Research Topics

What sensory ecology might learn from landscape ecology?

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What sensory ecology might learn from landscape ecology?

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Editorial: What sensory ecology might learn from landscape ecology?

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Editorial on the Research Topic
What sensory ecology might learn from landscape ecology?

Increasing awareness by the mid-20th century that the spatio-temporal heterogeneity of the environment has a crucial impact on the flow of both matter and energy at various scales (population, community, and ecosystem level) gave rise to Landscape Ecology as ecological discipline (Naveh and Lieberman, 1984; Forman and Godron, 1986). Almost contemporarily, science witnessed the dawn of Sensory Ecology (Ali, 1978; Lythgoe, 1979; Huber and Markl, 1983; Barth, 1986; Dusenbery, 1992; recent review in Willemart, 2023), which focuses on understanding information flow in the environment (signal generation, propagation, perception, and interpretation). Just like the flow of matter and energy, information flow is far from constant owing to spatio-temporal variations of the biotic and abiotic environment (Endler, 1993; Pijanowski et al., 2011). These natural fluctuations have driven the evolution of plastic sensory systems in animals (Pyza, 2013; Maruska and Butler, 2021). However, the efficiency of information flow is threatened by rapid human modifications of the environment by increasing the acoustic, chemical, and visual noise, thereby interfering with the information necessary for communication and orientation (Pijanowski et al., 2011; Riffell et al., 2014; Duarte et al., 2021).

The present Research Topic is an effort to integrate Sensory Ecology and Landscape Ecology, calling attention to the importance of considering environmental heterogeneity in investigations of sensory adaptations of animals. A crucial first step is to measure the variation in a particular sensory landscape. In their contribution, Nilsson et al. highlight the importance of quantifying the distribution of light reaching animals' eyes in different environments. The authors demonstrate the association of vertical light gradients with weather conditions, time of day, and season. This information is vital for species that primarily use vision for finding suitable habitats, foraging, and for social interactions. Many animals, however, rely on chemical information in the environment, which is strongly influenced by the variability of air speed and direction. Analyzing air movement dynamics in a tropical dry forest in Costa Rica, DePasquale et al. found that air speed and turbulence increased with height above ground, peaked at midday, and may be lower in late than early successional parts of the forest. Species that use olfaction as primary source of information may have adapted to and even exploit this predictability of air movement patterns.

Spatio-temporal variations in the sensory environment are certainly the dominant driver of the evolution of sensory systems. Using statistical methods to control for effects of phylogenetic proximity and repeated measurements in their data sets, Huang et al. found strong evidence that relative eye size across six snake families from Taiwan changes with habitat type (bigger in terrestrial than aquatic snakes) and activity pattern (bigger in diurnal than nocturnal snakes). Thus, low light conditions associated with both aquatic and nocturnal lifestyles may have facilitated the evolution and/or improvement of sensory modalities alternative to vision, as is well-known in fish. Weakly electric fish, for instance, generate discharges of their electric organs to sense their environment and to communicate. In addition to an increased electrical activity during night time, Mucha et al. observed elevated electric organ discharges in visually complex habitats (floating vegetation in dense swamps) during the day in two species from Uganda. These findings emphasize the importance of spatio-temporal heterogeneity in light intensity concerning the use of different sensory modalities in these animals.

The evolution of signals goes hand in hand with the evolution of the sensory systems of receivers. The main drivers are sexual selection, competition, and predation. Despite their species specificity, signals may vary between and even within populations, as is the case with floral colors of a plant population in the Atacama Desert. Martínez-Harms et al. suggest that different color phenotypes, associated with different pigment compositions, are perceived differently by pollinators. This, eventually, enhances cross-pollination among individuals of the same phenotype and drives diversifying (positive) selection. By contrast, Yeager and Barnett found no evidence for positive selection in aposematic signal variation in a poison frog population from Ecuador. The authors argue that phenotype variation has not been reduced due to a weak purifying (negative) selection on a signal that is highly conspicuous to mates, rivals, and predators. In addition to sexual selection, competition, and predation, signal divergence between environments may be due to spatio-temporal variations in biotic and abiotic variables. In their contribution, Schirmer et al. show divergent color patterns in butterfly assemblages from two neighboring biomes in northeastern Brazil. The authors argue that darker wings in species from the rainforest are, presumably, associated with increased parasite-pressure, whereas lighter wing colors in the tropical dry forest may be an adaptive response to an elevated need for thermoregulation in this biome.

Although crucial for our understanding of the evolution of sensory systems and signals, information on spatio-temporal variations of the environment is frequently challenging to obtain. In their review, Chhaya et al. advocate the use of long-term acoustic monitoring to assess both the structure and the dynamics of acoustic communities (ensemble of vocalizing species in the environment), thereby providing real-time information on species distributions and movements. Similarly, Gonzales et al. propose long-term visual monitoring through remote sensing tools to map floral resource isolation and to investigate changes of resource patches over time. Such long-term monitoring techniques are key to identify anthropogenic changes in the sensory landscape that cause disturbances of information flow in the environment.

Human actions interfere with the environment at multiple levels. Anthropogenic climate change, for instance, increases the frequency of prolonged periods of excessive heat. Perl et al. investigated the impact of such heat waves during the final stage of pupal development on the behavior of a bumble bee species. The observed negative effects on vision, mechanoreception, olfaction, and taste show how human disturbances may alter the sensory systems of bumble bees and, thus, the way they perceive the environment. Yet, anthropogenic interference is not restricted to alterations of sensory systems. Signaling, as well, may be compromised in human-changed landscapes. Koneru and Caro demonstrate multiple ways of how visual signaling in animals is influenced by anthropogenic environmental changes. Human impacts range from alterations in pigment production through dietary changes to increasing colouration-background mismatches through changes in climate and landscape. Nair and Balakrishnan discuss how changes in the sensory environment interfere with the transmission and reception of acoustic sexual signals in katydids. In their study, the reduction of available signaling sites, owing to anthropogenic habitat modifications, provoked sub-optimal clustering of the males, thereby increasing competition over females.

Over the past decades, the impact of anthropogenic disturbances on ecosystem functioning has become a hot topic in Ecology. This Research Topic highlights the importance of integrative approaches, uniting Landscape Ecology and Sensory Ecology, to comprehend how natural and anthropogenically-driven environmental variations shape information flow and, eventually, natural selection in animals. Key questions for future research to answer in this context are: (1) To what extent do spatio-temporal variations in the abiotic environment at different geographic scales affect signal propagation? (2) To what extent do spatio-temporal variations in the biotic and abiotic environment drive the differentiation of sensory niches among animals? (3) Which environmental cues do animals use for decision-making, such as microhabitat choice? (4) How does anthropogenic interference influence the generation, propagation, reception, and discrimination of sensory information? We hope that this compilation of manuscripts stimulates new research in this direction, studying the sensory challenges for animals in a rapidly changing word.

Author contributions

MH, FG, PM, and DP drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Community Bioacoustics: Studying Acoustic Community Structure for Ecological and Conservation Insights

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The diversity of animal acoustic signals has evolved due to multiple ecological processes, both biotic and abiotic. At the level of communities of signaling animals, these processes may lead to diverse outcomes, including partitioning of acoustic signals along multiple axes (divergent signal parameters, signaling locations, and timing). Acoustic data provides information on the organization, diversity and dynamics of an acoustic community, and thus enables study of ecological change and turnover in a non-intrusive way. In this review, we lay out how community bioacoustics (the study of acoustic community structure and dynamics), has value in ecological monitoring and conservation of diverse landscapes and taxa. First, we review the concepts of signal space, signal partitioning and their effects on the structure of acoustic communities. Next, we highlight how spatiotemporal ecological change is reflected in acoustic community structure, and the potential this presents in monitoring and conservation. As passive acoustic monitoring gains popularity worldwide, we propose that the analytical framework of community bioacoustics has promise in studying the response of entire suites of species (from insects to large whales) to rapid anthropogenic change.

Keywords: community bioacoustics, signal space, acoustic community, passive acoustic monitoring, conservation

INTRODUCTION

With the ongoing global crisis of biodiversity loss, passive acoustic monitoring has gained popularity in both aquatic and terrestrial environments (Magurran et al., 2010; Blumstein et al., 2011; Parks et al., 2014; Linke et al., 2018; Sugai et al., 2019b), employing sound to monitor wildlife. Animal sounds are diverse and varied, and play a critical role in advertising territories and attracting mates (Marler and Slabbekoorn, 2004; Bradbury and Vehrencamp, 2011), as well as echolocation in some species (Griffin, 1958). Acoustic signal parameters and signaling strategies are influenced by noise from biotic and abiotic sources, structural features of the environment (Morton, 1975; Marten and Marler, 1977; Wiley and Richards, 1978; Wiley, 1991; Aubin and Jouventin, 1998; Nemeth et al., 2002; Blumenrath and Dabelsteen, 2004; Brumm and Slabbekoorn, 2005; Bee and Micheyl, 2008; Brumm and Naguib, 2009; Ruppé et al., 2015; Templeton et al., 2016), and the morphological constraints imposed by emitter geometry (Podos, 2001; Riede et al., 2006; Kounitsky et al., 2015). In order to compare signal parameters of different species, Nelson and Marler (1990) articulated the concept of "signal space," where signal parameters could be quantitatively represented. Multiple factors, both evolutionary and contemporary, may influence how signals are distributed in signal space. For example, acoustic competition (i.e., competition between signalers to communicate without interference) is predicted to lead to divergence in signal parameters of coexisting species, particularly those that are closely related, such that each species in an assemblage occupies a unique region in signal space [birds: (Planqué and Slabbekoorn, 2008; Luther, 2009; Krishnan and Tamma, 2016; Krishnan, 2019a; Chitnis et al., 2020), anurans: (Littlejohn, 1959; Duellman and Pyles, 1983; Chek et al., 2003), insects: (Schmidt et al., 2013; Jain et al., 2014)]. In a scenario where competition drives signal evolution, sympatric species may also signal at different times [birds:(Ficken et al., 1974; Fleischer et al., 1985; Popp et al., 1985; Brumm, 2006; Luther, 2008; Planqué and Slabbekoorn, 2008), anurans: (Sugai et al., 2021b), fish: (Ruppé et al., 2015)] or locations in space [birds: (Nemeth et al., 2002; Chitnis et al., 2020), anurans: (Hodl, 1977), insects: (Diwakar and Balakrishnan, 2007; Jain and Balakrishnan, 2012), bats: (Kennedy et al., 2014)]. The distributions of species in signal space represent a "signature" of each community, a definitive pattern that can be monitored to track ecological changes. These "acoustic communities" represent the sum of all sound-producing species in an aquatic or terrestrial landscape (Drewry and Rand, 1983; Duellman and Pyles, 1983; Luther, 2009; Sueur and Farina, 2015; Farina and James, 2016). Community bioacoustics integrates the signal space of an acoustic community with information on species composition, diversity and signaling activity to study spatiotemporal community dynamics (i.e., seasonal and spatial variation in species composition or phylogenetic diversity) (Luther, 2008, 2009; Krishnan, 2019a; Lahiri et al., 2021). The term "acoustic community structure" (**Table 1**) adapts the concept of ecological community structure, employing acoustic parameters and signaling activity as measurable traits to examine the distribution of sound-producing species in signal space, together with measures of species composition and phylogenetic diversity. The wealth of data generated by passive acoustic monitoring efforts promises valuable ecological insight, both into the distributions and movements of individual species as well as community-level processes such as seasonal turnover.

Here, we discuss how ecological processes drive acoustic community structure, and propose using acoustic communities to track ecological change. This serves to facilitate non-invasive biodiversity monitoring and conservation informed by behavior, ecology and natural history. The ubiquity and diversity of animal acoustic signals renders them promising indicators of ecosystem health (Sueur and Farina, 2015; Sugai et al., 2019b). Most conservation-oriented studies using passive acoustics typically use either single-species monitoring, or index-driven approaches. We suggest that biodiversity monitoring programs incorporate information on acoustic community composition and spatiotemporal dynamics (Desjonquères et al., 2018; Gasc et al., 2018; Krishnan, 2019a) to effectively and rapidly study species distributions and community turnover. This serves both as an additional use of passive acoustic data, as well as an alternative to other approaches in the field, depending on the needs of conservation. Community bioacoustics is thus potentially valuable to ecologists and conservation biologists working across scales from individual species to entire ecosystems.

CONCEPTUAL FOUNDATIONS

Before examining how acoustic communities inform ecological studies, it is important to understand how ecological processes drive patterns in acoustic community structure (Krishnan and Tamma, 2016; Sugai et al., 2021a). To illustrate this concept, we use some examples from some of the main hypotheses in the field. For effective communication, a high signal-to-noise ratio ensures improved detection by the receiver, thus benefiting the signaler (Endler, 1992; Wollerman and Wiley, 2002; Hart et al., 2015; Vélez et al., 2017). However, signal efficacy is reduced by masking interference from competing sound sources, such as other nearby signalers (Schwartz and Wells, 1983; Narins, 1992; Greenfield, 1994; Aubin and Jouventin, 1998; Bee and Micheyl, 2008; Balakrishnan et al., 2014). Divergence of signal properties reduces masking, enables segregation of competing sound streams (MacDougall-Shackleton et al., 1998; Krishnan, 2019b), and thus reinforces species recognition and premating isolation between close relatives (Nelson, 1988, 1989; Grant and Grant, 1996; Qvarnström et al., 2006). In diverse animals [crickets: (Schmidt et al., 2013), cicadas: (Shieh et al., 2015), aquatic insects: (Gottesman et al., 2020), fish: (Ruppé et al., 2015; Bertucci et al., 2020), anurans: (Drewry and Rand, 1983; Duellman and Pyles, 1983; Narins, 1995; Chek et al., 2003), birds: (Kirschel et al., 2009b, 2020; Krishnan and Tamma, 2016; Chitnis et al., 2020), bats: (Heller and von Helversen, 1989; Kingston et al., 2000; Luo et al., 2019), primates: (Braune et al., 2008)], closely related sympatric species exhibit divergent signals, partitioning the acoustic resource to minimize acoustic competition. Each species is therefore predicted to occupy a unique region or "niche" in the acoustic resource, a hypothesis extrapolated from ecological niche theory (Hutchinson, 1957; Holt, 2009). This realized "acoustic niche" (Farina et al., 2011; Pijanowski et al., 2011b), in the truest sense also includes partitioning at the level of receiver perception (Luther, 2008; Lemmon, 2009; Seddon and Tobias, 2010; Amezquita et al., 2011) and measurement of signal propagation distance (Marten and Marler, 1977; Nemeth et al., 2002). The presence of unintended recipients may alter signaling behavior (Ryan et al., 1982; Zuk et al., 2006). All of these factors influence the volume over which the signal elicits a response, or its "active space" (Brenowitz, 1982; Lohr et al., 2003; Bradbury and Vehrencamp, 2011), and are thus important in measuring overall signal discriminability. However, it is not always possible to investigate receiver partitioning in the field, and most studies of signal partitioning are, therefore, concerned with divergence at the level of signal parameters and the spatiotemporal distribution of signalers.

TABLE 1 | Definitions and discussion of important terms related to community bioacoustics.

Signal space	An abstract multidimensional space described by the spectral and temporal features of acoustic signals. More divergent signals are further apart in signal space. Signal space may be represented at multiple scales, including for the entire community (see below).
Signal partitioning	Signal divergence, putatively to minimize competitive overlap along multiple axes. This includes divergent signal parameters, which manifests as overdispersion in signal space. Additionally, signalers may exhibit temporal or spatial segregation in signaling activity.
Acoustic community	The sum total of all signaling species in a study site. As with an ecological community, acoustic communities may be defined at different spatial and taxonomic scales.
Community signal space	The signal space of the entire acoustic community, where each species occupies a specific volume, thus representing the diversity of signals within the community.
Acoustic community structure	The organization of an acoustic community, including distribution of species in signal space, spatial and temporal organization of signaling species, species composition, measures of signaling activity and phylogenetic diversity.

Competition, however, is not the only factor influencing signal evolution, and thus acoustic traits within communities of species. For example, habitat constraints on sound transmission are predicted to drive signal convergence toward parameters optimal for transmission in a certain habitat (the acoustic adaptation hypothesis, where acoustic signals adapt toward maximized transmission in a particular habitat) (Morton, 1975; Marten and Marler, 1977; Wiley, 1991; Snell-Rood, 2012). In bats, where time-frequency parameters of echolocation pulses have a significant effect on perception and navigation, species occupying the same habitat may possess convergent signals (Parsons and Jones, 2000; Jones and Holderied, 2007). Other vocalizations may also converge owing to conserved function, including alarm calls that are critical for survival (Marler and Slabbekoorn, 2004; Braune et al., 2008). Acoustic adaptation remains debated in the field (Ey and Fischer, 2009), and more studies are necessary to elucidate how ecological processes drive acoustic signal evolution. Patterns of divergence or convergence of signals may also be driven by other factors. For example, sexual selection may drive signal divergence by female choice (Seddon et al., 2013), whereas communication between competing species (extended communication networks) may drive signal convergence (Tobias et al., 2014). Alternatively, neutral processes may drive entirely different patterns in signal evolution, such as signals resembling each other on account of phylogenetic relatedness (Wilkins et al., 2013). Thus, although we discuss patterns of divergence or convergence as examples to illustrate our analytical framework, we note that the drivers of these patterns are diverse. Regardless of this, the patterns in acoustic community structure may themselves provide valuable information, and we use the examples discussed above to illustrate the utility of this approach.

