodrilidae), and several European *Eisenia* species (Lumbricidae) have been extensively studied for their bioluminescence [25–31]. Two types of bioluminescence with different chemical, physical, and biological features were detected in earthworms [30]. Most of the luminescent species have

flavin-derivative bioluminescent substances stored in granule-filled coelomic mucocytes. The light production occurs when coelomic fluid (mucus) is discharged. However, the body cavity of earthworms only becomes luminous while dying because their coelomocytes break up inside the body. Oppositely, Siberian *Fridericia heliota* and an unidentified *Henlea* species (both in Enchytraeidae) can glow from body walls, whereas the former does not even have glowing mucus. The luminophore in the luciferin of *F. heliota* has been determined to be a tyrosine derivative. All luminescent substances known so far are also fluorescent under UV light and have similar emission spectrums with those of bioluminescence [30].

Several flavin derivatives have been isolated from both luminescent and nonluminescent earthworms, wherein riboflavin (vitamin B2) in the unbounded state was found in their coelomocytes [32]. The coelomic fluid of luminescent *Eisenia lucens* fluoresces yellow-green light initially and turns into blue when bioluminescence ceases. In contrast, fluorescent color changing did not occur in nonluminescent *E. fetida.* It suggests that some product of the luminescence reaction changes the color of fluorescence. It is thus postulated that the incapability of bioluminescence in nonluminescent earthworms is due to their lack of a certain component to convert riboflavin into lumiflavin in the oxidative system [29].

Riboflavin exists in the cytoplasm of coelomocytes with different contents among earthworm species of various genera [33]. The secreted mucus from intersegmental pores and mouths also produces fluorescence [29, 34, 35]. Since riboflavin is essential to the regeneration, the stem cells responsible for regeneration accumulate more riboflavin than other cells thus inducing stronger autofluorescence [36].

The coelomic fluid also yields species-specific intensity of fluorescence. For example, *Eisenia andrei* and *E. fetida*, which are hard to differentiate morphologically, each carries a specific fluorescence fingerprint in the cell-free coelomic fluid [37]. A broader taxon examination of the coelomocyte-derived fluorescence proved its value in supravital species identification of morphologically resembling earthworms (**Figure 2**) [38].

2.4 Nematoda

Living nematodes from 15 selected genera have been found exhibiting pale yellow to green fluorescence from their intestines, spicules, and lips in microscopic observation under both blue (450–490 nm) and UV (365 nm) epi-illumination [14]. Aging nematodes tend to have stronger fluorescence than juvenile individuals. The intensity of fluorescence could be an indicator of age and viability of nematodes [14]. When the nematodes were dying or killed by physical or chemical treatment, blue fluorescence burst from the intestine into the entire body in a wave. This dramatic phenomenon is known as "death fluorescence" [39]. The blue fluorescent substance was inferred to be lipofuscin, which is stored in intestinal lysosome-related

Figure 2.
A nonluminescent earthworm (from Taiwan) emitting yellow-green fluorescence (right) under 365 nm UVA torch. By CTR.

organelles and is cumulative with age [14]. Later research identified anthranilic acid glucosyl esters (derived from tryptophan, not lipofuscin) to be the source of death fluorescence [39]. This revealed that organismal death of nematodes has a similar mechanism to necrotic propagation in mammals [39].

2.5 Onychophora

Research on the fluorescence of velvet worms is limited. Weak cyan fluorescence in vitro was seen on the outer zone of the cuticle specimens embedded in frozen sections of South African *Peripatopsis moseleyi* (Euonychophora: Peripatopsidae), but not on claws or jaws [40]. In a previous work on the same species, however, neither its cuticles nor fresh slime expelled fluorescing [41].

2.6 Arthropoda

Land-dwelling arthropods are found in all four subphyla: Chelicerata, Myriapoda, Hexapoda, and Crustacea (paraphyletic). A wide array of terrestrial arthropods has been reported fluorescing upon UV excitation [41–49]. No fluorescent land crustacean has yet been reported, though crab eye lenses are known to be fluorescent.

2.6.1 Chelicerata

Chelicerates are featured by the absence of antennae and jaws and the presence of chelicerae as their feeding appendages. Terrestrial groups are exclusively in class Arachnida and consist of mites (order Acariformes), ticks (Parasitiformes), harvestmen (Opiliones), camel spiders (Solifugae), hooded tickspiders (Ricinulei), pseudoscorpions (Pseudoscorpiones), scorpions (Scorpiones), whip scorpions (Uropygi), tailless whip scorpions (Amblypygi), and spiders (Araneae). Fluorescent species have been recorded in at least eight out of the 10 orders (**Figure 3**), and more are awaiting future research [41–45].

Two fluorescent sources are known in chelicerates: excitation of cuticular fluorophores like beta-carboline and coumarin in hyaline layer, and integumentary/hemolymph fluorophores like tyrosol [5, 43, 50]. The former occurs only in extant scorpions and horseshoe crabs and extinct sea scorpions (Eurypterida), whereas the latter is prevalent in the other chelicerates which lack the hyaline layer.

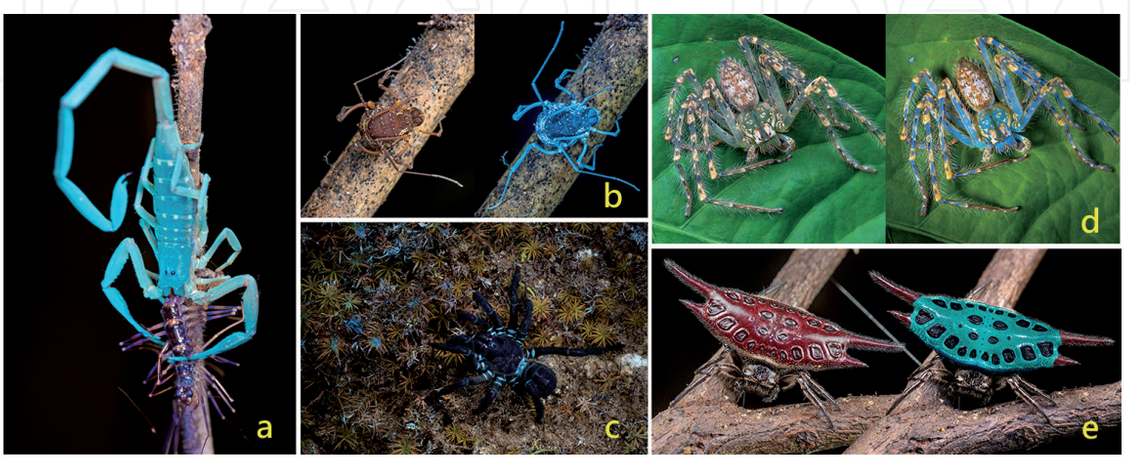

Figure 3.
Biofluorescence in chelicerates. (a) bark scorpion Lychas scutilus *(Scorpiones: Buthidae); (b) harvestmen (Opiliones); (c) trapdoor spider* Liphistius malayanus *(Megalomorphae: Liphistiidae); (d)* Gnathopalystes *huntsman spider (Araneomorphae: Sparassidae); (e)* Gasteracantha *spiny orb-weaver spider (Araneomorphae: Araneidae). All animals from Singapore, under flash light/365 nm UVA torch. By Nicky Bay.*

The cuticular fluorescence is much stronger than hemolymph one and remains unfaded for years in museum specimens. The presence of the hyaline layer and accompanied UV fluorescence has been suggested to be a plesiomorphic (ancestral) trait in Chelicerata [43]. In this scenario, UV fluorescence either has no adaptive significance for scorpions or just serves as a function common to scorpions, horseshoe crabs, and eurypterids. Therefore, it might not be an adaptation of scorpions for land living [43, 50]. Alternatively, UV fluorescence in scorpions has evolved a new adaptation deviated from its initial role in their marine ancestors and relatives. For example, body fluorescence serving as a shelter-finding indicator has been proposed [51]. Recently several species of *Chaerilus* scorpions (Chaerilidae) were discovered to be non UV fluorescent and independent of their various living environments (in soil, epigean, or cave-dwelling) [52]. This exception provides an insight into the long-debated role of fluorescence in scorpions. The loss of fluorescence is surely an evolution novelty in scorpions, but its irrelevance to habitat types rendered the adaptationist explanation questionable (see [53]).

UV fluorescence is widespread across families among spiders [41, 45, 54]. There are two suborders of spiders: Mygalomorphae (e.g., tarantulas) and Araneomorphae (modern spiders). Mygalomorphae resembles the ancient form in morphology: covered with tergites on the dorsum and fluoresces only from intersegmental membranes and appendage tips [54] (**Figure 3**). On the other hand, fluorescence occurs in many araneomorph families, especially in the highly derived Entelegynae [45, 54] (**Figure 3**) family. Most araneomorph spiders have lost their tergites, permitting UV light to penetrate the integument, triggering hemolymph fluorescence [51]. As far as is known, only a few species fluoresce through the entire body. Among body parts, fluorescence occurs most commonly in eyes and joints, moderately in abdominal hemolymph, and least in the cephalothorax. It appears that all araneomorph spiders fluoresce to various degrees and each species has its species-specific emission of fluorescence [45]. Spider eggs of both suborders examined thus far are all fluorescent. Egg sacs also fluoresce, though common but not universally [54]. Web silk emits weak yellow-green fluorescence under UV light [55].

UV fluorescence has evolved repeatedly in spiders' evolution and is liable to keep changing among species. Fluorescent patterns and fluorophores also differ among families [45]. Its function in spiders, however, is unclear. The evolution of fluorescence has been postulated to be driven by pray-predator interaction, sexual selection, or photoprotection [56–60]. Most of the spiders have poor vision and largely rely on mechanical and chemical cues for predation and mating. Fluorescence is unlikely to function as intraspecific signals. Instead, fluorescence may help spiders blend into ambient environment and thus reduce perception by their prey and predators [45, 56]. Jumping spiders (family Salticidae) have excellent vision. Many species show sexual dichromatism, and these males have elaborate mating dances. Some marking patches in males carry dancing-relevant fluorescent signals. Experiments provided convincing evidence that the dynamic fluorescent signals play a vital role for jumping spiders' mate choice [58, 59]. Another postulated function for UV fluorescence in spiders is for protection from UV radiation [60]. A similar hypothesis had been proposed earlier for the evolutionary origin of fluorescence proteins in corals [61]. A transcriptome analysis of spiders has suggested that they are unable to synthesize melanin, a common light-absorption pigment almost universally present in all organisms [62]. Melanin in insects and crustaceans operates additionally as an innate immune system [63]. It appears compelling that UV fluorescence is a photoprotective mechanism for araneomorph spiders which do not have melanin and tergite. Recently, however, melanin has been confirmed to be present in spiders, thus diminishing the validity of this hypothesis [64].