A FRAMEWORK TO QUANTIFY ACOUSTIC COMMUNITY STRUCTURE

The first step in understanding acoustic community structure is to quantify the acoustic traits of all species in a community. The concept of signal space provides a means to quantitatively interrogate whether the signals of coexisting species overlap. Broadly, signal space encompasses spectral and temporal properties of a sound (**Figure 1**), by quantifying parameters of a vocal unit, for example, a note (parameters include peak frequency and note duration, to name a few) and representing them as a point in multidimensional space. The

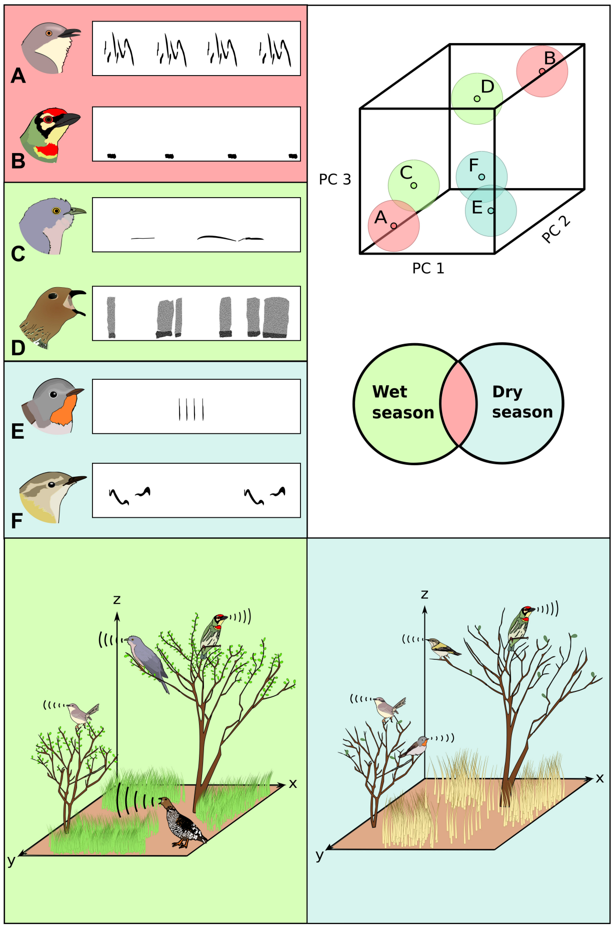


FIGURE 1 | Signal space (top right), derived using principal components analysis on acoustic signal parameters of diverse species (**A–F**, top left) enables study of acoustic communities across spatiotemporal scales, including different seasons (color codes). Signal space for each species is represented as the volume occupied by the entire repertoire (larger circles, with the smaller point representing the centroid). Bottom panels: Acoustic community structure varies along both spatial and temporal axes (color codes represent different seasons). This figure loosely depicts some bird species from an acoustic community in western India for illustrative purposes, demonstrating seasonal turnover, with species replacing each other, and spatial segregation, with each species singing from a preferred location in space (Krishnan, 2019a).

entire repertoire forms a cloud of such points, the volume of which represents each species' signal space (Nelson and Marler, 1990), which is constrained both by physical characteristics and the transmissibility of sound (Wilkins et al., 2013). Signal space of diverse sensory signals can be examined at scales ranging from individual species to entire communities or lineages, analogous to a functional trait space in community ecology (Endler, 1992; Stoddard and Prum, 2008; Luther, 2009; Krishnan, 2019a; Chitnis et al., 2020; Krishnan et al., 2020; Lahiri et al., 2021). As an outcome of diverse ecological processes, an acoustic community exhibits organization within signal space. Using the tools of community ecology and community phylogenetics, we may obtain a series of metrics that quantitatively represent the acoustic community, and we lay these out below.

At the outset, it is important to quantify species diversity and signal space of any acoustic community. For the first, a census of species detected in an acoustic dataset, together with measures of how frequently their vocalizations are detected, provides an estimate of community diversity. In order to quantify community signal space, a call library consisting of the signals of each species is digitized to extract signal parameters (Luther, 2009; Krishnan, 2019a; Lahiri et al., 2021), which are then ordinated using multivariate methods such as principal components or linear discriminant analysis. Thus, all sounds in a community are represented as points in signal space. Ideally, the digitized signals should represent intra- and interspecific variability in signals within the population under consideration, and may be obtained either directly from passive recordings or by making focal recordings to build a call library before passive sampling. The latter is more reliable in poorly studied areas, as it enables researchers to match vocalizations to species before passive sampling.

Signal partitioning is an example of a process which is predicted to lead to overdispersion, where points representing the different acoustic signals are further apart than expected by chance (Chek et al., 2003). The converse is true in case of signal convergence, where clustering around a central tendency is predicted. Clustering may also arise due to phylogenetic relatedness of coexisting species, as the signals of close relatives are likely to resemble each other (i.e., they are phylogenetically conserved) (**Figure 2**), and quantifying the phylogenetic signal in acoustic traits provides a way to test these hypotheses. Studies have uncovered significant overdispersion in some bird acoustic communities (Luther, 2009; Krishnan, 2019a; Chitnis et al., 2020). These studies hypothesize that acoustic competition may drive the structure of the acoustic community by divergence of time-frequency parameters. Other studies, however, suggest that environmental filtering (for example, acoustic adaptation to habitat features) may result in community convergence instead (Planqué and Slabbekoorn, 2008; Cardoso and Price, 2010). These examples illustrate how the various drivers of signal evolution may lead to distinct patterns in the signal space of a community.

In order to statistically examine overdispersion versus convergence in signal space, studies typically employ null model tests (Chek et al., 2003; Chitnis et al., 2020; Sugai et al., 2021a). Null model tests in community ecology compare observed interspecific trait differences or co-occurrence patterns to those in randomly distributed "null" communities (Harvey et al., 1983; Gotelli, 2000; Gotelli and Entsminger, 2001; Gotelli and McCabe, 2002). These tests thus inform us whether species are more likely to co-occur, or certain community trait values are more likely to exist than expected by chance. When studying acoustic communities, null model tests are employed to detect whether species are further apart or closer in signal space than expected

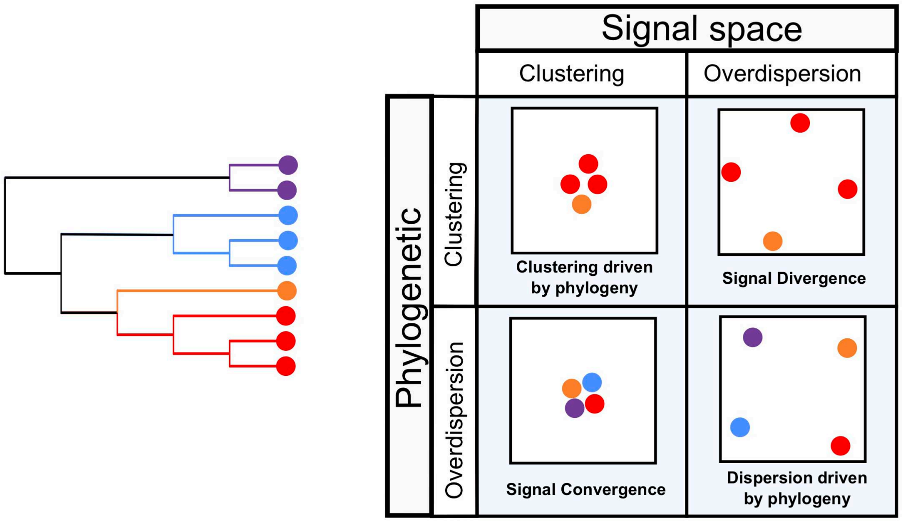


FIGURE 2 | Community phylogenetic analyses and signal space. Phylogenetic information is an important component in our understanding of acoustic community assembly and structure. In a hypothetical, simplified series of communities drawn from the phylogeny on the left [inspired by the principles of community phylogenetics (Webb, 2000; Cavender-Bares et al., 2004)], phylogenetic overdispersion or clustering combined with similar patterns in signal space suggest that phylogenetic relationships drive acoustic community structure (top left and bottom right squares), whereas other patterns may be driven by signal partitioning or convergence, among other processes (top right and bottom left).

by chance (Chek et al., 2003). Interspecific distances greater than those in the null communities are consistent with overdispersion, or signal divergence, whereas clustering is consistent with signal convergence or phylogenetic conservatism (Planqué and Slabbekoorn, 2008; Cardoso and Price, 2010; Schmidt et al., 2013; Tobias et al., 2014).

With detailed phylogenetic information now available for many taxa that produce acoustic signals (Jetz et al., 2012; Shi and Rabosky, 2015), the tools of community phylogenetic analysis (Webb, 2000; Cavender-Bares et al., 2004; Kembel et al., 2010) help us test hypotheses about the role of processes such as competition or environmental filtering in driving the distribution of points in signal space (**Figure 2**). These methods combine quantification of community phylogenetic structure and diversity with trait space patterns to understand community assembly and organization. For example, overdispersion in signal space coupled with phylogenetic clustering is a pattern consistent with signals of close relatives within a community diverging, potentially to minimize competitive overlap. Conversely, phylogenetic overdispersion coupled with clustering in signal space is consistent with the hypothesis of community convergence. Measures of community phylogenetic dissimilarity and beta-diversity (Ives and Helmus, 2010) may also be used to quantify species turnover in acoustic communities. Although care should be applied when interpreting patterns in community phylogenetics (Gerhold et al., 2015), these analyses provide a preliminary understanding of the relationship between phylogenetic structure and signal space (Lahiri et al., 2021). Additionally, by comparing phylogenetic community structure to different trait spaces, we may investigate the contribution of indirect effects (such as morphological divergence) to acoustic community structure (Krishnan and Tamma, 2016). These indirect effects may be important, particularly in light of the relationship between morphological characters and acoustic traits. Phylogenetic analysis thus opens up important avenues in community bioacoustics, and when combined with trait data, provides insight into the organization and turnover of acoustic communities.

SPATIAL AND TEMPORAL DIMENSIONS OF THE ACOUSTIC COMMUNITY

Divergent signal parameters represent only one aspect of acoustic signal partitioning. In addition, many organisms also signal at different times (temporal partitioning, distinct from the temporal properties of the notes themselves, which are used to calculate signal space) or locations in space (spatial partitioning). Temporal partitioning may involve fine-scale adjustments to signal timing and repetition rate, resulting in interdigitation of the signals of different individuals [birds: (Cody and Brown, 1969; Ficken et al., 1974; Fleischer et al., 1985; Popp et al., 1985; Brumm, 2006)]. Alternatively, both terrestrial and aquatic species may simply signal at different times of the day from each other, or even in different seasons [birds: (Luther, 2008; Krishnan, 2019a), bats: (Adams and Thibault, 2006; Mancina et al., 2012), fish: (Ruppé et al., 2015; Bertucci et al., 2020), marine mammals: (De Vreese et al., 2018)]. Further research is required to understand if diel-scale temporal partitioning results indirectly from ecological separation in foraging time, or by adjustments to the presence of competing signalers. Spatial partitioning, on the other hand, involves divergence in the locations from which the signal is emitted (Hodl, 1977; Bee, 2008). Segregation in space or stratification of singing heights have been documented in diverse taxa [insects: (Diwakar and Balakrishnan, 2007; Jain and Balakrishnan, 2012), birds: (Nemeth et al., 2002; Chitnis et al., 2020), bats: (Kalko and Handley, 2001; Kennedy et al., 2014)]. This pattern is putatively linked to maximizing sound transmission at a particular location (Miller and Degn, 1981; Dabelsteen et al., 1993; Nemeth et al., 2002; Marler and Slabbekoorn, 2004; Mathevon et al., 2005; Barker and Mennill, 2009; Kirschel et al., 2009a; Sprau et al., 2012; Núñez et al., 2019), or to indirect partitioning as a result of other ecological processes (Jain and Balakrishnan, 2012; Kennedy et al., 2014; Chitnis et al., 2020). For example, competition for resources may also drive signal partitioning as a by-product (Aldridge and Rautenbach, 1987; Norberg and Rayner, 1987; Kingston et al., 2000; Kingston and Rossiter, 2004; Siemers and Schnitzler, 2004; Kirschel et al., 2009b; Mancina et al., 2012; Krishnan and Tamma, 2016; Roemer et al., 2019). On the other hand, acoustic adaptation is hypothesized to lead to convergence, where species with similar signals occupy similar regions in physical space to maximize sound transmission (Boncoraglio and Saino, 2007). Patterns such as these highlight how distinct ecological processes may both directly and indirectly (Wilkins et al., 2013) drive signal partitioning, and thus acoustic community structure, along multiple axes. These axes range from signal parameters (and thus distributions of species in signal space) to the spatial and temporal distribution of signaling species.

Finally, the radiation and propagation patterns of emitted sound are an oft-ignored aspect of signal partitioning. Radiation patterns are shaped dynamically by the morphology of the sender aperture (Feng et al., 2012; Kounitsky et al., 2015), and emitted sounds are directed by the aim of the head (Ghose and Moss, 2003). Directionality of the signal impacts the perceived amplitude (Naguib, 1995), and aiming competing signals away from each other may further reduce interference, enabling signalers to avoid overlap in their active spaces. Signal amplitude is highly consequential for signal perception and attention (Dantzker et al., 1999; Greenfield and Rand, 2000; Brumm and Todt, 2003; Patricelli et al., 2007; Yorzinski and Patricelli, 2010), and for targeting biosonar in bats (Surlykke et al., 2012; Jakobsen et al., 2013). However, relatively few studies have quantified signal directionality and aiming in the field. This is in part because one requires an array of calibrated microphones, as well as estimates of distance from the source, head aim, horizontal and vertical angles to measure sound directionality accurately in the field (Blumstein et al., 2011). By quantifying signal directionality, the principles of engineering acoustics are directly applied in ecological studies to, for example, understand whether behavioral changes (such as aiming of signals in different directions) reduce the overlap of competing signals in certain environments. In addition, the use of multiple microphones to study these patterns may also prove useful in estimating the relative numbers of different signaling species using triangulation (Blumstein et al., 2011; Mennill et al., 2012). Although sound propagation patterns may differ in aquatic environments, similar general principles and techniques may be used to quantify both signal partitioning (Ruppé et al., 2015; Bertucci et al., 2020) and acoustic community structure (Hannay et al., 2013; De Vreese et al., 2018; Desiderà et al., 2019; Mooney et al., 2020). Using community structure and composition, including the distributions of species in signal space, we may obtain valuable ecological and conservation insight from non-invasive acoustic data, and we explore these possibilities in the sections below.

ACOUSTIC COMMUNITY STRUCTURE AS A BAROMETER OF ECOLOGICAL CHANGE

The patterns and processes discussed above imply that each acoustic community is likely to possess a distinct structure, driven both by the diversity of signals (and their distributions in signal space) as well as the spatial and temporal distributions of signaling species. Because communities are not static, ecological changes (spatial and temporal) are likely to be reflected in acoustic community structure, and we may thus use this framework to quantify and detect these changes. For example, seasonal changes such as hibernation or migration may alter the composition and phylogenetic diversity of both terrestrial and marine acoustic communities (Hannay et al., 2013; Putland et al., 2017; De Vreese et al., 2018; Krishnan, 2019a). Additionally, acoustic communities may exhibit small and large-scale spatial heterogeneity, because habitat preferences of signaling species drive differences in distribution (Van Parijs et al., 2001; **Figure 3**). Quantifying signal space helps us understand seasonal dynamics and habitat occupancy of acoustic communities, providing real-time information on species distributions and movements. This data potentially enables us to detect arrivals and departures both along migration routes and wintering grounds by measuring turnover in the acoustic community. Further studies could then use multi-year acoustic data to quantify the effects of extensive urbanization or shipping traffic on the migration and community structure of birds, bats and marine mammals. Human impacts are predicted to profoundly affect migrant numbers and timing of arrival, and community bioacoustics provides an early warning of these changes for terrestrial and aquatic ecosystems (Sanders and Mennill, 2014). Studies have quantified phenology (Buxton et al., 2016), or seasonal ecological patterns in bird communities over large geographical areas. Many studies of this variety utilize ecoacoustic or soundscape indices to quantify broad changes in sound profiles (Villanueva-Rivera et al., 2011; Sueur and Farina, 2015). Indices typically either quantify attributes of a recording, or compare recordings to each other in a manner similar to quantifying beta-diversity (Villanueva-Rivera et al., 2011; Desjonquères et al., 2020). Although useful for rapid assessments, they do not provide species-level information, and may sometimes provide conflicting results with census methodologies (Lellouch et al., 2014; Krishnan, 2019a). Thus, studies operating at multiple scales should combine the use of ecoacoustic indices with more detailed analyses of the kind we propose here. The quantification and utility of soundscape indices has been covered in multiple reviews and publications (Farina et al., 2011; Pijanowski et al., 2011a,b; Sueur and Farina, 2015; Gasc et al., 2017; Buxton et al., 2018a,b; Gibb et al., 2019), and the two approaches are compared and contrasted in **Table 2**. Thus, although our review focuses more on the use of acoustics to study community ecology and change, using the acoustic community as a unit, a combination of different approaches may prove fruitful for future studies.

For instance, by combining indices with census of changes in species composition, together with change in the community signal space, we can detect the effects of migration on community structure (Krishnan, 2019a). The strength of community bioacoustics lies in providing detailed single-species information as well as data on overall community diversity (**Table 2**). Gathering these data across seasons or multiple years provides a non-invasive way to track temporal community change. In addition to migration, many bat species enter hibernation in inclement weather, resulting in their absence from the acoustic community. Hibernating bats are very sensitive to environmental changes (Fenton, 2012; Nocera et al., 2019). Long-term community acoustic data potentially provides information on whether individual species are declining, on changes to hibernation timings, and changes in overall community diversity over time.