2.6.2 Myriapoda

Myriapoda comprises four orders: Chilopoda (centipedes), Diplopoda (millipedes), Symphyla, and Pauropoda. UV fluorescence is currently known only in the first two taxa [5, 41, 46, 47, 65]. Several centipedes and millipedes are luminescent [26, 46, 47, 66–68]. This raises the question of whether fluorescence and luminescence in myriapods are a mechanistic link for the production of light, whereby the fluorescent substance is the ultimate light emitter through energy transfer as in GFP [7, 46].

The sublittoral centipede *Orphaneous brevilabiatus* (Geophilomorpha: Oryidae) can discharge yellowish bioluminescent slime while walking, leaving a shiny trace with a fruit odor lasting from a few seconds to about 2 minutes [66, 67]. The glowing slime is secreted from coxal glands, and the light results from a luciferin-luciferase interaction. The slime does not show fluorescence [66]. The other glowing centipedes are found in five families, mostly in order Geophilomorpha, and share more or less similar bioluminescent behaviors, with variations in slime colors or luminescent intensity [67]. The slime is apparently not for intraspecific visual signaling since geophilomorph centipedes are eyeless. Rather it is a defensive mechanism against predators [67]. Weak UV fluorescence has been reported to occur in some nonluminescent *Cormocephalus* species (Scolopendromorpha: Scolopendridae) [41].

In millipedes, only some 10 out of the 12,000 described species are luminescent [47, 67]. Co-occurrence of photoluminescence and chemiluminescence has been documented in North American *Motyxia* species (also known as Sierra luminous millipedes, in Xystodesmidae) but is unclear for the other glowing millipedes (distribution in Asia and Pacifics, e.g., *Spirobolellus* in Spirobolidae, and *Salpidobolus* (*Dinematocricus* in most references) in Rhinocricidae). *Motyxia* millipedes glow spontaneously or by physical stimulation, with a bright greenish-white hue throughout the entire body [67]. A bioluminescent substance was isolated from *M. sequpiae* and identified to be 7,8-dihydropterin-6-carboxylic acid [69]. This compound is unstable outside of the cuticle, leading to pterin-6-carboxylic acid which is also found in the cuticle of *M. sequoiae*. Both compounds are UV fluorescent, showing emission peak at 505 and 450 nm, respectively. The emission spectrum of 7,8-dihydropterin-6-carboxylic acid in vivo and in vitro is very close to the bioluminescence of *M. sequoiae* (peaked at 495 nm), and Kuse et al. suggested it as the light emitter [65]. Pterin-6-carboxylic acid was later found in a non-bioluminescent xystodesmid, the Japanese train millipede *Parafontaria laminata armigera*. It fluoresces upon direct UV excitation and gives off a blue emission [65, 68]. Autofluorescence is widespread in diplopod orders like Spirobolida, Siphonophorida, and Polydesmida (**Figure 4**) [46].

Based on the evidence so far, the mechanisms of light production in glowing jellyfish and *Motyxia* millipedes seem different. The chemiluminescence in the latter produces a longer wavelength of light than that of fluorescence but reversely so in the former. Though the luminophore and fluorophore in *Motyxia* are biochemically related, their mechanistic interaction in photogenesis is unclear (see Section 3.1).

Bioluminescence in millipedes has been demonstrated to be an aposematic signal. Glowing fake millipedes were found to have a much lower predation rate than non-glowing ones in a field experiment [70]. It was postulated that the evolution of luminescence in *Motyxia* may have initially been triggered by a harsh environment to deal with metabolic stress and then was later repurposed for aposematism [47]. Biofluorescence, on the other hand, appears to play an insignificant role ecologically or ethologically, if any at all [5].

Figure 4.
A non-luminescent millipede (from Singapore) fluorescing in blue hue under flash light/365 nm UVA torch. By Nicky Bay.

2.6.3 Insecta

Insects are the most diverse animals, not only in terms species richness but also in morphology, physiology, behaviors, and ecological niches. Bioluminescence has evolved in some beetle families (Elateridae, Lampyridae, and Rhagophthalmidae), flies (in Keroplatidae or Mycetophilidae s. lat.), and springtails (order Collembola, six-legged arthropods allied to insects) [26, 71]. South American roaches *Lucihormetica* (Dictyoptera: Blaberidae) were once reported to be luminescent [72] but are actually fluorescent [73, 74].