Heterogeneity in habitat and species distributions across a landscape is also putatively reflected in a corresponding heterogeneity of acoustic communities (Luther, 2009). Acoustic monitoring has proved very useful in understanding occupancy of species which are hard to detect using traditional survey methods (Weller and Baldwin, 2012; Kalan et al., 2015; Campos-Cerqueira and Aide, 2016; Law et al., 2018; Gibb et al., 2019; Abrahams and Geary, 2020), and this provides a starting point to quantify spatial structure in the acoustic community. Occupancy, or the proportion of sites occupied by a species of interest, provides a quantitative estimate of the distribution and spread of a species. Dynamic multispecies occupancy models enable quantitative description of spatial heterogeneity in the acoustic community, as well as detection of the effects of environmental change on the relative distributions of species (Dorazio et al., 2010; Iknayan and Beissinger, 2018). There is a need for more large-scale multispecies occupancy studies using acoustics, in order to comprehensively quantify spatial community structure. This, together with metrics of community beta-diversity (Avolio et al., 2019), enables site-by-site spatial diversity comparisons. We may then test two contrasting hypotheses: does signal similarity drive spatial segregation in species distributions, or does habitat structure indirectly structure the acoustic community (Sugai et al., 2021a)? Similar analyses have also proved useful in understanding how isolation on islands influences the acoustic community, and thus signal partitioning in birds (Robert et al., 2019). This study framework therefore has great value in understanding the relationship between biogeography and acoustic community structure. Combining data from multiple recorders with triangulation algorithms will enable estimates

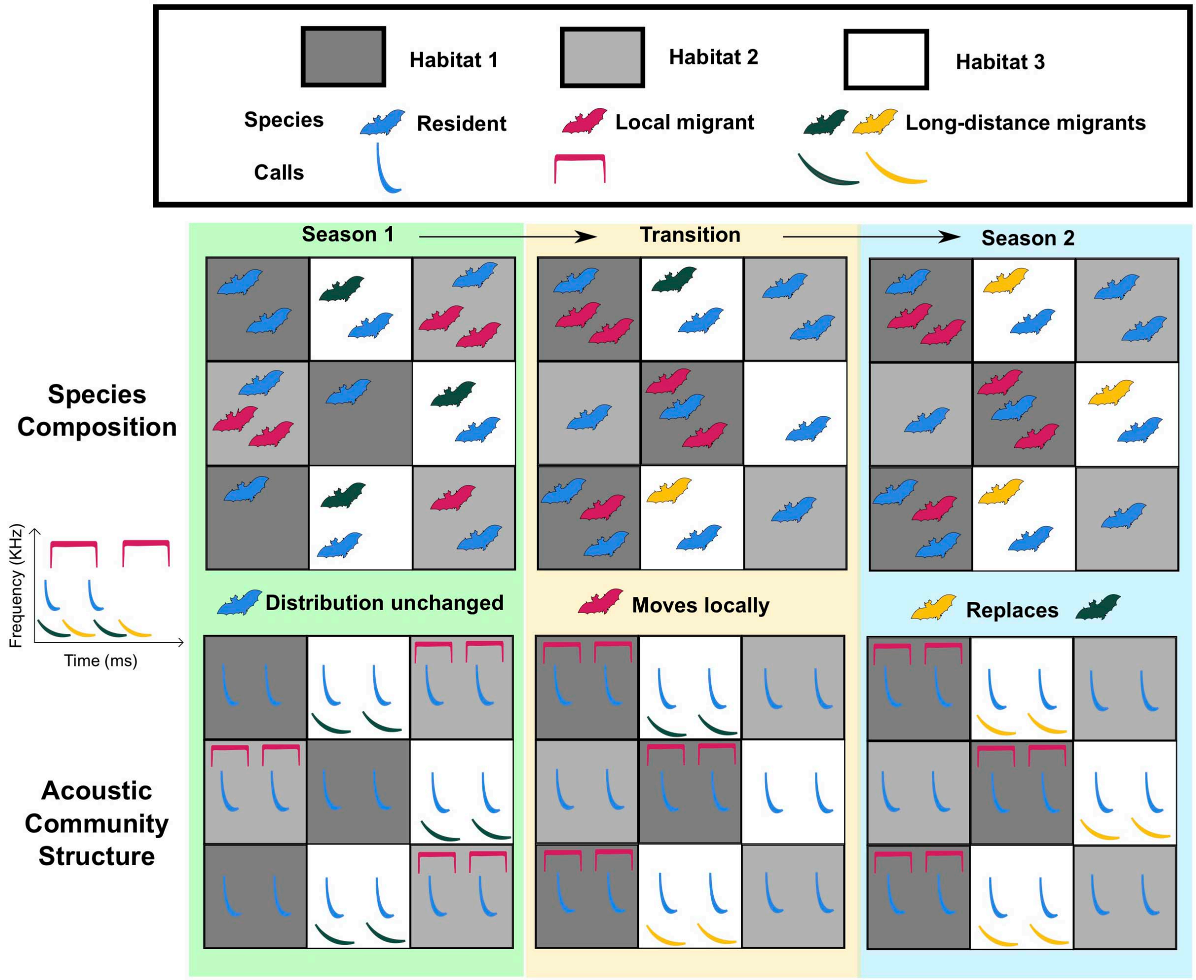


FIGURE 3 | Ecological changes are reflected in the acoustic community. Spatiotemporal change in a hypothetical bat acoustic community (consisting of resident and migrant species). Each habitat within this landscape is represented by a square, with colors representing different habitat types. Each bat species calls at a distinct frequency (see key on the left), and species composition in each cell changes with seasonal transitions (top row). Recorders placed in each square are predicted to detect spatiotemporal changes in acoustic community structure (bottom row), serving as a framework for long-term monitoring. Similar changes may also occur due to hibernation or seasonal differences in signaling activity.

of density and relative numbers of each signaling species in the community, and even open up the possibility of tracking local movements of taxa such as marine mammals (Blumstein et al., 2011).

CURRENT LIMITATIONS IN COMMUNITY BIOACOUSTICS, AND POSSIBLE SOLUTIONS

In spite of the usefulness of community bioacoustics, certain limitations must be considered as areas for future streamlining and improvement. Firstly, scientific studies undertaking biodiversity inventories including passive acoustics should be mindful of taxa that are silent or infrequently vocal, and use a combination of survey methods to detect all species (Darras et al., 2018; Wheeldon et al., 2019). For example, relatively silent birds such as storks, or fruit bats that do not produce echolocation calls, may be more reliably detected by visual surveys. Thus, the acoustic community is best treated as a surrogate of the ecological community, and as an indicator of its diversity rather than an exact inventory. Secondly, censusing massive acoustic data to quantify community structure can be labor-intensive. Recent advances in automated species recognition provide hope of a streamlined analytical pipeline (Mac Aodha et al., 2018; Ulloa et al., 2018; Stowell et al., 2019). However, in understudied tropical regions, comprehensive call libraries are a relatively recent phenomenon, and there is a global paucity of information on many taxa (Chakravarty et al., 2020; Desjonquères et al., 2020). More data is needed to improve automated recognition, particularly in high-noise environments where the sounds from multiple vocal species may result in masking that interferes with many recognition algorithms. In the meantime, researchers in these regions should

TABLE 2 | Comparison of the information provided by quantifying acoustic community structure using census methodology versus quantifying acoustic diversity using ecoacoustic indices.

	Census-based measurements of acoustic community structure	**Measuring ecoacoustic indices from audio files**
What are these measurements?	The composition and organization of species in an acoustic community, over space and time.	Metrics of diversity in the acoustic spectrum calculated directly from recorded sound files.
What is calculated?	Involves quantifying signal space, species and phylogenetic diversity, and measurements of signaling activity for the acoustic community.	Involves calculating indices of acoustic complexity within an audio recording, or between recordings from different sites.
What sounds do they measure?	Census methodology only includes biotic sounds.	Indices may be influenced by both abiotic and anthropogenic sound as well as biotic sounds.
What sort of data do they provide?	Provides single-species spatiotemporal data as well as community-level information on phenomena such as seasonal migration.	Provides broad information on soundscapes and overall spatiotemporal changes in the acoustic spectrum at the level of landscapes.
What additional data is required?	Census of large datasets requires a reference library for identification.	Analyses are carried out directly on recordings and do not use a reference library.
How might these different analyses support conservation?	Provides natural history information for detecting movements and quantifying distributions of suites of species, and monitoring both individual threatened species and entire ecosystems.	Provides rapid, large-scale assessment at a relatively coarse resolution, typically at the landscape level.

consider randomized subsampling of large datasets, (Krishnan, 2019a) which may provide a more tractable method to census the acoustic community. This approach will also reduce the computational power required.

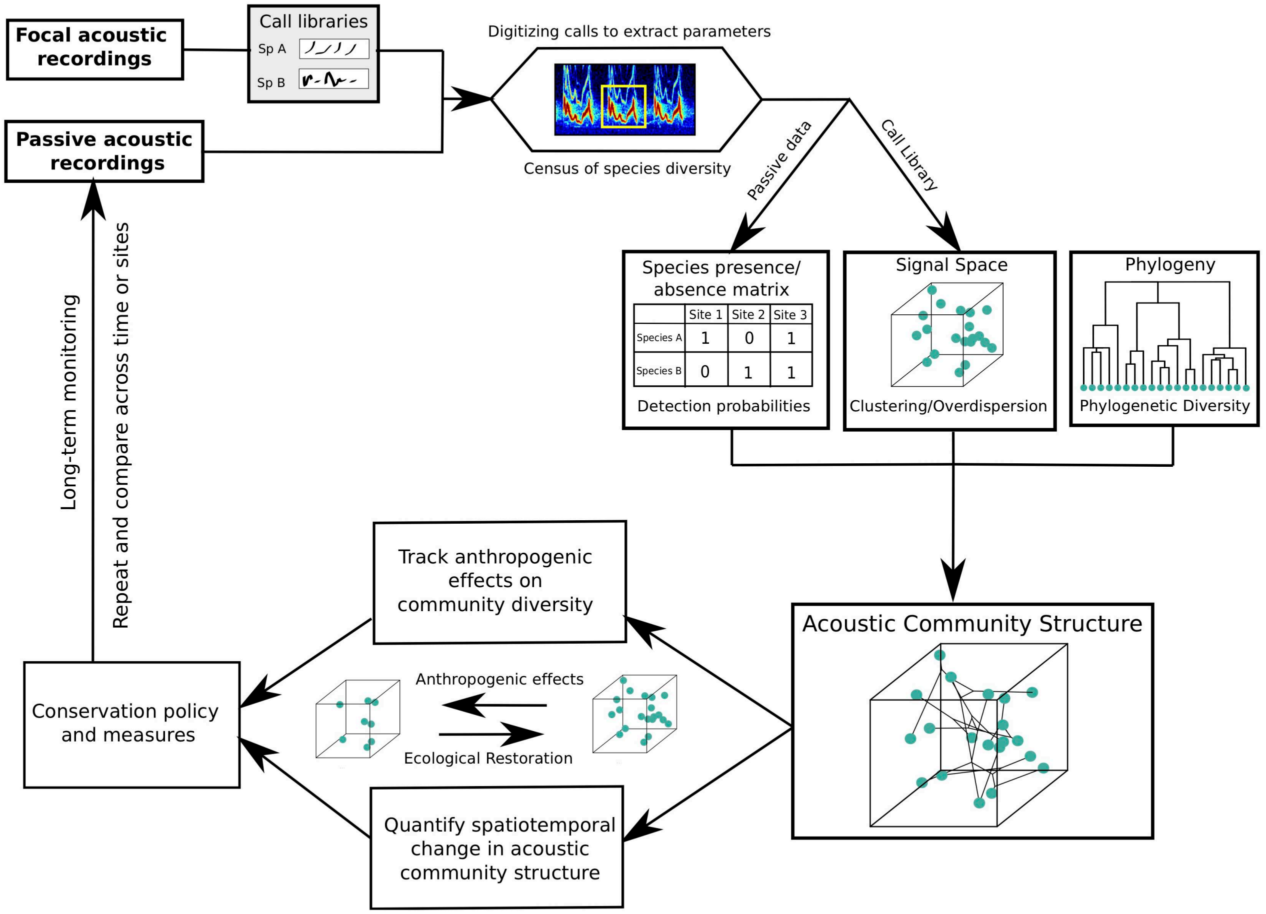
Another issue generally faced by passive acoustic studies is correcting for detectability in estimates of occupancy and community composition (Darras et al., 2016). This is particularly relevant for bats and dolphins, whose ultrasonic signals attenuate quickly over distance in a frequency-dependent manner. Low signal-to-noise ratios further accentuate this problem, reducing detectability of calls (Sugai et al., 2019a). Thus, false absences of higher-frequency species must be accounted for in any study, potentially by correcting the data to account for differences in detectability (Meyer et al., 2011) or by matching visual and acoustic survey data (Richman et al., 2014). Playback experiments using speakers may provide a detection space for each species in an environment, which can then be implemented in an occupancy-based model. Combining this method with triangulation, as described earlier, may help refine estimates and even provide relative abundance data for each species in a community (Mennill et al., 2012). This is an important area of future research into expanding the possibilities of acoustic monitoring.

SYNTHESIS: COMMUNITY BIOACOUSTICS IN LONG-TERM BIODIVERSITY MONITORING AND CONSERVATION

As the Anthropocene brings with it rapid changes in the earth's biodiversity, long-term biodiversity monitoring is the need of the hour to inform policy and conservation (Magurran et al., 2010). Non-invasive monitoring methods, in particular, are powerful sources of continuous ecological data. For this reason, passive acoustic monitoring has increased in popularity both in marine and terrestrial environments. Human activities such as land-use change could fundamentally alter spatiotemporal acoustic community structure (Zuk et al., 2006; Gasc et al., 2018; Torrent et al., 2018; Pillay et al., 2019), at the extreme resulting in the loss of natural sounds (Pijanowski et al., 2011a; Sueur et al., 2019). Quantifying these effects is very important in developing conservation policy for diverse ecosystems, from forests and deserts to the ocean depths. Studying how anthropogenic change influences spatiotemporal community composition could inform conservation planning and management, by serving both as an early warning system and as a barometer of the extent of ecological change.

As recorders become increasingly cost-effective (Hill et al., 2019), the potential for acoustic monitoring, particularly in tropical countries that harbor most of the world's biodiversity, is tremendous (Wrege et al., 2017). With these developments comes a need for capacity-building not just in the collection of data, but also in building robust analytical frameworks that support science and conservation planning. As a highly biodiverse tropical country, the example of India serves to illustrate the global promise of community bioacoustics, and the need for more studies utilizing these methods. There are still relatively few passive acoustic studies in India (Diwakar et al., 2007; Buxton et al., 2018a; Krishnan, 2019a; Lahiri et al., 2021), and most acoustic sampling relies on call counts or focal recordings (Khaling et al., 1998; Ghose et al., 2003; Agnihotri et al., 2014; Purushotham and Robin, 2016). For lesser-known Indian taxa such as bats, comprehensive call libraries are few (Raghuram et al., 2014; Wordley et al., 2018; Chakravarty et al., 2020). Expanding the study of acoustic community structure will thus be extremely valuable in understanding and conserving global biodiversity. The ability to rapidly collect large amounts of data in the absence of observers sets passive acoustics apart from focal community sampling (Haselmayer and Quinn, 2000; Abrahams, 2019). Even when deploying passive recorders for single-species surveys, a wealth of community-level information may be obtained. Most studies we have illustrated in this review, however, focus largely either on single species, or use indices for broader comparisons. By studying acoustic community dynamics, deriving a community signal space based on acoustic parameters, and quantifying the diversity of communities in different habitats, we can build a comprehensive tapestry of signaling species across landscapes. This enables us to obtain information both on individual species, as well as entire communities, using the same datasets as other passive acoustic studies. For example, by recording the acoustic community, we may identify the habitat preferences of each individual species (as detailed above), compare different sites to measure differences in community composition and diversity across different habitat types, and also track change in the overall community (and individual species) over time. Therefore, if anthropogenic activity alters the distribution of a certain species, this will be detected by passive recorders, and will also change community composition over time. By measuring the effect of anthropogenic changes on community turnover (for example, beta-diversity as a response variable), we can obtain valuable ecological information across scales.

For threatened species, acoustic community structure, diversity and dynamics at known sites is a useful barometer of ecosystem health. Studies using acoustics to date primarily focus on presence-absence of specific species, or on occupancy patterns, as detailed earlier. However, community-level data provides considerably more in the way of potential opportunities for conservation insight. For example, we can identify what species typically co-occur with a certain threatened species in the acoustic community, and the patterns of community structure in its preferred habitat. This could potentially be used to identify new priority sites for survey, reintroduction or conservation of these species, and to evaluate the success of reintroduction and restoration efforts (Lewis et al., 2021). Sites that possess the requisite suite of co-occurring species may be suitable for reintroduction of a threatened species, and community structure may be monitored after reintroduction, to measure whether it returns to a pattern similar to the source site. Such efforts are particularly relevant in insular ecosystems, where eradication of invasive species and translocation of native species is being



	Site 1	Site 2	Site 3
Species A	1	0	1
Species B	0	1	1

FIGURE 4 | A generalized work flow highlighting the utility of community bioacoustics in long-term monitoring and conservation. By combining a quantification of community signal space with phylogenetic analyses and passive acoustic census of spatiotemporal community diversity, we may track ecological changes, inform conservation measures and also gauge their success over time.

undertaken to restore ecological communities (Borker et al., 2020). Acoustic community structure could provide a well-defined, easily quantified metric of the success of these efforts in restoring native insular communities of birds, frogs, insects, and other signaling animals.

Community bioacoustics will further enable quantification of long-term change in global ecosystems. This, in turn, will help understand how human-induced environmental changes impact global biodiversity, from densely populated cities to the ocean depths. Although approaches driven by indices are gaining popularity in passive acoustic studies, there is also great benefit in approaches that provide species-level, natural history-driven information that is of direct use in informing conservation policy. Community bioacoustics, and specifically acoustic community structure, is an approach that provides this information, from the entire community down to individual species (Krishnan, 2019a; Pillay et al., 2019). By quantifying the distributions and volume occupied by species in community signal space, together with phylogenetic diversity (Lahiri et al., 2021; **Figure 4**), field researchers may compare them across environmental conditions or grades of anthropogenic impact. For instance, we can compare signal spaces across different land-use types, to examine how the distributions of species within signal space change. This enables a non-invasive estimate of how land-use change impacts community diversity, phylogenetic diversity and the composition of communities of sound-producing species. The same data can also be used to track how detections of individual species change across land-use types. These comparisons thus provide critical data on acoustic community structure, which reflects the ecological impacts of anthropogenic change. By broadening the use of community bioacoustics, we can thus apply an interdisciplinary understanding of acoustic signaling to inform effective global biodiversity monitoring and conservation.

AUTHOR CONTRIBUTIONS

VC, SL, MJ, and AK contributed to figure conception and design. All authors listed made substantial, direct and intellectual contributions to the text, and approved this manuscript for publication.

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Ecological Constraints on Sexual Selection in a Human-Modified Landscape

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Anthropogenic activities are changing the sensory landscape, interfering with transmission and reception of sexual signals. These changes are leading to alterations in mating behaviour with consequences to fitness. In systems where mate-finding involves long-distance signalling by one sex and approach by the other sex, the spatial distribution of signallers can have implications for male and female fitness. Spatial distribution of signallers is typically determined by an interplay of multiple factors, both ecological and evolutionary, including male competition, female choice and resources, such as calling and oviposition sites. We investigated the possible influence of resource distribution (signalling sites) on the strength and direction of sexual selection acting on false-leaf katydid *Onomarchus uninotatus* males, signalling in a human-modified landscape in the Western Ghats, India, a biodiversity hotspot. The landscape has changed from evergreen forests to plantations owing to human settlements. We first determined the spatial distribution of calling males and of available calling sites, which are trees of the genus *Artocarpus*, in the landscape. Using the information on male spacing, call transmission and hearing thresholds, the perceptual spaces of male signals were computed to understand the acoustic environment of calling males and females. It was found that both calling males and females could hear calls of males from neighbouring trees with a probability of 0.76 and 0.59, respectively. Although calling males were found to be spaced apart more than predicted by chance, significant overlap was seen in their acoustic ranges. Clustering of males enables females to easily sample multiple males, facilitating mate choice, but is detrimental to males as it increases competition for females. Using simulations, we determined the optimal spatial distributions of *O. uninotatus* males for female choice, and for reduction of male competition, given the signalling site distribution. The observed distribution of signallers was then compared with the hypothetical optimal distributions to examine the drivers of signaller spacing. Spacing of calling males in the field was found to be not optimal for either males or females. Resource distribution was found to limit the effectiveness of sexual selection drivers in pushing male spacing toward fitness optima of males or females.

Keywords: acoustic signals, acoustic range, female mate choice, male competition, Orthoptera, spatial distribution, anthropogenic disturbances

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INTRODUCTION

In many animal species, mate-finding involves signalling by one sex and approach by the other (Bradbury and Vehrencamp, 1998), wherein the environment can have a direct bearing on the success of individuals in finding a potential mate (Römer, 1998). Anthropogenic disturbances to the environment can interfere with different sensory and physiological processes in organisms, affecting the expression, transmission, and reception of signals (Kern and Radford, 2016; Gurule-Small and Tinghitella, 2018). As a result, this can affect the perception of signals and mate-choice decisions of the receiver, leading to changes in the intensity of intra- and inter-sexual competition (Bent et al., 2021; Pilakouta and Ålund, 2021). For example, Candolin et al. (2007) showed the effect of reduced visibility in the breeding habitats of the three-spined stickleback *Gasterosteus aculeatus* owing to eutrophication. There was an increase in the courtship effort of males but without a proportional increase in mate attraction. In the red mason bee, *Osmia bicornis,* temperature has been shown to affect odour plumes, influencing female choice (Conrad et al., 2017). This, in turn, can affect the overall strength and direction of sexual selection.

The two components of sexual selection, male competition and female mate choice (Darwin, 1871), can act differently on traits in terms of the direction and strength of selection (Qvarnström and Forsgren, 1998; Moore and Moore, 1999; Candolin, 2004; Wong and Candolin, 2005; Yang and Richards-Zawacki, 2021). Female mate choice is expected to select for "superior males" with competitive advantage over other males, conferring direct or indirect benefits to the female (Qvarnström and Forsgren, 1998; Wong and Candolin, 2005). Under this assumption, male competition and female mate choice can favour the same traits, leading to reinforcing selection on these traits (Andersson, 1994; Berglund et al., 1996; Wiley and Poston, 1996). However, in cases where selection favouring male traits introduces costs on females, the interplay between the two forces can be counteracting (Qvarnström and Forsgren, 1998; Moore and Moore, 1999; Bussiere, 2002; Pitnick and García-González, 2002; Bonduriansky and Rowe, 2003).Therefore, studying these mechanisms singly or in isolation can lead to incomplete information about sexual selection on a trait (Hunt et al., 2009).

Understanding the possible effects of anthropogenic disturbances on the overall strength of sexual selection on a trait requires looking into effects of environmental change on the different mechanisms of sexual selection. There is a paucity of studies that have looked into the effect of human disturbances on these mechanisms separately to understand the cumulative effect of these changes on sexual selection.

Male orthopteran insects use long-range acoustic signals (calling songs) with species-specific temporal and spectral features for mate attraction (Robinson and Hall, 2002). These signals are used by females to locate conspecific males (Alexander, 1967). Intraspecific variation in these call features allow females to discriminate among signalling males and possibly exercise choice (Brown et al., 1996; Greenfield, 1997; Wagner, 1998; Brown, 1999; Gerhardt and Huber, 2002). The relative spacing of signalling males and females and the acoustic range of signals (distance at which the average sound pressure level of male calling song attenuates to the level of male/female hearing threshold) determines whether males and females are able to hear neighbouring callers. The detection ranges of their signals often extend beyond average inter-male distances, leading to overlaps in the acoustic ranges of callers (Mhatre and Balakrishnan, 2006; Ritz and Köhler, 2007). Such overlaps in acoustic ranges can have negative fitness consequences for the advertising sex, a fact that is well substantiated by both theoretical analysis (Forrest and Green, 1991; Forrest and Raspet, 1994) and empirical evidence (Arak et al., 1990; Greenfield, 1994; Farris et al., 1997).

Spacing apart is one of the strategies that male callers can employ to increase their success in attracting females by reducing masking of their signals: calling males in many systems space apart to avoid overlaps in their acoustic ranges (Whitney and Krebs, 1975; Campbell and Shipp, 1979; Bailey and Morris, 1986; Deb and Balakrishnan, 2014). This also reduces competition, enabling signallers to broadcast within a range free of competitors (Arak and Eiriksson, 1992; Deb and Balakrishnan, 2014). However, in many species, calling males also form clustered aggregations (Gerhardt and Huber, 2002; Mhatre and Balakrishnan, 2006; Ritz and Köhler, 2007).

Female mate choice is invoked as an explanation for the evolution of spatial clustering of advertising males. Females use different features of male acoustic signals to exercise choice (Latimer and Schatral, 1986; Forrest and Green, 1991; Tuckerman et al., 1993; Brown et al., 1996) and typically prefer call features with greater acoustical energy, such as longer call bout lengths and higher call rates (Ryan and Keddy-Hector, 1992; Brown, 1999). These features could be indicators of male quality due to the higher costs involved in their production (Loher and Dambach, 1989; Scheuber et al., 2003a,b). Exercising mate choice involves searching and sampling of potential mates, which has associated time and energetic costs, and risk of predation (Gwynne, 1987; Heller and Arlettaz, 1994; Bradbury and Vehrencamp, 1998; Raghuram et al., 2015; Rosenthal, 2017). Simultaneous sampling of signallers can help minimise search costs, and female mate choice can therefore be expected to drive spatial clustering of signallers, facilitating quick and easy assessment of potential mates (Morales et al., 2001; Murphy and Gerhardt, 2002; Murphy, 2012). This premise has been used to develop many theoretical models to explain the evolution of lekking systems (Bradbury, 1981; Gibson et al., 1990; Gibson, 1992).

An important ecological factor that can dictate spacing behaviour is the spatial distribution of resources such as food, potential mates, oviposition, and signalling sites (Emlen and Oring, 1977; Greenfield and Shelly, 1985; Shelly et al., 1987; Arak and Eiriksson, 1992; Hews, 1993). Male grasshoppers, *Ligurotettix coquilletti* distribute spatially according to the food plants most attractive to females (Greenfield and Shelly, 1985; Shelly et al., 1987). Spatial distribution of katydid *Tettigonia viridissima* and *Amblycorypha parvipennis* males was shown to be affected by availability of singing sites higher than the surroundings, which increases the acoustic range of their signals (Shaw et al., 1981; Arak and Eiriksson, 1992). Changes to spatial distribution of resources owing to human-induced changes in the landscape can affect communication in species, leading to changes in space use by organisms to facilitate communication.

Spacing behaviour of advertising males in acoustically communicating species has been well studied in anurans and orthopterans (Cade, 1981; Telford, 1985; Campbell, 1990; Dyson and Passmore, 1992; Bourne, 1993; Gerhardt and Huber, 2002; Mhatre and Balakrishnan, 2006; Ritz and Köhler, 2007; Deb and Balakrishnan, 2014). Most of these studies have examined male competition, female mate choice and resources as the key factors, albeit in isolation. There are only a few studies that have looked at a combination of these factors dictating male spacing (Shaw et al., 1982; Weidemann et al., 1990; Arak and Eiriksson, 1992; Allen, 1995) and none that have incorporated all three.

In this study, we examined the role of all three factors in spacing of signallers in an acoustically signalling, paleotropical false-leaf katydid species, *Onomarchus uninotatus* (Rajaraman et al., 2015), in a human-modified landscape. Since spacing behaviour can be an outcome of multiple factors, the role of each can be dissected by simulating different hypothetical spatial distributions of signallers, given the actual distribution of resources in the natural habitat, and then examining the implications of the different possible spatial distributions of calling males for male competition and female mate choice. This allows us to examine the optimal spatial distribution(s) that maximise male and female fitness, respectively and whether the actual distribution of signallers observed in the field benefits males and/or females.

We first examined the spatial distribution of *O. uninotatus* signallers in their natural habitat and compared it with the distribution of available calling sites. If calling males are not spaced randomly with respect to the distribution of calling sites, other factors such as male competition and/or female mate choice can be postulated to play a role in the spacing behaviour observed in the field. We then simulated different spatial distributions of calling males with varying minimum nearest neighbour distances using the actual distribution of available calling sites. For all simulated distributions, the number of females within the acoustic range of individual calling males and proportion of females that could hear multiple males were calculated, to quantify the implications of male spacing for male competition and female choice respectively. The theoretical optimal spatial distributions for both were determined and compared with the observed spatial distribution of males in the field to understand the effect of the two drivers (male competition and/or female choice) on male spacing behaviour, as well as the effect of resource distribution on the strength of sexual selection drivers in this species.

MATERIALS AND METHODS

Study System

Onomarchus uninotatus (Serville 1838) is a canopy inhabiting, paleotropical false-leaf katydid species (Subfamily Pseudophyllinae: Family Tettigoniidae: Order Orthoptera). It has been described in Northeast India (Srinivasan and Prabakar, 2012), Southwestern India (Diwakar and Balakrishnan, 2007), China (de Jong, 1946), Malaysia (Heller, 1995), Indonesia (de Jong, 1946), and Australia (de Jong, 1946). Males have often been observed to call singly from *Artocarpus* trees (Nair, 2020, Ph.D. thesis). Both males and females feed on *Artocarpus* leaves, with females using the trees for oviposition as well. *O. uninotatus* has one breeding season per year, from November to April. The study was conducted in Kadari village in Karnataka, India (13°21′N–75°08′E) during the peak breeding season from January to May 2015 and 2016.

Spatial Structure of Calling Males

Host Plant Preferences

Random acoustic sampling of calling males was conducted in the study area during the peak calling hours (19:00–00:30 h). Calling *O. uninotatus* males were located by listening and the species of the tree on which the call was heard was recorded. The use of tree species by calling males was compared with the abundance of different tree species in the study area by calculating the selection ratio (proportion used/proportion available) (Manly et al., 2002) to examine preference for particular tree species as calling sites.

For abundance count of tree species in the study area, Girth at Breast Height (GBH) of tree species on which callers were found calling based on observations during random acoustic sampling was used as a criterion to define relevant calling sites. A distribution of GBH of the trees on which the callers were found was obtained by random sampling of around 50 trees of each plant species on which males were found calling. However, in the case of one of the tree species, *Vateria indica*, only 17 trees where available in the study area. From this distribution, a GBH cut-off of 20 cm was taken for the abundance count of trees, as only around 1% of the sampled trees had GBH < 20 cm (**Supplementary Figure 1**).

The area under study (1.35 km^2) was gridded using Mapsource, QGIS, and Google Earth into 112 grids (each 111 m × 109 m). About 58 grids out of 112 were selected randomly for the abundance count of trees. A 300 m transect walk was carried out in each grid and the number of trees of *Artocarpus* spp. and non-*Artocarpus* spp. (with GBH ≥ 20 cm) within 5 m on each side of the transect was recorded. At every obstacle encountered, a random angle was chosen using a magnetic compass to continue the transect walk. Walking at the borders of the grid was avoided. The relative abundance of *Artocarpus* spp. and non-*Artocarpus* spp. was compared with the relative use of *Artocarpus* versus non-*Artocarpus* tree species as calling sites to examine preference of *O. uninotatus* for *Artocarpus* species.

Distribution of Host Plant Species

GPS locations of all the *Artocarpus* trees in the study area (1.35 km^2) were obtained using a GPS receiver (Garmin eTrex Vista HCx, United States) and the trees were also physically marked with individual codes using paint. A spatial map of the GPS locations of all the *Artocarpus* trees in the area was then obtained through QGIS 2.8.2. The spatial distribution of *Artocarpus* trees was analysed using a Clark and Evans test (Clark and Evans, 1954), which uses the nearest neighbour distances in a spatial point pattern as a measure of spatial relationships.

The test compares the observed nearest neighbour distances with the expected nearest neighbour distances if the points were distributed randomly. The expected nearest neighbour distribution is generated by constructing the smallest possible rectangle over all points. If the mean observed nearest neighbour distribution is significantly lower than the expected mean for a random distribution, the test indicates a clustered distribution (Clark and Evans, 1954; Campbell, 1990).

Calling Site Fidelity Across Nights

Fifteen calling males were located in the canopy of *Artocarpus* trees, captured and marked on the pronotum with unique three-colour codes using non-toxic paint markers (Edding 780, Germany). The marked callers were released back at the site of capture. The tree codes and caller identification codes and GPS locations of trees were recorded. Each tree on which a marked caller was released was acoustically sampled for seven consecutive nights and, if a call was heard from the tree, an attempt was made at identifying the caller in the canopy.

Spatial Distribution of Calling Males in the Field

The positions and identities of trees on which males were heard calling were recorded in the entire area under study. Over 16 nights, a total of 773 (83.5% of total trees) trees were sampled in the area under study for positions of callers during the peak calling hours (19:00–00:30 h). A cut-off of 25 min (obtained from calling effort data: refer to **Supplementary Methods** and **Supplementary Figure 2**) was used as the sampling time for each *Artocarpus* tree to determine whether a calling male was present on the tree. On an average around 47 trees were sampled each night and calling males on the sampled trees were located.

The spatial map of the callers in the study area was obtained using observations of the positions of the callers recorded on all 16 nights, which was then overlaid on the map of the *Artocarpus* trees using QGIS 2.8.2. It should be noted here that the exact GPS location of a caller within the canopy of the tree was not possible to attain. Therefore, the GPS location of the tree was taken as caller location, under the assumption that the caller was located at the centre of the tree. The distribution of the nearest neighbour distances of the trees was then compared with the nearest neighbour distribution of callers, and a randomisation test was performed using R (R Core Team, 2018) to investigate whether the callers were distributed randomly with respect to the trees or were clumped or dispersed. The observed difference between the mean nearest neighbour distance of trees and callers was compared to the generated expected difference between the mean nearest neighbour distance of trees and callers if the callers were to space randomly on trees. The distribution of the expected difference was generated using a randomisation test in R (R Core Team, 2018). Randomisation test was also performed on nearest neighbour distances of calling males and trees sampled on each night separately, as the *Artocarpus* tree distribution in the entire study area is a heterogeneous distribution, with high clustering in some pockets and more dispersed in others. Therefore, the nearest neighbour calculations were also performed separately for each night, as pooling of the nearest neighbour distances for the entire area can obscure any localised pattern in the spatial distribution of calling males. The test was performed using R (R Core Team, 2018).

Acoustic Environment of Calling Males and Females

Using the spatial map of calling males in the study area, acoustic ranges of calling males with respect to females were constructed. Acoustic range is represented as a circle with the caller as the center and radius defined by the distance at which the average sound pressure level of the male's call at the center, around 71.9 ± 0.5 dB (Rajaraman et al., 2018), attenuates to the threshold of female hearing (35 dB SPL: Rajaraman et al., 2018). The average transmission distance for *O. uninotatus* calling song was computed using average sound pressure level at source, the SPL attenuation curve for the *O. uninotatus* call in the habitat (Jain, 2010) and average hearing threshold of females. To examine the acoustic environment of calling males, median overlap in the acoustic ranges along with the proportion of callers in the overlap areas was determined using R (R Core Team, 2018), by calculating the area of intersection in the acoustic ranges constructed around each point using gBuffer() and over() function from the rgeos package and sp package, respectively. To examine if the calling males could hear each other, the number of caller locations that lie within the areas of intersection of constructed acoustic ranges were then calculated in R (R Core Team, 2018).

In case of females, it was not possible to obtain their actual locations in the study area as they are acoustically silent. Therefore, two assumptions were made with respect to female locations: (1) one female to every available *Artocarpus* tree and (2) females located on randomly picked *Artocarpus* trees with an adult sex ratio of 1:1 (172 males and females). For the second assumption, multiple runs with random distributions of females were carried out in R (R Core Team, 2018). Proportion of females that might hear a male in an adjacent tree (in addition to the male in their tree) was then calculated under the two assumptions (R Core Team, 2018) by getting the female locations that are within the acoustic range of more than one male.

Factors Affecting Male Spacing: Simulations

Using the measured *Artocarpus* tree locations, different caller distributions were simulated in R (R Core Team, 2018) with different minimum nearest neighbour distances, ranging from 5 to 50 m. This was done by sampling tree positions which were at a minimum specified distance from each other. In this way, 10 spatial distributions of calling males were obtained with minimum nearest neighbour distances of 5, 10, 15, 20 25, 30, 40, 45, and 50 m.