Fluorescence seems ubiquitous in most, if not all, insect orders and occurs not only in the insect body but also in their eggs, egg cases, silks, exuviae and other products (**Figure 5**) [41, 48, 49, 73, 75]. Fluorescent materials include pterin, flavin, and kynurenine derivatives, chitin, resilin, and luciferin (thiazole derivative), among many others [5, 48, 76–80]. Since chitin is autofluorescent, insects with weakly sclerotized cuticles tend to show stronger fluorescence than those that are heavily armored-like beetles [49]. Eyes, markings on body or wings (usually white, yellow, or cyan in color), and joints are the most frequent fluorescent parts [41, 48, 81–84]. Fluorescence in fireflies will be addressed in the next section.

Fluorescence may play roles in intra- and interspecific communication as suggested in butterflies, moths, damselflies, and dragonflies [48, 82, 83, 85], but it is premature to make a conclusion about their function at present [85]. Autofluorescence of resilin, chitin, and some other substances could just be an epiphenomenon and have no adaptation value.

2.7 Chordata

Traditionally, chordates constitute three subordinate groups: lancelets (Cephalochordata), tunicates (Tunicata), and vertebrates (Vertebrata). Terrestrial forms are exclusively vertebrates. UV fluorescence in land vertebrates was first noted in some plumage areas of Australian parrots and later found prevalent in most parrot species worldwide as well as in some other bird species [5, 85–88]. A few fluorescent amphibians (South American tree frog *Hypsiboas punctatus* [Anura: Hylidae]), reptiles (African and Madagascan chameleons [Squamata: Chamaeleonidae]), and mammals (North America flying squirrels *Glaucomys* species [Rodentia: Sciuridae]) were discovered recently [13, 89, 90]. Mouse skin emits red fluorescence that peaks at 674 nm, and the fluorophore is found to be derived from food [91]. In addition, autofluorescent substances like keratin, collagen, and enamel, among others [15],

Figure 5.
Biofluorescence in various insects or their products. (a) stick insect Necroscia punctata *(Phasmatodea: Heteronemiidae); (b) long-horned grasshopper (Orthoptera: Tettigoniidae); (c) true bug's nymphs and eggshells (Hemiptera: Pentatomidae); (d)* Paralecanium *scale insect (Hemiptera: Coccidae); (e)* Aspidomorpha *tortoise beetle (Coleoptera: Chrysomelidae); (f)* Cerosterna *long-horned beetle (Coleoptera: Cerambycidae); (g) larvae of* Macrolycus *netwinged beetle (Coleoptera: Lycidae); (h) Red slug caterpillar* Eterusia aedea *(Lepidoptera: Zygaenidae); (i) cocoon of a lichen moth (Lepidoptera: Erebidae). (a-g) from Singapore, under flash light/365 nm UVA torch, by Nicky Bay; (h-i) from Taiwan, under white light/395 nm near-UVA torch, by JML.*

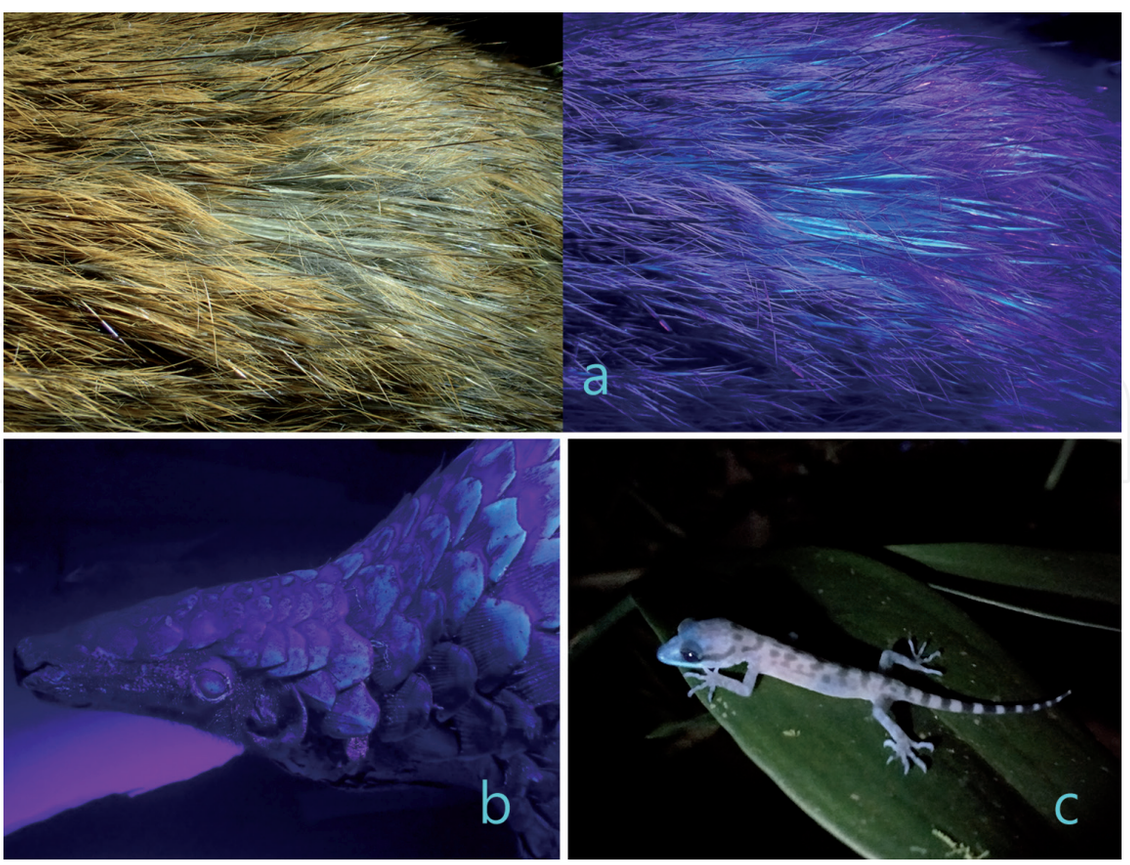

Figure 6.
Biofluorescence in mammals and reptile. (a) Coxing's white bellied rat Niviventer coninga *(Rodentia: Muridae), with fluorescent setae. (b) Chinese pangolin* Manis pentadactyla *(Pholidota: Manidae), with fluorescing keratin scales. (c) Kinabalu bow-fingered gecko* Cyrtodactylus baluensis *(Squamata: Gekkonidae), with bone-based fluorescence. (a-b) Taiwan specimens in NMNS collection, under white light/395 nm near-UVA torch; (c) from Sabah, Malaysia, under 365 nm torch. By JML.*

also lead to UV fluorescence in vertebrate claws, nails, hairs, scales, skin, teeth, and bones to various extents (**Figure 6**). Known vertebrate fluorophores include fulvins, carotenoids, spheniscin, hyloins, pheophorbides, etc. [5, 13, 91].

Intraspecific communication through UV fluorescent signals has been proposed in parrots, frogs, and chameleons [13, 85, 87, 89, 92]. Under natural illumination, fluorescence found on the yellow crown of the budgerigar *Melopsittacus undulatus* (Psittaciformes: Psittaculidae) may enhance visual perception. Both male and female parrots prefer to be associated with fluorescent potential mates over nonfluorescent ones which had been treated with sunblock gel [87]. Pearn et al. [93], however, did not note any fluorescence-linked difference in mate choice. In the frog case, Taboada et al. demonstrated that the emission spectrum of fluorescence from lymph and skins matches the sensitivity of night vision in amphibians and considerably enhances brightness of the individuals under twilight and nocturnal scenarios. This may increase their detectability by conspecifics while remaining cryptic to their predators in a dusk environment [13]. In regard to chameleons, they usually live in more closed habitats like forests which have a higher relative component of ambient UV light [94]. They fluoresce in blue color (around 430 nm) which contrasts well with the green and brown background reflectance of forests. Their fluorescent bony tubercles are sexual dimorphic and may play some roles in sexual selection [89]. Stuart-Fox et al. showed that display colors of chameleons occupying more shaded environments have a relatively higher UV component [95].

3. Biofluorescence of fireflies

Researches of fluorescence are extremely scant in comparison to bioluminescence of fireflies in all scientific fields. Most were infrequent studies done in the twentieth century and made only limited progress. We will make an extensive review of related fluorescent substances first and then report our field observations of externally visible fluorescence in fireflies.

3.1 Biofluorescence in vitro, a historical review

Similar to annelids and millipedes, photoluminescence coexists with chemiluminescence in luminous firefly species wherein their luminophores are simultaneously fluorophores (e.g., oxyluciferin). Other fluorescent substrates, like resilin and chitin, are widespread in Lampyridae and irrelevant to photogenesis.

A firefly-related fluorescent substance was extracted from the thoracic and abdominal photogenic organs of a click beetle species (Elateridae: *Pyrophorus noctilucus*) from West Indies and was named pyrophorine by Dubois in the late nineteenth century [96, 97]. It showed similar chemical and physical properties with those of esculin, a glucose compound, and did not interact with luciferase to produce light. Dubois suggested that pyrophorine may have a resonance property which can transform the absorbed invisible rays into visible light, thus intensifying the animal's luminescence which he called "condensed light" [96]. Similar ideas were welcomed by contemporary scientists, but Dubois disagreed on solar radiation being the energy source of condensed light as was commonly thought [100]. The idea was falsified by Coblentz who demonstrated nonoverlapping spectra of pyrophorine fluorescence and firefly luminescence [97, 98]. Dubois persisted his hypothesis of "condensed light" [99].

A similar substance was later extracted from North American fireflies and was named luciferesceine. McDermott regarded it an incidental material irrelevant

to photogenesis in fireflies since the substance was also found in nonluminescent species [100]. Dubois agreed to use luciferesceine consistently rather than pyrophorine [99]. Unfortunately the identity of luciferesceine or pyrophorine remained unknown until now, though a pteridine with ribityl residue was inferred [76, 80]. Another fluorescent substance was extracted and purified from the Japanese firefly *Luciola cruciata* [80]. Its property is markedly different from luciferesceine. It was determined to be 8-methyl-2,4,7(1H,3H,8H)-pteridinetrione, a pteridine named luciopterin [76, 80].