For each simulated distribution of calling males, two outcomes were computed: (1) average number of females that were within only one male's acoustic range (as a measure of male competition) and (2) proportion of females that could hear more than one male caller (as a measure of female mate choice). Signalling in a zone free from neighbouring signallers reduces the number of females that are within acoustic ranges of multiple callers, thereby increasing a male's probability of getting a mate. Therefore, measuring the average number of females that are within only one male's acoustic range, would serve as a measure of fitness for males in the system. An aggregated distribution of males would increase the probability of occurrence of females in the overlap areas of signals of multiple callers, facilitating simultaneous sampling. Measuring the proportion of females in the population that are in the acoustic overlap areas enables us to study the type of spatial structure of signallers that benefits females in the system.

For female locations, two sets of simulations were carried out, based on the following assumptions: (1) one female to each tree and (2) females randomly located on the *Artocarpus* trees with an adult sex ratio of 1:1. A point to be noted here is that since the spatial distribution of available calling sites (*Artocarpus* trees) is constant for all simulations, the number of calling males changes in inverse relation with minimum nearest neighbour distance.

The two measured outcomes, (1) average number of females per male within only one male's acoustic range (for male competition) and (2) proportion of females that could hear more than one caller (for female mate choice), were plotted against the minimum nearest neighbour distances of calling males, for each simulated distribution of calling males, to understand the implications of spatial distribution of signallers on male competition and female choice.

RESULTS

Host Plant Preferences, Calling Site Fidelity, and Distribution of Host Plant Species

About 93.3% of the *O. uninotatus* calling males (28 out of 30) were found on trees of the genus *Artocarpus*. The use of *Artocarpus* species as calling sites was not however mirrored in their proportional availability. *Artocarpus* made up only around 2% of the total number of trees within the study area (121 out of

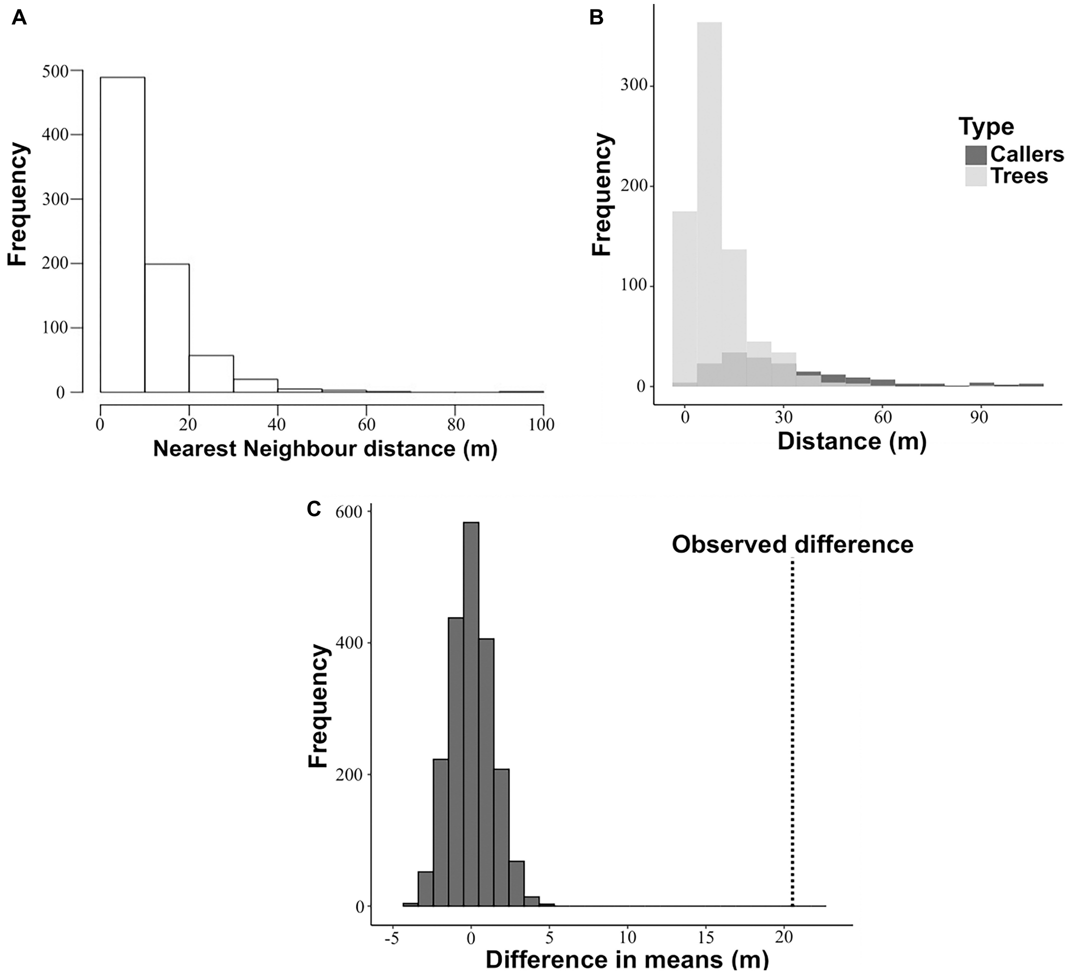

FIGURE 1 | Comparison of nearest neighbour distances of calling males and trees pooled across 16 nights. **(A)** Distribution of nearest neighbour distances of *Artocarpus* trees across all plots (n = 773 trees). **(B)** Distributions of trees and callers superimposed. **(C)** Results of randomisation test. The observed difference between the mean nearest neighbour distance of trees and the mean nearest neighbour distance of callers is marked on the plot by the vertical dotted line.

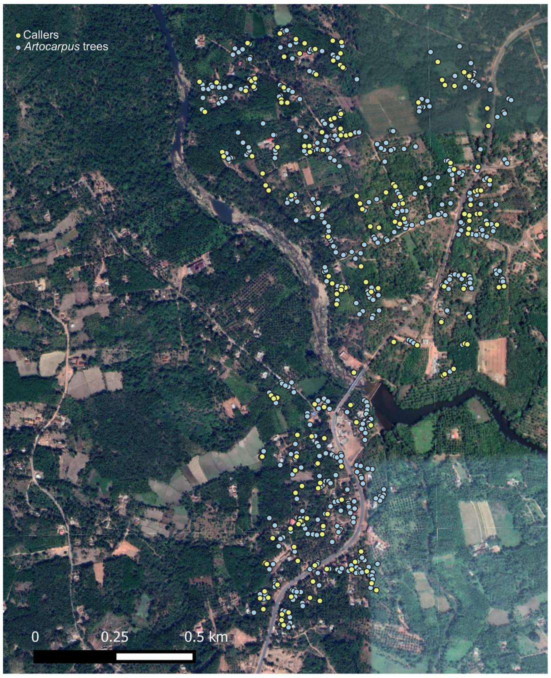

FIGURE 2 | Map of *Artocarpus* tree locations and calling male locations in the study area.

5148). The selection ratio (proportion used/proportion available) for *Artocarpus* spp. was found to be 39.7 and for non-*Artocarpus* spp. was found to be 0.06. This implies that the *O. uninotatus* males strongly prefer *Artocarpus* trees as calling sites.

Out of the 15 marked males, data could be obtained only for five males either because no call was heard during the sampling period from the tree across all days of sampling or because the caller was not visually accessible. These callers were found to maintain their calling sites (trees) across nights for an average of 78% ± 14.7% (mean ± SD) of the nights when a call was heard from the tree.

The distribution of nearest neighbour distances of *Artocarpus* trees was right skewed, with smaller distances dominating the distribution (**Figure 1A**). The Clark and Evans test showed that the distribution of *Artocarpus* trees in the study area was highly clustered ($R = 0.51$, $z = -13.07$, $P < 0.005$).

Spatial Structure of Calling Males

The spatial locations of calling males in relation to the *Artocarpus* tree distribution ($n = 773$ trees) in the study area is shown in **Figure 2**. A total of 172 calling males were located in the study area. A visual comparison of the distribution of nearest neighbour (NN) distances of trees and that of callers revealed that the NN distances of callers were on average greater than of trees (**Figure 1B**). This suggests that calling males are dispersing and spacing out more than would be expected if they were to occupy trees randomly. To statistically test for overdispersion of calling males, data were pooled across nights and the spatial distributions of trees and callers were analysed using a randomisation test (**Figure 1C**). Callers were found to be spacing themselves apart since the observed difference between the mean NN distances of callers and of trees was found to be significantly higher than the expected difference if the callers were to distribute themselves randomly with respect to trees (**Figure 1C**).

The mean nearest neighbour distance of callers per night was 31.6 ± 3.8 m (mean ± SE, $N = 16$) and the mean nearest

TABLE 1 | Nearest Neighbour analysis of spatial structure of calling males sampled on each night.

Date	*N*	Mean (NN_callers) (m)	Mean (NN_trees) (m)	ΔNN_{obs} (m)	ΔNN_{random} (m)	*P*
11-2-16	24	20.59317	7.506995	13.08618	−0.01140278	<0.01
12-2-16	15	18.25106	6.918921	11.33214	−0.01030851	<0.01
13-2-16	63	31.90049	9.338901	22.56159	−0.1007387	<0.01
29-2-16	53	28.23726	10.99214	17.24512	0.04288597	<0.01
1-3-16	63	35.33399	28.1429	7.191091	−0.6003124	>0.05
2-3-16	63	77.22267	10.53343	66.68925	−0.2550797	<0.01
3-3-16	36	38.63384	7.547321	31.08652	0.1033788	<0.01
4-3-16	73	26.61114	11.65886	14.95228	0.08821305	<0.01
5-3-16	39	33.40278	52.96623	−19.5635	1.814847	>0.05
8-3-16	42	41.27977	15.40007	25.87969	0.02734286	<0.01
9-3-16	76	33.49636	11.48015	22.01621	−0.04979648	<0.01
10-3-16	31	18.13496	7.439043	10.69592	−0.1123728	<0.01
11-3-16	68	20.716	8.736724	11.98008	−0.07482763	<0.01
12-3-16	48	21.19353	9.385668	11.80787	−0.07610984	<0.01
13-3-16	54	48.47345	7.183478	41.28998	−0.05828568	<0.01
14-3-16	25	12.92842	47.39184	34.46342	−0.271807	<0.01

N, total number of trees sampled; Mean (NN_callers), average nearest neighbour distance of the callers in the sampling unit sampled that night; Mean (NN_trees), average nearest neighbour distances of trees in the area sampled that night; ΔNN_{obs}*, observed difference between the nearest neighbour distances of callers and trees in the area sampled that night;* ΔNN_{random}*, expected difference [generated using R (R Core Team, 2018)] between nearest neighbour distances of callers and trees in the plot sampled that night if callers were randomly distributed. Two out of 16 nights (highlighted in red) showed a random pattern of distribution of callers.*

neighbour distance of trees on each night was 15.7 ± 3.6 m (mean ± SE, $N = 16$). Randomisation tests were also performed for each of the 16 nights separately, where observed difference between the nearest neighbour distances of callers and trees in the area sampled that night (ΔNN_{obs}) was compared with the expected difference between nearest neighbour distances of callers and trees in the area sampled that night if callers were randomly distributed (ΔNN_{random}). The males were seen to be distributing themselves non-randomly and spacing apart (**Table 1**). On fourteen out of sixteen nights, the observed difference in the nearest neighbour distances of callers and trees was found to be much higher than the expected difference. Two out of 16 nights (highlighted in red) showed a random pattern of distribution of callers (**Table 1**). This could be because the nearest neighbour tree distances in these two areas were much larger than other areas and were either almost equal to or more than the average nearest neighbour distance (32 m) that the callers were seen to maintain (**Table 1**).

Acoustic Environment of Calling Males and Females

The proportion of females that could hear more than one male when one female was assumed to be found on every *Artocarpus* tree was 0.59 (**Figures 3A,B**). Interestingly, this value did not change if the females were assumed to be randomly distributed, with a 1:1 sex ratio, where the median value was found to be 0.6 (**Figure 3B**). Therefore, there is a high chance that, given this spatial structure of callers and habitat, females can potentially hear multiple males irrespective of their relative distribution. There was considerable overlap in the acoustic ranges of the calling males. The median overlap in the acoustic ranges of males was 24.4% (IQR = 7.57–51.6%) with maximum overlap of 96% and minimum overlap of 0.024% (**Figure 3C**).

Factors Affecting Male Spacing

Under both assumptions for female locations, implications of male spacing for males and females showed opposite trends with increasing minimum nearest neighbour distances between calling males (**Figures 4A,B**). When females were one to each tree (**Figure 4A**), the proportion of females who could hear multiple males, a situation conducive for mate choice, starts showing a decline once the minimum nearest neighbour distance between males exceeds 25 m. On the other hand, for males, average number of females per male that are confined to acoustic range of only one male, increases with increasing distance between males. The number of females exceeds 1 when the minimum nearest

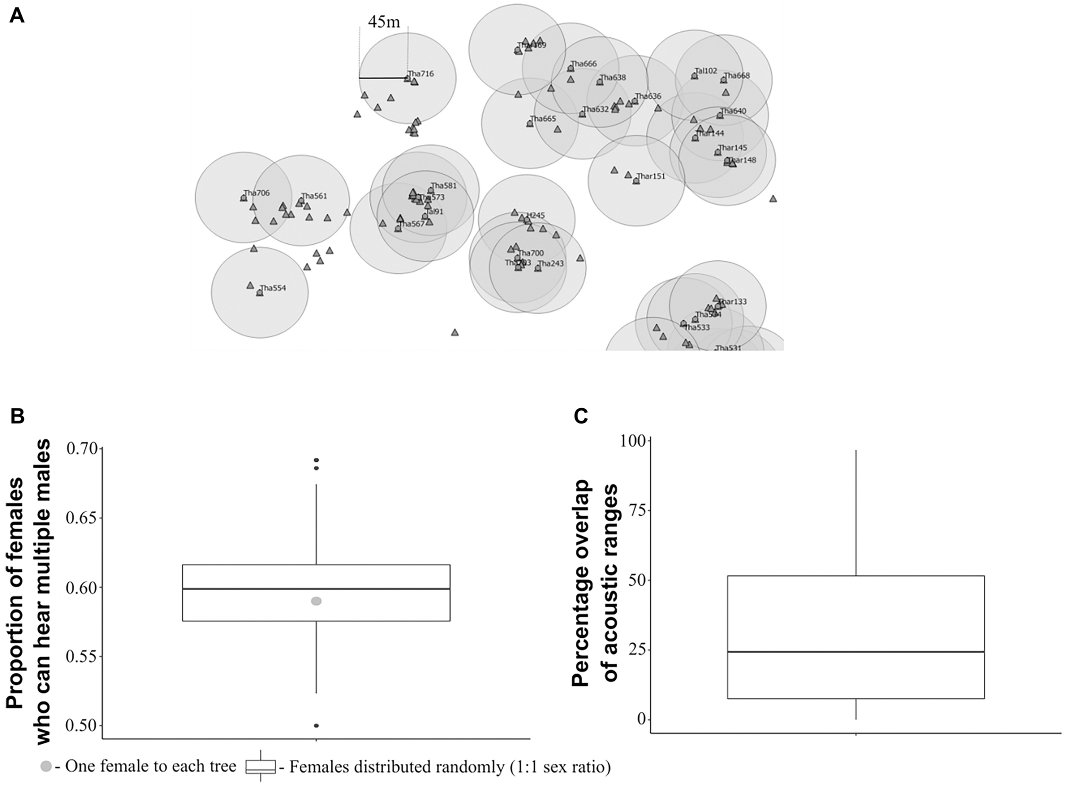

FIGURE 3 | Acoustic environment of calling males and females. **(A)** Figure depicting the acoustic ranges of a small sample of callers (taken from the spatial map of callers), with the callers (circles) as the centres of circles, the radius of which is defined by the distance at which the average SPL of the male's call drops to the female hearing threshold. Triangles indicate assumed locations of individual females, where females are assumed to be one per each tree. **(B)** Proportion of females that could potentially hear more than one male (apart from the male on their tree) under the two assumptions: (1) One female to each tree ($n_{females} = 773$, $n_{males} = 172$), (2) Females randomly distributed on trees ($n_{females} = 172$, $n_{males} = 172$); **(C)** Percentage overlap in acoustic ranges of males (n = 172).

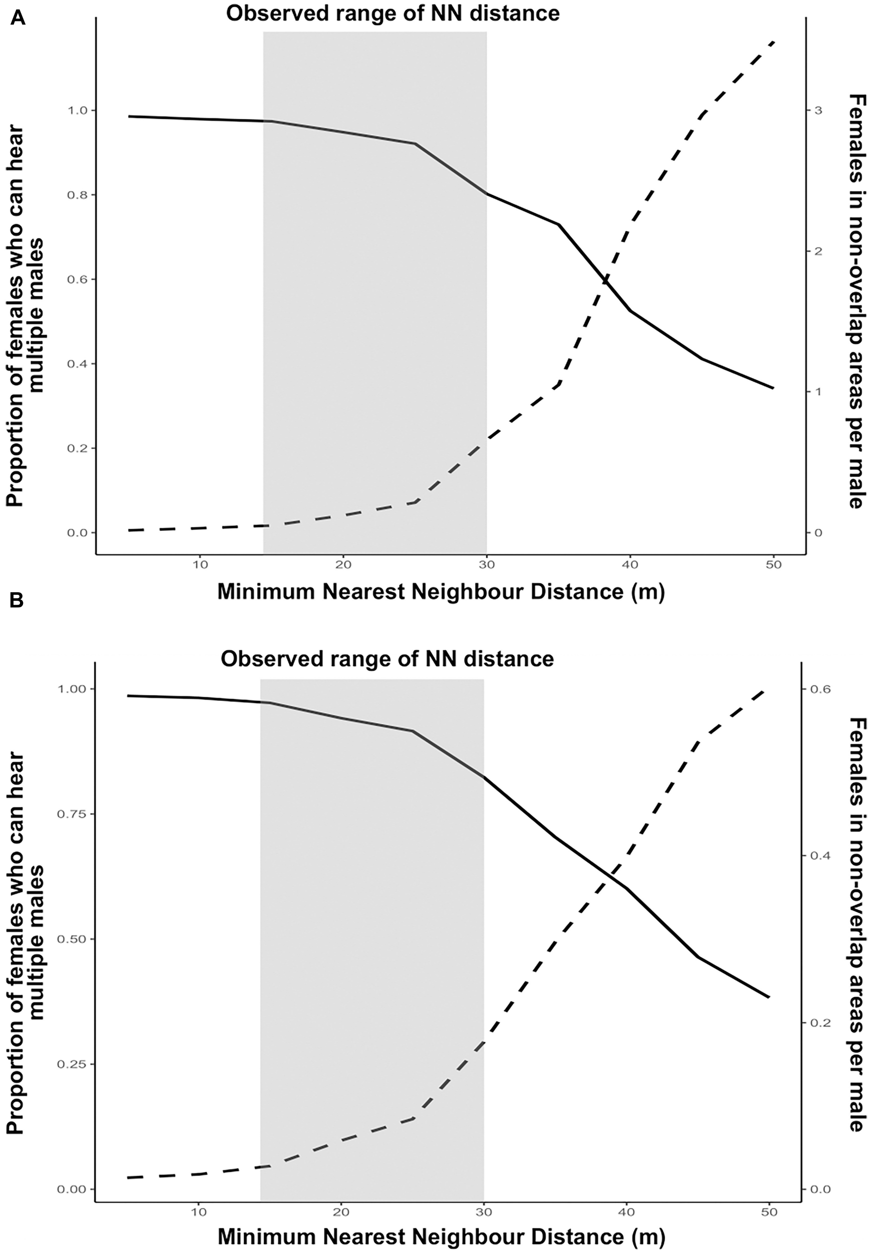

FIGURE 4 | Effect of male spacing on fitness of calling males and females under the assumptions of **(A)** one female to each *Artocarpus* tree and **(B)** females randomly distributed on *Artocarpus* trees with 1:1 sex ratio. Dotted lines represent females in non-overlap areas (male fitness) and solid lines represent proportion of females that can hear multiple males (female fitness/mate choice). The shaded region represents the observed range of distribution of nearest neighbour (NN) distances maintained by calling males in the field.

neighbour distance between males is 35 m or more (**Figure 4A**). Therefore, by increasing their inter-individual distances, callers can increase their probability of getting a mate, reducing male–male competition. As expected, male competition is pushing the spatial structure of callers to a more dispersed distribution. On the other hand, female choice seems to be pushing the signaller distribution to a more clustered distribution. With decreasing nearest neighbour distances of callers, proportion of females in the population that can hear multiple males approaches 1, indicating that at highly clustered distribution of calling males, nearly every female in the population can hear multiple males. The observed range of nearest neighbour distances maintained by maximum number of callers in the field (15–30 m), lies in between the two optima. This indicates a counteracting effect of male competition and female choice on male spacing behaviour in this species.

When females are randomly distributed with 1:1 sex ratio, the trend does not change much for females. However, for males, the average number of females per male that are within only one male's acoustic range does not even reach one, even when the males are at least 50 m apart (**Figure 4B**).This means that when females are distributed randomly on *Artocarpus* trees with 1:1 sex ratio, probability of securing a mate is low for calling males even when males are spacing apart.

DISCUSSION

Conflicting Selection on Male Spacing?

Onomarchus uninotatus calling males displayed non-random spacing and were found to be overdispersed. However, unlike other systems where males space apart to avoid or reduce overlaps in their signal ranges (Whitney and Krebs, 1975; Campbell and Shipp, 1979; Deb and Balakrishnan, 2014), there was considerable overlap observed in the acoustic ranges of *O. uninotatus* calling males. This points to a possible role of other factors such as female sampling or resource clustering in the observed spacing behaviour of calling males.

Manipulating the spacing between callers through simulations indicates that the two mechanisms of sexual selection have conflicting effects on male spacing behaviour, with different selective optima for nearest neighbour distances between callers. Calling males benefit more in terms of reduced competition (measured here as number of females confined to acoustic ranges of only one male) when the males maintain a minimum distance of 35 m or more: in the case of one female to each tree, males have at least one female in their acoustic ranges that is not in the overlap area of multiple callers. For females, on the other hand, a clustered assemblage of males with less than 10 m nearest neighbour distances, is conducive for simultaneous sampling of multiple calling males. In the natural population, the nearest neighbour distance maintained by maximum number of callers lies in the range of 15–30 m which is in between the two optima. This range, however, does not seem to be equally advantageous for both males and females. Whereas a high proportion of females (∼0.9) are privy to multiple calling males at a time, males are unable to avoid competition. On average, males do not have even one female that is confined to the acoustic range of only one caller.

Resource Distribution as a Constraint on the Strength of Sexual Selection Drivers

The landscape under study has been transformed from evergreen forests to plantations owing to human settlement, leading to a clustered distribution of *Artocarpus* trees, which are planted by humans. This clustering of *Artocarpus* trees in the landscape seems to have differential effects on female choice and male competition. From the field data, it can be seen that females are able to hear multiple males and the probability of being able to hear a calling male from an adjacent tree is not affected by the spatial distribution of the females. This is due to the aggregated distribution of *Artocarpus* trees to which the females are limited. This, combined with large acoustic ranges of the calling males (∼45 m), results in most of the *Artocarpus* trees falling within the acoustic range of one or more callers. Therefore, irrespective of the female distribution, the possibility of a given female hearing a caller on another tree is high.

Females have been shown to use calling songs for discriminating among males and show preferential attraction for certain call features (Hedrick, 1986; Tuckerman et al., 1993; Gerhardt and Huber, 2002). Best-of-n and threshold strategy, two popular models for mate-sampling, show reduced fitness with increasing search costs (Real, 1990). In *O. uninotatus*, which is a canopy-inhabiting katydid, movement across trees in response to male calls could put females under high predation risk. Raghuram et al. (2015) observed, in flight cage experiments, that the predatory bat, *Megaderma spasma*, a known predator of *O. uninotatus*, responded 100% of the time to tethered females in flight as opposed to about 30% of the time to male calls. Therefore, females would benefit from an aggregated distribution of males as it increases their access to multiple males without having to sequentially sample the callers, which would require them to fly between multiple trees.

Males, on the other hand, can increase the probability of attracting a mate by choosing calling positions which would result in multiple *Artocarpus* trees falling within their acoustic ranges, as females are also confined to *Artocarpus* trees for the purposes of foraging and oviposition (Nair and Rajaraman, personal observations). However, given that the calls have a large acoustic range of 45 m and are in an area with clumped distribution of calling sites, a caller simultaneously runs the risk of overlap of his acoustic range with other callers in the vicinity. Males bear a fitness cost when they have overlapping acoustic ranges with other callers (Arak et al., 1990; Farris et al., 1997; Mhatre and Balakrishnan, 2006). This suggests that males should avoid overlap altogether to reduce competition. However, it can be seen from the data that males are unable to avoid acoustic overlaps.

From the simulations, it can be seen that, with a minimum nearest neighbour distance of 40 m or more, the average number of females per male in the non-overlap areas increases to ∼3, when there is one female to each tree. However, at a minimum nearest neighbour distance of 50 m, given the clustered distribution of *Artocarpus* trees, the number of calling males reduces to 141, whereas in the wild the number of calling males recorded were 172. Therefore, the distribution of calling sites seems to be placing a constraint on the extent to which males can space apart and avoid overlap.

These results show that anthropogenic changes can affect sexual selection on traits, with the effect varying between the two components of sexual selection. Human disturbances can influence the sexes differently and this highlights the importance of partitioning the effects of these disturbances on the different mechanisms of selection. It also points to the importance of interaction between evolutionary and ecological factors in shaping phenotypes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical clearance for carrying out this project was obtained from the Institutional Animal Ethics Committee (IAEC), Indian Institute of Science (Project No. CAF/Ethics/519/2016).

AUTHOR CONTRIBUTIONS

RB and AN designed the study, interpreted the results, and wrote the manuscript. AN performed the field observations and simulations and carried out the data analyses. Both authors gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.802078/full#supplementary-material

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Size of Snake Eyes Correlates With Habitat Types and Diel Activity Patterns

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Eye size influences visual acuity, sensitivity, and temporal resolution and is a result of vertebrate adaptation to the environment. The habitats of snake species are diverse, ranging from fossorial, terrestrial, arboreal, to aquatic. They also demonstrate a variety of behavioral and physiological characteristics, such as activity time, feeding patterns, and prey detection. In this study, we comparatively investigated how the relative eye size (i.e., eye diameter vs. head width) associated with the ecological (i.e., habitat), behavioral (i.e., diel activity pattern, foraging strategy), and physiological traits (i.e., the presence of pits), respectively, across six snake families from Taiwan. Among the traits we examined, we found that terrestrial and/or diurnal snakes tended to exhibit the larger relative eye size, indicating the evolutionary responses of eye size to changes in habitat types and activity patterns, respectively, while no evidence of how foraging strategies and the presence of pits affected snake eye size was found. Our findings not only shed light on the adaptive significance of the visual system in diversifying the behaviors and the environments exploited in snakes, but also underline the interactive effects of multidimensional evolutionary attributes (e.g., behavior, ecology, physiology and phylogeny) on the evolution of optimal visual performance.

Keywords: snake eye size, diel activity patterns, habitat type, allometric growth, phylogenetic regression

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INTRODUCTION

Vision is a crucial sensory system connecting organisms to their environments. Eye size, which positively associates with the amount of photoreceptors in eyes (Land and Nilsson, 2002), determines visual acuity, sensitivity, and temporal resolution (Roaf, 1943; Land and Nilsson, 2002). Such a morphological trait can further account for the functional mechanisms of how species adapt to the environmental lights given their physical limitations. For example, organisms with the larger eyes may possess better foraging efficiency due to the improved visual resolution and/or the greater light sensitivity (Land and Nilsson, 2002; Thomas et al., 2006; Ausprey et al., 2021); yet, they may be imposed by higher energy expenditure simultaneously [e.g., the increased body weight (Laughlin, 1995), the development and maintenance of a large number of nerve cells (Laughlin et al., 1998), and/or susceptibility of eye (Harper, 1988)]. Thus, eye size may represent an ideal indicator of the visual requirements underlying the evolutionary tradeoffs between visual functions and energy expenditure.

In many vertebrates, the absolute eye size often varies allometrically with body size-associated traits [e.g., body length (Burton, 2008), body weight (Howland et al., 2004), head size (Kirk, 2006)], while the relative eye size tightly corresponds to ecological and behavioral attributes of species, such as habitat types (Liu et al., 2012; Thomas et al., 2020), diel activity patterns (Liu et al., 2012; Schmitz and Higham, 2018), and foraging strategies (Thomas et al., 2006; Lisney and Collin, 2007). Currently, the evolution of eye size has been studied thoroughly in many taxa [e.g., fishes (Caves et al., 2017; Vinterstare et al., 2020), frogs (Shrimpton et al., 2021), geckos (Werner and Seifan, 2006), birds (Thomas et al., 2006; Burton, 2008) and mammals (Kirk, 2006; Heard-Booth and Kirk, 2012; Nummela et al., 2013)]. However, it is relatively underexplored how the eye size across different snake species is shaped by the environment, facilitating their demands for behavioral activities and foraging strategies (Katti et al., 2019). For example, it is unclear whether the size of eyes is different in the snakes adopting active and sedentary foraging strategies. Also, do snakes modify the investment in eye size, given the high physiological costs incurred, if they are able to sense environmental cues using the additional sensory system (e.g., pit for infrared perception)?

Snakes have shown the remarkable ability to adapt to diverse environments (Greene, 1997). They heavily rely on visual cues when evaluating the environment (Danaisawadi et al., 2016; Schraft and Clark, 2019) and have been suggested to possess dichromatic vision (Bittencourt et al., 2019). Many of them possess rod and cone opsins (Simões et al., 2015, 2016) and thus are able to perceive different light intensities and colors (Bittencourt et al., 2019). In Colubridae, nocturnal species had larger eyes than diurnal species and species that inhabit arboreal habitats tend to have larger eyes compared with terrestrial or semiaquatic species (Liu et al., 2012). This suggested that the intensity and the propagation of lights provided by different environments can drive the evolution of snake eyes. In the watersnakes that heavily live on frogs, it has been found that the eye size was positively associated with the proportion of frogs in the diet, suggesting that larger eyes benefited the foraging ability of the snakes (Camp et al., 2020). Given the importance of vision in the foraging behavior of snakes, understanding the evolution of snake eyes can thus provide us deeper insights into the diversification of eye morphology and its role in regulating predator-prey interactions across different environmental gradients.

In this study, we examined how the habitat types, behavioral patterns (i.e., foraging strategies, diel activity patterns), and physiological conditions (i.e., the presence of pits) associate with the eye size across multiple snake families, respectively. Based on the potential visual requirements in the given environmental conditions and/or behavioral strategies, we predicted that (1) terrestrial and arboreal snakes should have larger eyes than aquatic snakes; (2) snakes that actively hunt prey have larger eyes than those that ambush; (3) diurnal snakes have larger eyes than nocturnal snakes; and (4) snakes with pits tend to have smaller eyes.

MATERIALS AND METHODS

We examined the specimens of 33 snake species deposited in the National Museum of Natural Sciences (NMNS) in Taiwan. All of the species inhabit mainland Taiwan and the surrounding islands. The systematic classification included the earliest differentiation of the blind snake family (one species; Typhlopidae) and the Xenodermidae (one species), the Viperidae (five species), the Homalopsidae (two species), the Pseudaspididae (one species), the Elapidae (six species), and the latest differentiation of the Colubridae (17 species). The sample size of each species ranged from 3 to 207 individuals, for a total of 1,176 individuals.

We identified the habitat type, the diel activity pattern, the foraging strategy, and the presence of pits of each snake species based on Tu (2004) (**Table 1**). We measured the diameter of the left eye and head width of the specimens using a vernier scale (accurate to 0.1 mm; electronic vernier calipers; code number 500–138; model: CD-8″ BS, [Mitutoyo (Mitutoyo) Corporation, Japan]. We defined the eye diameter as the maximal diameter of the visible spectacle as the horizontal width of the inner edge of the eye contact from the nearest scale of the eye, and the head width as the widest maximal distance between the cheeks when looking down on the head (**Supplementary Figure 1**). Since the eyes of blind snakes are vestigial (Greene, 1997), we excluded the measurement of blind snakes from the subsequent analyses.

We estimated the effect of the specific ecological/behavioral/physiological factors on the relative eye size (i.e., regressed by the head size) among the six snake families using Bayesian general linear mixed-effect models. The models allowed us to take accounts of the phylogeny and repeated measurements simultaneously. The model was derived as below:

$$\ln\left(Y_{ij}\right) = \alpha + \left(\gamma_p + I_{1i} + I_{2i}\right) + \left(\beta + L_{1i} + L_{2i}\right)\ln\left(X_{ij}\right) + c_p \ln\left(X_{ij}\right) + \varepsilon_{ij}$$

with the following priors:

$$\alpha,\ \beta, \gamma, c \sim T\,(\mathrm{DF} = 3)\,,$$

$$\sigma_I,\ \sigma_L \sim\ T_+\,(\mathrm{DF} = 3)\,,$$

$$\mathrm{Cor}\,(I)\,,\ \mathrm{Cor}\,(L)\ \sim \mathrm{LkjCholesky}\,(1)\,,$$

$$\sigma_\varepsilon \sim\ \mathrm{Scaled}\ T_+\left(\mathrm{DF} = 3,\ \mathrm{scale} = 2.5\right),$$

where Y and X denote eye diameter and head width; i and j denote species and different samples within species, respectively, α and β denoted the overall intercept and slope, respectively, ε_{ij} denoted the random errors; I and L denoted the random intercept and slope of ith species. The fixed effect, γ_p, including (1) snake family (i.e., Colubridae, Elapidae, Homalopsidae and Viperidae), (2) habitat types (i.e., seawater, freshwater, terrestrial and arboreal), (3) diel activity patterns (i.e., diurnal, cathemeral

TABLE 1 | Summary of the behavioral characteristics, head width and eye diameter of the 33 snake species from Taiwan used in this study.