Firefly luciferin, commonly known as D-luciferin, is a thiazole derivative ($C_{11}H_8N_2O_3S_2$) [76, 80, 101, 102]. It is fluorescent as a crystalline form or in aqueous solution and shifted its excitation peak from 327 to 395 nm in strong basic solution without changing emission peak at 530 nm [102]. The ultimate product of photogenesis, oxyluciferin, is the real light emitter in firefly bioluminescence [103–107]. Oxyluciferin is extremely unstable, and scientists have used several analogues to study its biochemical and photochemical properties and photogenic mechanism. Their tautomers give different fluorescent emission of spectrum in different pH environments. Keto-form oxyluciferin fluoresces in red in vitro (λmax ~ 635 nm) but turns into yellow green (λmax ~ 530 nm) with a blue shift when binding with luciferase in reaction. The latter is identical to the emission of the bioluminescent spectrum. It is postulated that the intermolecular interactions and polarity affect the color of emission of oxyluciferin [106, 107]. Shimomura indicated that the fluorescence spectrum measured after the light emission, even for only a few milliseconds, cannot be considered the fluorescence spectrum of the light emitter [104].

Smalley et al. noted that there were two fluorescent compounds localized in the firefly photogenic organs, one in the photocyte granules and the other in the dorsal layer of the lanterns [108]. The former fluoresces dimly with a cyan-green emission (λmax between 510 and 540 nm) and glows brightly when in basic solution. The latter emits a cyan fluorescence (λmax between 510 and 520 nm) which disappears quickly when exposed to water. The former is likely luciferin but the latter remains unclear.

A red fluorescent material isolated from *Photinus pyralis* was reported by Metcalf in the earth in the 1940s but not identified [109]. Fluorescent pigments in paper chromatography were popularly applied in invertebrate taxonomy as a diagnostic character in species and genus in the 1950s–1960s [23, 77]. Wilkerson and Lloyd detected 51 fluorescent compounds by paper chromatography from 13 North American firefly species of 5 genera (*Photinus*, *Micronaspis*, *Pyrectomena, Pyropyga*, and *Photuris*). Each genus did have its own specific pigment contents. [110].

3.2 Biofluorescence in vivo, a macroscopic observation

Not much externally visible fluorescence in fireflies has been documented. In addition to Smalley and her colleagues' findings [108], we found only two related studies [111, 112].

Blue fluorescence resulting from resilin in lenses of compound eyes of *P. pyralis* was reported in 1970 [111]. Actually this protein is common and widespread in arthropod eye lenses [113]. This matches our observation that compound eyes are among the most frequently fluorescing body parts of fireflies under UV illumination, giving a cyan or blue emission.

Recently Yiu and Jeng reported an interesting case of fluorescence in a paedomorphic (neotenic or "larviform") female *Oculogryphus* firefly in Hong Kong [112]. The female glowed in yellow-green light from a pair of photogenic organs in the abdomen and emitted cyan fluorescence through the whole body under UV

illumination. This combination is unique to terrestrial animals, even in luminescent groups. It appears that the two systems are mechanistically independent and share some commonness with millipedes in having a shorter wavelength of light emission in biofluorescence than in bioluminescence.

This case triggered our curiosity to explore the secret world of biofluorescence in fireflies. By using UVA or near-UV torches (λmax 365 or 395 nm) in the field and laboratory, we demonstrated that UV fluorescence is widespread across most of the luminescent firefly groups in East and Southeast Asia (**Figures 7** and **8**). In addition to blue fluorescence from resilin in eye lenses, photogenic organs consistently emit cyan fluorescence to naked eyes, no matter if they are glowing/flashing or not. Fluorescence is brighter when the photogenic organs are glowing (**Figure 7a–j**). The fluorescent substance in photogenic organs is likely luciferin as previously suggested [108]. Alcoholic or dry specimens have much weaker fluorescence in their photogenic organs (e.g., **Figure 7k**). The other body parts frequently fluorescing include head capsule (blue emission probably by resilin as seen in eye lenses), intersegmental membrane, and weakly sclerotized cuticles in both adults and larvae (pale blue emission likely autofluorescence of chitin or cuticular proteins) [49]. In contrast, diurnal genera like some *Pyrocoelia* (part), *Vesta*, *Lucidina*, *Pristolycus*, and *Drilaster* species that carry conspicuous or highly contrasting coloration do not display noticeable UV fluorescence except in vestigial photogenic organs if they have them (**Figure 7l–n**).