Species	Hab.	Act.	For.	FP	*N*	HW mean ± SD (mm)	ED mean ± SD (mm)
Colubridae							
Ptyas dhumnades	T	D	Ac	W/o	13	11.44 ± 3.566	6.53 ± 1.357
Ptyas major	A	D	Ac	W/o	9	12.26 ± 2.691	3.34 ± 0.609
Ptyas mucosa	T	D	Ac	W/o	5	18.86 ± 3.591	6.18 ± 0.783
Ptyas korros	T	C	Ac	W/o	57	17.76 ± 3.171	7.13 ± 0.867
Elaphe carinata	T	C	Ac	W/o	83	15.05 ± 5.063	4.09 ± 1.405
Elaphe taeniura	T	C	Ac	W/o	6	24.04 ± 8.545	6.33 ± 0.991
Oreocryptophis porphyraceus	T	N	Ac	W/o	4	11.79 ± 2.997	3.18 ± 0.408
Boiga kraepelini	A	C	Ac	W/o	6	13.68 ± 3.394	4.29 ± 0.626
Lycodon ruhstrati	T	N	Ac	W/o	7	9.35 ± 3.139	2.34 ± 0.548
Lycodon rufozonatus	T	N	Ac	W/o	22	13.51 ± 3.581	3.17 ± 0.460
Oligodon ornatus	T	D	Ac	W/o	4	7.70 ± 2.123	2.46 ± 0.661
Oligodon formosanus	T	C	Ac	W/o	9	11.11 ± 1.993	2.89 ± 0.203
Trimerodytes annularis	F	N	Ac	W/o	80	18.56 ± 6.552	3.26 ± 0.929
Rhabdophis tigrinus	T	D	Ac	W/o	3	12.42 ± 2.567	4.03 ± 1.455
Fowlea piscator	T	D	Ac	W/o	79	14.49 ± 4.242	3.64 ± 0.677
Amphiesma stolatum	T	D	Ac	W/o	3	5.72 ± 1.065	2.80 ± 1.047
Pseudagkistrodon rudis	T	C	Ac	W/o	3	16.39 ± 8.702	4.25 ± 1.553
Elapidae							
Hydrophis melanocephalus	S	D	Ac	W/o	28	12.90 ± 3.171	2.02 ± 0.395
Emydocephalus ijimae	S	D	Ac	W/o	7	14.65 ± 1.852	2.70 ± 0.428
Laticauda semifasciata	S	C	Ac	W/o	53	21.04 ± 6.687	3.15 ± 0.772
Laticauda colubrina	S	C	Ac	W/o	144	19.35 ± 4.000	3.30 ± 0.524
Bungarus multicinctus	T	N	Ac	W/o	207	15.55 ± 2.536	2.29 ± 0.335
Naja atra	T	D	Ac	W/o	98	24.43 ± 4.609	4.30 ± 0.530
Pseudaspididae							
Psammodynastes pulverulentus	T	C	Ac	W/o	13	9.00 ± 1.986	3.23 ± 0.426
Homalopsidae	T						
Myrrophis chinensis	F	N	Ac	W/o	65	13.37 ± 5.466	1.89 ± 0.473
Hypsiscopus plumbea	F	C	Ac	W/o	3	9.59 ± 4.812	1.93 ± 0.558
Viperidae							
Protobothrops mucrosquamatus	T	N	Am	W/	19	16.34 ± 1.433	3.07 ± 0.354
Trimeresurus stejnegeri	T	N	Am	W/	37	16.67 ± 3.399	3.38 ± 0.399
Deinagkistrodon acutus	T	N	Am	W/	36	28.46 ± 3.399	3.99 ± 0.619
Daboia siamensis	T	C	Am	W/o	36	22.38 ± 4.359	3.82 ± 0.493
Pareas formosensis	T	N	Ac	W/o	9	7.36 ± 0.834	3.04 ± 0.471
Xenodermidae							
Achalinus formosanus	T	N	Ac	W/o	9	6.32 ± 1.867	1.25 ± 0.189
Typhlopidae							
Indotyphlops braminus	T	N	Ac	W/o	19	4.24 ± 0.654	0 ± 0

(Continued)

TABLE 1 | (Continued)

Hab, Habitat; T, terrestrial; A, arboreal; S, seawater; F, freshwater. Act, daily activity pattern: D, diurnal; C, cathemeral; N, nocturnal. For, foraging type: Ac, active; Am, ambush; FP, facial pit; HW, head width; ED, eye diameter.

The species are listed in the same order as in ***Figure 1****. The information of habitat types, diel activity patterns, and foraging strategies were based on* Tu (2004)*.*

and nocturnal), (4) foraging strategies (i.e., active and ambush), or (5) the presence of pits (present and absent), were examined, respectively, in the models. We also included the interaction term between the fixed factor examined and the head width, $c_p\ln(X_{ij})$, where c_p denoted the interaction effect.

Including the two parameters, I_1 and L_1, allowed us to take account of the intraspecific variation caused by repeatedly measuring multiple snake individuals within the same species. The other two parameters, I_2 and L_2, allowed us to adjust the proportionality coefficients and allometric growth indices of each species, eliminating the problem of false duplication of relatives. The covariance matrix adopted in this study was based on the most approximate affinity tree published in Figueroa et al. (2016) (see **Supplementary Figure 2**; Hadfield and Nakagawa, 2010). Given that four species from our study were not included in Figueroa et al. (2016), we substituted them by the most phylogenetically closest species (**Supplementary Figure 2**). We yielded the final results using the averaged models, where we were able to integrate the information of the posterior probabilities produced by the models including and excluding the interactive effects [i.e., $c_p\ln(X_{ij})$].

To better illustrate the effect of each of the fixed factors on the eye size of snakes, we introduced the term, expected eye diameter, to allow us to directly compare the eye size. The magnitude of expected eye diameter was extracted, based on the linear regression of the allometry of eye size vs. head width, at the point where the head width equaled 14.84 mm (i.e., the mean head width among all the snake species). We excluded the blind snake, *Indotyphlops braminus*, from all of the analyses (except for morphological description) because the eye size was very close to zero. We also excluded the other two species, *Achalinus formosanus* and *Psammodynastes pulverulentus*, from the analysis of the history of eye size evolution due to the small species number within the respective family.

The models were estimated using the Bayesian Markov chain Monte Carlo method with the R-based brms suite (Bürkner, 2018). Each linear model was tuned with 10,000 warm-up Markov chains. Thereafter, we ran 10,000 Markov chains and used the results as the posterior probability distribution of each parameter. To conduct the significance tests, we compared the posterior distribution of each parameter among the categorical levels of the targeted fixed factor, and calculated the median (hereafter, M), 95% highest density interval (HDI), and two-tailed P_{MCMC}. The significant statistical difference between the two categories was recognized if the corresponding $P_{MCMC} < 0.05$, and vice versa.

RESULTS

Phylogeny

There was a significant negative allometric growth of eye diameter vs. head width in snakes (**Figures 1A,D**), since the overall median ($M = 0.469$) and 95% HDI [i.e., (0.361, 0.562)] were significantly smaller than 1 ($P_{MCMC} < 0.001$). The allometric growth (i.e., the slope) was the maximum in the Colubridae and the weakest in the Elapidae (**Table 2A**). Multiple comparisons indicated that the scaling coefficients were significantly higher in the Colubridae than in the Elapidae, but no significant differences existed between the other families (**Table 2A**). In the comparison of scaling coefficients, both Viperidae and Colubridae were significantly higher than the family Homalopsidae (**Table 2B** and **Figure 1B**). In comparing the expected eye diameters, significant differences were observed among the four groups (**Table 2C** and **Figures 1C,D**). The Colubridae have the largest eyes relative to head width, followed by the Viperidae, followed by the Elapidae, and the family Homalopsidae was the smallest (**Table 2C**). Taken together, the higher allometric growth index and proportionality coefficient of the later-emerging Colubridae resulted in a significantly larger increase in eye diameter with head width than the other three taxa, and a significantly larger expected eye diameter than the other three taxa (**Figure 1D**).

Habitat Types

In terms of the correlation between eye diameter and head width and habitat type, the median of the allometric growth index was very similar and not statistically significant for the four habitat types ($P_{MCMC} > 0.997$; **Figure 2A**). In terms of the proportional coefficients, the magnitude of the terrestrial type was significantly higher compared with the freshwater type ($P_{MCMC} = 0.013$; **Figure 2B**) and marginally higher compared with the seawater type, respectively ($P_{MCMC} = 0.055$; **Figure 2B**). There was no significant difference in the proportional coefficients when comparing marine and freshwater types ($P_{MCMC} = 0.518$; **Figure 2B**) or the arboreal type with the other three types ($P_{MCMC} = 0.624$; **Figure 2B**). Similarly, in terms of the expected eye diameter, the magnitude of the terrestrial type was significantly higher compared with the freshwater type ($P_{MCMC} = 0.013$; **Figure 2C**) and marginally higher compared with the seawater type, respectively ($P_{MCMC} = 0.055$; **Figure 2C**). There was no significant difference in the expected eye diameter when comparing marine and freshwater types ($P_{MCMC} = 0.518$; **Figure 2C**) or the arboreal type with the other three types ($P_{MCMC} = 0.624$; **Figure 2C**). In summary, the relative eye diameter of aquatic snakes was significantly smaller than that of terrestrial snakes, and this phenomenon was mainly due to the difference in the ratio coefficients rather than the allometric growth index (**Figure 2D**).

Diel Activity Pattern

With regard to the correlation between eye diameter and head width and diel activity pattern, the median allometric growth index was higher for the diurnal type, but there was no statistically significant difference among the three types (**Figure 3A**). In terms of the proportionality coefficient, the coefficient for the diurnal type was the highest and significantly higher than that for the nocturnal type ($P_{MCMC} = 0.043$; **Figure 3B**), while the cathemeral type was at the intermediate level and did not differ significantly from the other two types (**Figure 3B**). In comparing the expected eye diameter, the median coefficient was the lowest for the nocturnal type ($P_{MCMC} = 0.038$; **Figure 3C**) and intermediate for the diurnal type ($P_{MCMC} = 0.038$; **Figure 3C**), which was not significantly different from the other two types (**Figure 3C**). In summary, the relative eye diameters of nocturnal snakes were significantly smaller than those of diurnal snakes, and this was mainly due to the difference in the scaling coefficients (**Figure 3D**).

Foraging Strategy and the Presence of Pits

We found no significant difference in the regression coefficients of the eye diameter when comparing the two foraging types (allometric growth index, $P_{MCMC} = 0.391$; proportionality coefficient, $P_{MCMC} = 0.469$; expected eye diameter, $P_{MCMC} = 0.581$; **Figure 4**). Also, we found no significant difference in the regression coefficients of the eye diameter when comparing the snakes with and without pit (allometric growth index, $P_{MCMC} = 0.350$; proportionality coefficient, $P_{MCMC} = 0.496$; expected eye diameter, $P_{MCMC} = 0.610$; **Figure 5**).

DISCUSSION

In this study, we demonstrated the important role of environmental pressures in driving the evolution of the eye size in snakes. Among the four families we examined, Colubridae exhibited the most apparent trend of evolutionary allometry of eyes. In addition, we showed that terrestrial and/or diurnal snakes have larger eyes, based on our comparative analyses, whereas foraging strategies and the presence of pits did not correlate with the relative eye size of the snakes we examined.

Phylogeny

Colubridae, the latest differentiated family examined in this study, have the larger relative eye size. This suggests that they may have developed better vision and/or relied more on the visual cues in engaging in the daily activities compared with the rest of more ancestral snake families. Such implications comply with the conventional thought regarding the evolutionary direction of snake vision. Snakes have improved the visual ability along with the evolutionary history. There might be up to five visual opsins present in the ancestral vertebrate (i.e., RH1, RH2, SWS1, SWS2, and LWS; Simões et al., 2015). While the snakes that belong to the basal lineage, scolecophidians, only express RH1 genes likely as an adaptation of fossorial habits, most of the stem snakes

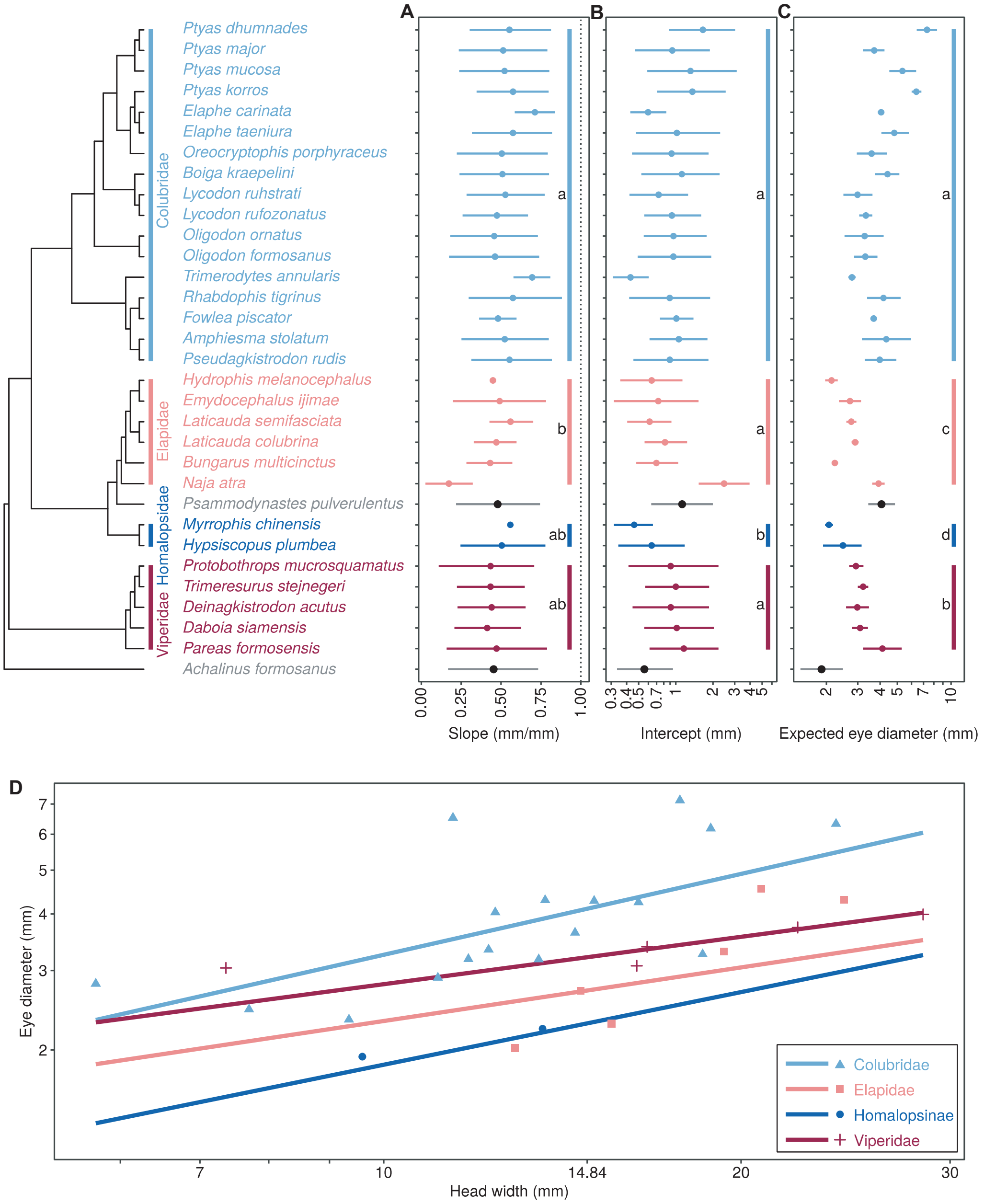

FIGURE 1 | Comparison of growth patterns of eye diameter and head width for different taxa, including allometric growth index **(A)**, proportionality coefficient **(B)**, expected eye diameter **(C)** and scatter diagram **(D)**. Different color dots and lines are used to represent the four major taxa. In subplots **(A–C)**, the dots and horizontal lines represent the median and 95% HDI of the posterior distribution, respectively, and lowercase letters indicate multiple comparisons between groups (no identical letters indicate significant differences). In **(D)**, the points indicate the mean head width and eye diameter within each species.

express the additional two opsin genes that enable dichromatic color vision (i.e., SWS1 and LWS) (Tu, 2004; Davies et al., 2009; Simões et al., 2015; Katti et al., 2019). The better visual ability, as the result of the development of larger eyes (Corral-López et al., 2017), could have further advanced the performance of how they explore and utilize resources in the environment. Snakes have generally been considered to evolve from slow, passive cavity-dwelling predators to agile, aggressive predators (Underwood, 1967). The larger eye size associated with the extremely high proportion of the active hunting strategy, at least among the Colubridae species examined here, may thus provides a hint on how the visual system could have facilitated the evolution of foraging strategies in this organism.

Habitat Type

Terrestrial snakes have a larger relative eye size than that inhabit the freshwater environments, while there was no difference in the relative eye size among the comparisons across other habitat

TABLE 2 | Summary of posterior distributions and multiple comparisons of scaling coefficients (A), proportionality coefficients (B) and relative eye diameters (C) among four clades.

	Family	Estimate	P_{MCMC} of multiple comparisons			
			Colubridae	**Elapidae**	**Homalopsidae**	**Viperidae**
(A)	Colubridae	0.541 (0.456, 0.627)	—			
	Elapidae	0.430 (0.340, 0.512)	0.049	—		
	Homalopsidae	0.531 (0.372, 0.696)	0.916	0.237	—	
	Viperidae	0.438 (0.305, 0.559)	0.110	0.896	0.343	—
(B)	Colubridae	0.948 (0.754, 1.169)	—			
	Elapidae	0.856 (0.661, 1.071)	0.504	—		
	Homalopsidae	0.543 (0.349, 0.768)	0.009	0.031	—	
	Viperidae	0.996 (0.682, 1.388)	0.783	0.415	0.012	—
(C)	Colubridae	4.066 (3.892, 4.248)	—			
	Elapidae	2.719 (2.628, 2.812)	<0.001	—		
	Homalopsidae	2.263 (0.198, 2.553)	<0.001	<0.001	—	
	Viperidae	3.240 (3.041, 3.460)	<0.001	<0.001	<0.001	—

Posterior medians and 95% highest density intervals are listed.

types. Our findings are inconsistent with the previous study that concluded that arboreal snakes had larger eyes than the terrestrial and semiaquatic snakes (Liu et al., 2012). This inconsistence may have occurred because the phylogenetic effects were not included in the analyses of the previous study.

Notably, the difference in the eye size between terrestrial and freshwater snakes were not generated by the allometry of eye size

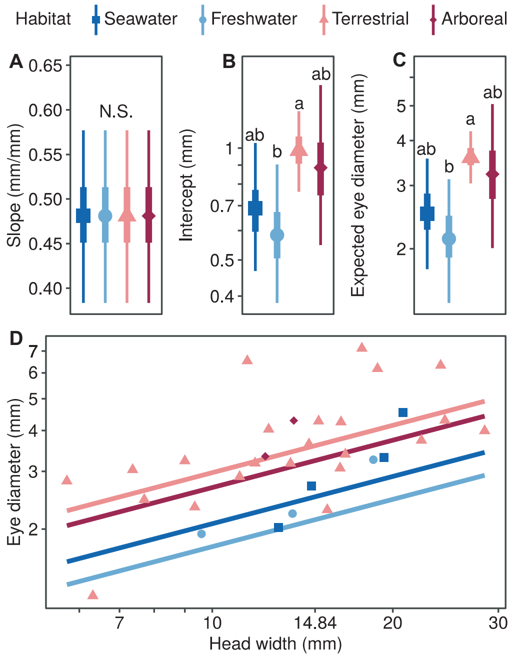

FIGURE 2 | Comparison of growth patterns of eye diameter and head width for different taxa, including allometric growth index **(A)**, proportionality coefficient **(B)**, expected eye diameter **(C)** and scatter diagram **(D)**. In subplots **(A–C)**, the symbols, vertical thick lines and vertical thin lines indicate the median, 50 and 95% HDI of the posterior distribution, respectively, and lowercase letters indicate multiple comparisons between groups (not having the same letter indicates a significant difference), while N.S. indicates no statistical difference. In **(D)**, the points indicate the mean head width and eye diameter within each species.