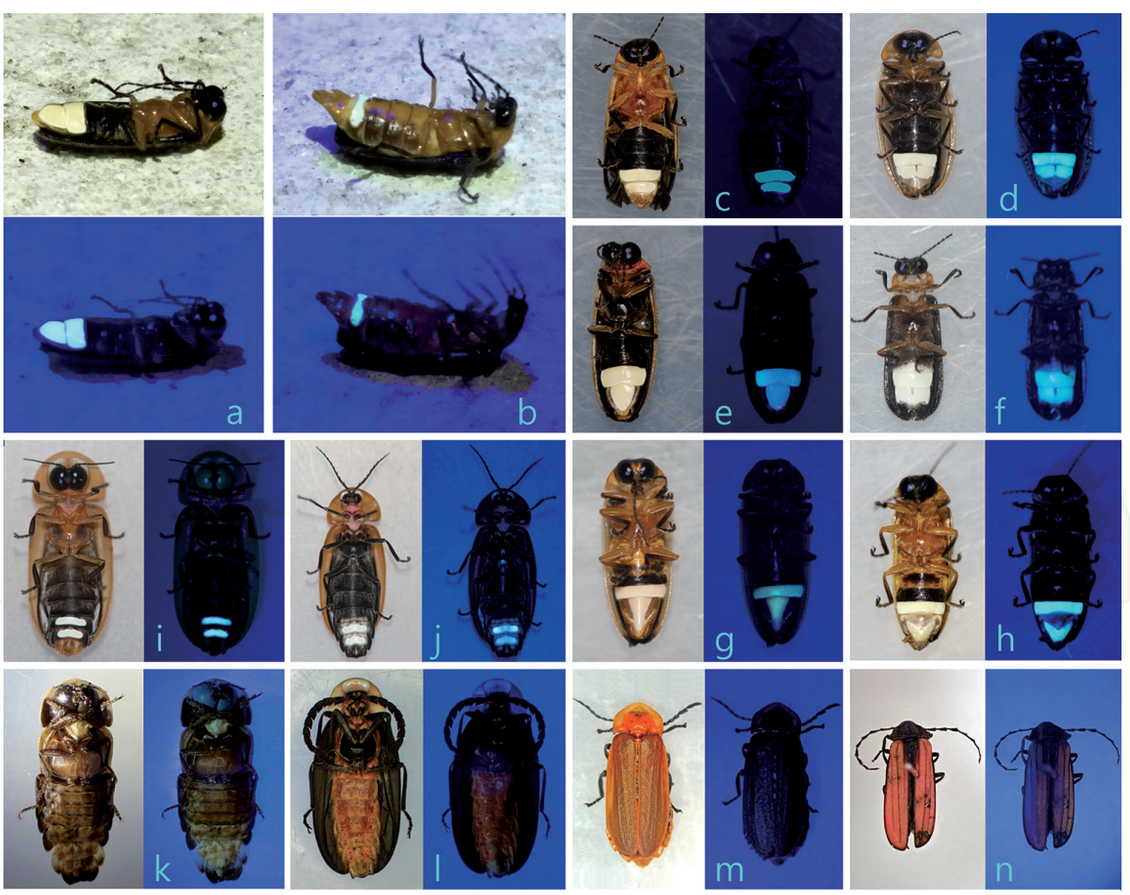

Figure 7.
Fireflies under white light/ UVA illumination. (a-b) Abscondita cerata *(T), m & f; (c)* Aquatica leii *(C), m; (d)* Asymmetricata ovalis *(M), m; (e)* Pygoluciola *sp. (C), m; (f)* Pyrophanes *sp. (M), m; (g)* Triangulara frontoflava *(M), m; (h)* Sclerotia substriata *(M), m; (i)* Diaphanes *sp. (M), m; (j)* Pyrocoelia bicolor *(M), m; (k)* Lamprigera tenebrosa *(I), m specimen; (l)* Pyrocoelia *sp. (C), m specimen; (m)* Vesta saturnalis *(C), m; (n)* Pristolycus kanoi *(T), m specimen; C=China, I=India, J=Japan, M=Myanmar, T=Taiwan; m=male, f=female. Taxonomically, (a-h) and (n) of Luciolinae, (i-m) of Lampyrinae. Ecologically, all but (l-n) are nocturnal. Under 365 nm or 395 nm near-UVA torch. By CTR and JML.*

Paedomorphic female fireflies are inferred to produce fluorescence throughout the whole body as in *Oculogryphus* because their soft cuticles constitute rich cuticular proteins which are autofluorescent [49, 108]. This is generally true, but the fluorescence is quite dim and neglectable compared to the fluorescence by photogenic organs. For example, *Diaphanes* and *Pyrocoelia* females which show moderate paedomorphism (**Figure 8a–b**) exhibit weak fluorescence through translucent cuticles. Highly paedomorphic females of *Rhagophthalmus* glowworms (Rhagophthalmidae) have a slightly brighter fluorescence (**Figure 8c**). The strong cyan fluorescence of *Oculogryphus* females appears to be an exception, though the paedomorphic degree of the females is approximate to that of *Lamprigera* and *Rhagophthalmus*. The fluorescence of *Oculogryphus* females likely results from luciferin dispersed in the whole body since they can glow bodywide. Some closely allied genera like *Stenocladius* and *Brachypterodrilus*, among others, which are capable of glowing through the whole body [114, 115], may be able to fluoresce in the same manner.

Interestingly, our team documented fluorescent pygopods in one peculiar *Pygoluciola* larva (**Figure 8d**). Pygopod is a pair of elastic and extractable suckers, serving locomotion and cleaning purposes in firefly larvae [115]. The said *Pygoluciola* larva was found near the water's edge in a mountain creek and fluoresced in a blue light under UV from its pygopods and intersegmental membranes. Surprisingly, fluids secreted by the pygopods were fluorescent as well. We examined several earth-living larvae of *Pyrocoelia* and *Diaphanes* fireflies in nearby environments and did not find a similar phenomenon.

Eggs still within females' body of a *Lamprigera* species in Taiwan exhibit vivid yellow-green fluorescence (**Figure 8e**). Its fluorescent substance is unclear since the light emission is noticeably different to the naked eye from that of luciferin, chitin, resilin, and other proteins.

Figure 8.
Paedomorphic females and larva of fireflies under white light/ UVA illumination. (a) Diaphanes *sp. (Myanmar); (b)* Pyrocoelia atripennis *(Japan); (c)* Rhagophthalmus jenniferae *(Taiwan); (d)* Pygoluciola *sp. (China), larva; (e)* Lamprigera yunnana *(Taiwan). Taxonomically, (a-b) and (e) of Lampyrinae, (c) of Rhagophthalmidae, (d) of Luciolinae. Ecologically, all are nocturnal. Notice the blue fluorescence of head and eyes in (b), and pygapod in (d); yellow green fluorescence of eggs inside female's body in (e). Under 365 nm or 395 nm near-UVA torch. By CTR and JML.*

3.3 Adaptive or not?

Noticeable UV fluorescence only occurs in luminescent fireflies and is very dim or nearly absent in diurnal and crepuscular species. Therefore, we have restricted the following discussion about the function of fluorescence to nocturnal fireflies only.

With few exceptions, luminescent fireflies are active from twilight to deep night [112, 115, 116]. During 1.3–2 hours from sunset to total darkness, the ambient light spectrum changes from long wavelength light (orange-red) dominant at sunset, to a mix of shorter wavelength light from sky (blue) and longer wavelength light from vegetation reflection (green) in twilight, to very dim blue light in early darkness, and ultimately to a dominant long wavelength light (moonless) or neutral (full moon) but low intensity at night [94, 116, 117]. In theory UV fluorescence of luminescent fireflies can occur only in twilight and early night when there is more ambient shorter wavelength light. However, we never see any fluorescing firefly with the naked eye in the field. Naturally induced UV fluorescence may happen very occasionally and is thus hard to be a reliable signal to learn or to perceive. In addition, firefly bioluminescence is much brighter and serves the duel purposes of courtship and aposematic signals. Relatively weak fluorescence is redundant as an extra signal system [112]. Furthermore, fluorescence carries no species-specific variation and thus has lower efficacy as a signal than bioluminescence does. This might also be true for firefly larvae which are night hunters and always display bioluminescence aposematism [118].

Can fireflies detect their own UV fluorescence? Recent studies provided inspiring but inconclusive cues. Most of the beetle families have three-opsin color visual system, corresponding to UV, blue wavelength, and long wavelength light (LW), respectively [119]. Transcriptome and phylogenetic analyses revealed that fireflies may have lost blue-sensitive opsin since divergence from the last common ancestor of the family [119, 120, 121]. Adult fireflies keep two visual sensitivity peaks: yellow-green light by LW opsin (λmax 550–580 nm) and near-UV light by UV opsin (λmax 360–420 nm) [121, 122]. As a result, fireflies would be insensitive to the most common emission spectra of blue and cyan fluorescence. However, several studies demonstrated that fireflies did respond to manipulation of blue light, changing their preference or flashing frequency [123, 124]. More studies are required in order to solve the discordance between genomics and ethology.

4. Conclusion

Biofluorescence occurs in all major land animal phyla and subgroups, with diverse fluorophores and performance. Co-occurrence of bioluminescence and biofluorescence is an interesting phenomenon existing in both marine and land animals and calls for more investigation. The GFP-like mechanism of fluorescence, however, has not been found in terrestrial luminescent animals. The latter emit a shorter or subequal wavelength of biofluorescence than that of bioluminescence like in glowing earthworms, millipedes, and fireflies but in reverse in the former [30, 65, 68, 102]. The light emission of luminescent land animals is the result of luciferase-luciferin interaction. The fluorescent substance may be biochemically related to the luminescent material, but does not contribute to the photogenesis given the evidence thus far [29, 65, 102–104].

The role of biofluorescence is disputed. Although some solid cases of birds and spiders are supported by experiment manipulations, most postulated functions

require further investigation. The new findings from nonfluorescent *Chaerilus* scorpions are worth a particular mention [52]. It provides a critical and inspiring example to reexamine the role of scorpions' fluorescence. The study of horseshoe crabs and the long-extinct sea scorpions [43] suggested that fluorescence is most likely an old trait that initially evolved in distant ancestors living in the sea and remained in most of the extant descends and is not necessarily an adaptation for land living scorpions. It is suggested that any assertion of adaptation should be considered under a phylogenetic framework [54]. Exceptional cases in the same or closely related groups may provide a good chance to test the postulated advantage.

Empirically, nocturnal animals or animals having cryptic living styles tend to exhibit UV fluorescence more frequently than animals that are diurnal or live in open environments (see [95]). This trend is clear in fireflies wherein only nocturnal and luminescent species exhibit noticeable UV fluorescence other than autofluorescence by chitin, resilin, cuticular proteins, etc. In contrast, diurnal or crepuscular fireflies, though having greater opportunities to be exposed to UV excitation, do not display fluorescence. It makes little sense that seldom induced UV fluorescence, if any, in the daytime or night, can serve as an efficient or reliable communication means for fireflies, either intra- or interspecifically.

It is presently premature to assert the role of fluorescence for nocturnal animals. We argue functionless should be the null hypothesis as classic scientific approach suggests. Further evidence from visual perception, physiological response, and particularly behavioral assay in both lab and field should be collected to test the hypothesis.

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Conflict of interest

The authors declare that there is no conflict of interest.

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