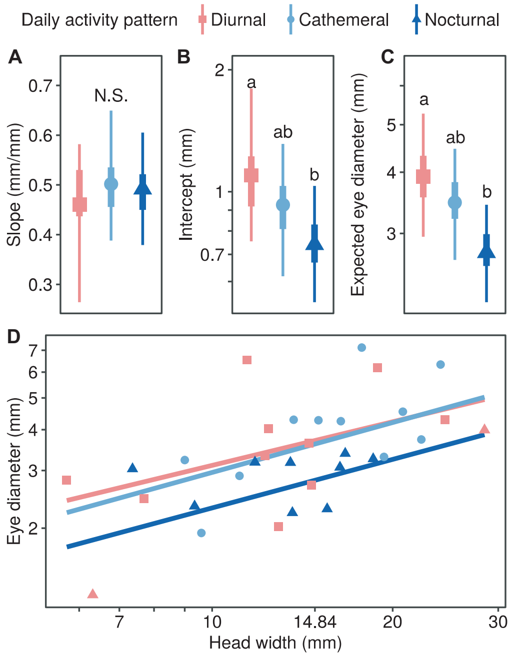

FIGURE 3 | Comparison of growth patterns of eye diameter and head width at different activity times, including allometric growth index **(A)**, proportionality coefficient **(B)**, expected eye diameter **(C)** and scatter diagram **(D)**. In subplots **(A–C)**, the symbols, vertical thick lines and vertical thin lines indicate the median, 50 and 95% HDI of the posterior distribution, respectively, and lowercase letters indicate multiple comparisons between groups (not having the same letter indicates a significant difference), while N.S. indicates no statistical difference. In **(D)**, the points indicate the mean head width and eye diameter within each species.

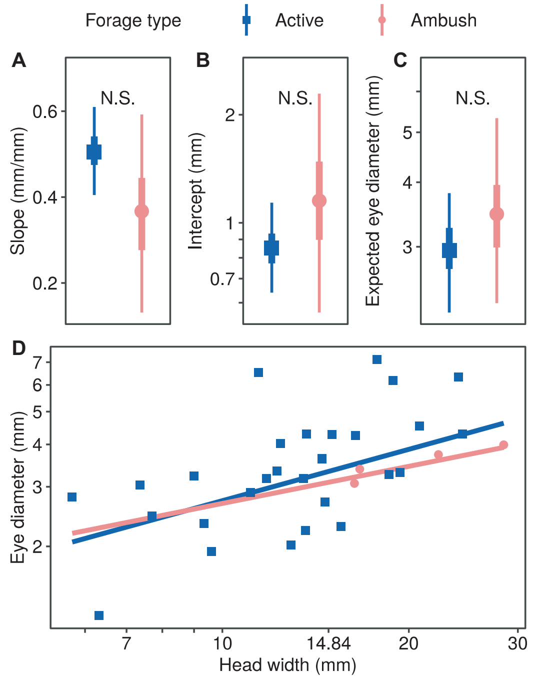

FIGURE 4 | Comparison of growth patterns of eye diameter and head width for different foraging modes, including allometric growth index **(A)**, scaling factor **(B)**, expected eye diameter **(C)** and scatter diagram **(D)**. In subplots **(A–C)**, the symbols, vertical thick lines and vertical thin lines indicate the median, 50 and 95% HDI of the posterior distribution, respectively, and lowercase letters indicate multiple comparisons between groups (not having the same letter indicates a significant difference), while N.S. indicates no statistical difference. In **(D)**, the points indicate the mean head width and eye diameter within each species.

vs. head width (i.e., the slope), but primarily by the absolute eye size (i.e., the intercept; **Figure 2**). Given that it is the absolute eye size that directly determines the visual functions and optical limits (Heesy and Hall, 2010; Veilleux and Kirk, 2014), the results suggests that it would require the freshwater snakes to either expand the body size dramatically or suppress the development of other vital organs in skulls in order to employ vision in the same level as terrestrial species. Such evolutionary strategies may not be as beneficial for the freshwater snakes, considering the high energetic costs potentially incurred and that information may be delivered more efficiently by other sensory systems (e.g., olfactory) than the visual system in aquatic environments due to limited illuminance.

Diel Activity Pattern

Our results showed that the eyes of nocturnal snakes appeared to be smaller than diurnal snakes, indicating that the diel activity pattern acts as the causal, behavioral mechanism, shaping the evolution of snake vision. Such a statement aligns with the conclusion derived by the two previous studies of Colubrid snakes (Liu et al., 2012; Hauzman et al., 2018) and one of them further showed that nocturnal snakes have reduced visual acuity and poorer spatial resolution power (Hauzman et al., 2018).

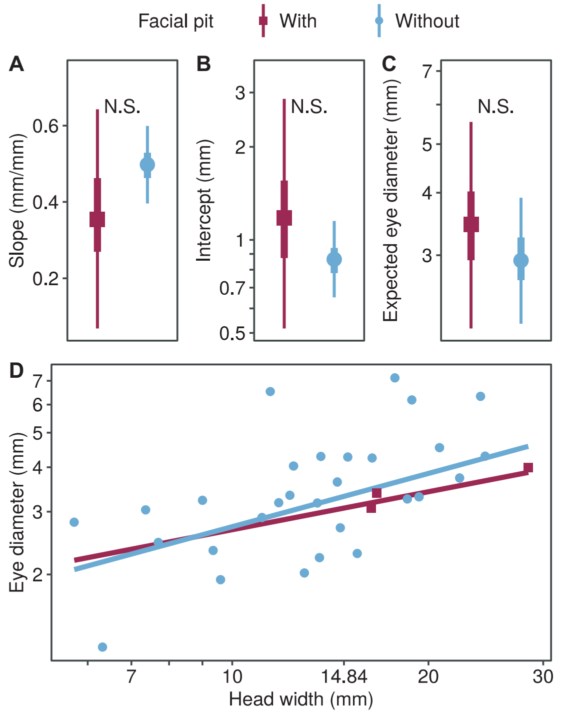

FIGURE 5 | Comparison of growth patterns of eye diameter and head width with and without pit, including allometric growth index **(A)**, proportionality coefficient **(B)**, expected eye diameter **(C)** and scatter diagram **(D)**. In subplots **(A–C)**, the symbols, vertical thick lines and vertical thin lines indicate the median, 50 and 95% HDI of the posterior distribution, respectively, and lowercase letters indicate multiple comparisons between groups (not having the same letter indicates a significant difference), while N.S. indicates no statistical difference. In **(D)**, the points indicate the mean head width and eye diameter within each species.

Despite the difficulty in navigating the environment in the dim-light condition, some nocturnal, highly mobile animals (e.g., geckos; Werner and Seifan, 2006), waterfowls (Thomas et al., 2006), or primates (Kirk, 2006) have shown to overcome the obstacles with the large eyes evolved. Distinctively, in our case, we showed how the less mobile animals (e.g., snakes) could have adopted the other evolutionary trajectory by adapting to the nocturnal activity pattern with other sensory systems complemented (Chen and Wiens, 2020). The majority of Viperidae are classified as nocturnal species in this study; this family has demonstrated the remarkable ability of exploiting the vomeronasal and the infrared sensing systems for discriminating prey species (Yang and Mori, 2021) and thermoregulatory (Krochmal et al., 2004). Our findings, regarding the eye size reduction in nocturnal snakes, thus yield a new hypothesis in terms of the evolutionary origin of multiple sensory modalities associated with the diel activity in snakes. Meanwhile, such a bipolar adaptive response of eye size across a broader taxonomic scale to the nocturnal environments also stresses the complexity of optimal sensory performance as the product of multidimensional evolutionary attributes (e.g., behavior, ecology, physiology, and phylogeny).

Foraging Strategy and the Presence of Pits

Both foraging strategies and the presence of pits showed no effect on the eye size based on the analyses, suggesting that snakes may have similar visual demands when exploiting either active hunting or ambush strategy. It also implies that the information perceived through the visual system is as important as that through the infrared system and, therefore, the presence of pits does not necessary relax the selection intensity in the evolution of snake eyes. Despite that we did not detect the significant effect of the two respective traits on the eye size, the results indeed generate some research directions worthwhile to examine subsequently. For example, considering the strong association between foraging strategies and the spatial structure of feeding habitats (Gilmour et al., 2018), as well as the evolutionary directions of the foraging strategy and optical ability cohered in snakes (Underwood, 1967; Tu, 2004; Simões et al., 2015; Katti et al., 2019), one may test whether the arboreal snakes tend to possess smaller eyes and adopt the ambush strategy disproportionally. By exploring how the behavioral, physiological, and ecological traits contribute to the evolution of eye size interactively, we are able to foster our ability to account for the evolution of innovative sensory systems and their role in promoting species adaptation across a broader geographical landscape.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by NMNSH002.

AUTHOR CONTRIBUTIONS

MC-WH and W-SH conceived the study. MC-WH measured all of the specimens. C-PL performed the statistical analyses. C-PL, C-CC, and W-SH led the writing. J-WL revised several sections with critical inputs in research discussions. All authors gave final approval for the publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.821965/full#supplementary-material

Supplementary Figure 1 | The measurement of snake eye diameter **(A)** and head width **(B)**.

Supplementary Figure 2 | The most approximate relatives of 31 snake species from Taiwan used in this study. The phylogenetic tree is adapted from Figueroa et al. (2016) based on the most approximate phylogenetic tree. The numbers to the right of the nodes indicate the support of the bootstrap method. Red texts indicate that species in this study could not be matched with Figueroa et al. (2016) and should be synonyms. Blue texts indicate species were not included in Figueroa et al. (2016), so they are represented by similar species.

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Heatwave-Like Events During Development Are Sufficient to Impair Bumblebee Worker Responses to Sensory Stimuli

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Heatwaves are increasingly common globally and are known to have detrimental impacts on animal morphology and behaviour. These impacts can be severe, especially if heatwaves occur during development, even on animals that can regulate the temperature of their developing young. The onset and duration of heatwaves are stochastic and therefore may affect all or only part of development. In the heterothermic bumblebee *Bombus terrestris,* elevated temperatures over the entirety of development cause morphological changes in adults, despite their capability to regulate brood temperature. However, the effects of heatwaves that occur during a short period of development are unclear. We test the impact of elevated developmental temperature during the latter fraction of development on the behaviour and morphology of adult worker *B. terrestris*. We show that exposure to elevated temperature over a portion of late development is sufficient to impair the initial behavioural responses of workers to various sensory stimuli. Despite this, exposure to elevated temperatures during a period of development did not have any significant impact on body or organ size. The negative effect of elevated developmental temperatures was independent of the exposure time, which lasted from 11–20 days at the end of the workers' developmental period. Thus, heat stress in bumblebees can manifest without morphological indicators and impair critical behavioural responses to relevant sensory stimuli, even if only present for a short period of time at the end of development. This has important implications for our understanding of deleterious climactic events and how we measure indicators of stress in pollinators.

Keywords: bumblebee, heatwave, behaviour, sensory system, reflex, *Bombus terrestris*

INTRODUCTION

For many animals, abnormal temperatures during development, either elevated or depressed, are known to have profound impacts on adult phenotype. This sometimes takes the form of phenotypic plasticity, such as the reptile temperature-dependent sex determination systems (Singh et al., 2020) or the spring/summer morphs of certain butterfly species (Gilbert, 2001). Other times, deviation from an optimum developmental temperature is negative, causing adult morphological deformation (Groh et al., 2004; Gerard et al., 2018), behavioural impairment (Van Damme et al., 1992; Tautz et al., 2003), deficient neurological development (Groh et al., 2004) and physiological disruption (Watkins, 2000; Singh et al., 2020).

Across exothermic vertebrates, non-adaptive deviations in adult phenotypes due to abnormal developmental temperatures have been well investigated when temperature stress occurs during the entirety of development (Burger, 1991; Van Damme et al., 1992; Watkins, 2000; Micheli-Campbell et al., 2011; Sfakianakis et al., 2011; Singh et al., 2020). Zebra fish embryos reared in warmer temperatures swim better as adults than their cooler compatriots, but show no discernible morphological differences (Sfakianakis et al., 2011). In contrast, hatchlings of the turtle *Elusor macrurus* from cooler incubation temperatures are the better swimmers (Micheli-Campbell et al., 2011; Singh et al., 2020). Even in insects, such as damselflies, exposure to elevated temperature throughout development has been shown to affect both wing shape and flight performance (Arambourou et al., 2017). The solitary bee *Osmia bicornis* suffers increased adult mortality, as well as decreases in prepupal weight, under hotter developmental temperatures (Radmacher and Strohm, 2011).

Exposure to elevated ambient temperatures has also been shown to affect bee colonies, albeit in a species-dependant manner. Honeybees that experience heat stress during development do not show any morphological indicators (Jones et al., 2005) (though see Groh et al., 2004 for reports of morphological disfigurement) but display learning and memory impairment (Tautz et al., 2003). Furthermore, adult honeybees reared under naturally occurring but undesirable temperatures had fewer mushroom body microglomeruli in the region dedicated to olfactory learning (Groh et al., 2004), suggesting that their capacity to learn olfactory stimuli would be impaired. Bumblebees subjected to heat stress over the duration of development have modified wing shapes as adults (Gerard et al., 2018), although it remains unknown if these or any other potential morphological variations have any behavioural consequences.

While we now have a good general understanding of how exposure to abnormal temperatures during the entirety of development affect a range of organisms, we still know little about the effects of abnormal temperature exposure during only a part of development. Understanding such effects, however, is becoming increasingly important if we are to understand how animals will be impacted by global warming. Global warming is increasing the frequency of heatwaves (Perkins-Kirkpatrick and Lewis, 2020) – prolonged periods of excessive heat (Perkins and Alexander, 2013; Perkins-Kirkpatrick and Lewis, 2020) – a weather phenomenon that may only impact an animal over a portion of their development. Animals like bumblebees that grow their colonies over spring and summer, when extreme heat events are most common, are particularly vulnerable to excessive temperature events. While the influence of elevated temperatures over a fraction of development is relatively unknown, investigations into the impacts of heatwaves specifically indicate they may have a profound effect on all organisms (Stillman, 2019).

Short-term heat-shock in insects often causes reproductive disruptions (Sales et al., 2018; Chen et al., 2019; Martinet et al., 2020), particularly impacting male fertility by altering spermatozoa (Sales et al., 2018; Martinet et al., 2020). In *O. bicornis*, exposure to elevated temperatures during pupation only disrupted male mate attraction behaviour (Conrad et al., 2017). In eusocial insects, capable of intranidal temperature regulation, the effect of partial developmental heatwaves has only been investigated in Africanized honeybees (Medina et al., 2018; Poot-Báez et al., 2020). A naturally occurring heatwave prior to pupation caused smaller adults (Poot-Báez et al., 2020) whereas a simulated heatwave during pupation affected wing shape, altered the strength of fluctuating asymmetry and lowered the age at onset of foraging (Medina et al., 2018).

The stochasticity of heatwaves means that eusocial insect larvae will be at different developmental stages at the onset of a heatwave and will therefore experience differential exposure to elevated temperatures. Having uneven exposure to elevated temperatures may still affect colony performance. Here, we begin to address the functional consequences of elevated temperatures during varied periods of mid and late development in the bumblebee *Bombus terrestris*. Bumblebees are an important model for this work because they tightly regulate both their colony (Schultze-Motel, 1991; Jones and Oldroyd, 2006; Gardner et al., 2007) and brood temperatures (Vogt, 1986; Weidenmüller et al., 2002). The proper development and function of sensory systems in bumblebees is essential for their pollination activities as they rely heavily on visual, olfactory, gustatory and mechanosensory information to navigate between flower patches and their colony (Dyer and Chittka, 2004; Orbán and Plowright, 2014; Wilmsen et al., 2017; Sprayberry, 2018) and to optimise their foraging (Spaethe et al., 2001; Chittka et al., 2003; Kulahci et al., 2008). Furthermore, bumblebees are already experiencing range shrinkage and population declines in both Europe (Kerr et al., 2015) and North America (Cameron et al., 2011; Kerr et al., 2015) as a result of a warming climate. The capacity for this genus to adapt to hotter temperatures may be physiologically constrained; *Bombus vosnesenkii* workers show no geographical variability regarding their critical thermal maximum (Pimsler et al., 2020), indicating a lack of local adaptation. We predicted that elevated temperatures over a portion of development, akin to a partial developmental heatwave, would impair behavioural responses to relevant sensory stimuli and cause morphological changes in adult worker bumblebees. Our results indicate that, despite having no discernible impact on morphology, partially elevated temperatures impair bumblebee responses to important sensory information, likely leading to decreased colony fitness.

MATERIALS AND METHODS

Animals

Eight *Bombus terrestris* (L., 1758) colonies from Koppert (Berkel en Rodenrijs, Netherlands) were reared in the dark in climate control cabinets (Panasonic MIR 123L, Tokyo, Japan). The colonies (consisting of 1 queen, 19–40 workers – the variation was due to the initial colony state – and all brood cells) were kept in plastic boxes (**Supplementary Figure 1**) filled with unscented cat litter to absorb humidity. Individuals from each colony were reared and experimented upon concurrently and the colonies were randomly assigned to treatments on arrival to the laboratory. Four colonies were kept in one incubator maintained at an air temperature of 26 ± 0.07°C and another four colonies were kept in another incubator at an air temperature of 33 ± 0.5°C. Climate loggers (Lascar Electronics, EasyLog EL-USB-2, Whiteparish, United Kingdom) recording temperature and humidity every 10 s were placed in each incubator (outside of the colonies). Two additional loggers recorded the air temperature and humidity inside two of the four colonies by means of small wire probes (attached to data loggers placed inside the cabinets but outside the colonies) that were placed beside the brood cells. At the end of the experiment, the data was downloaded from the loggers and average temperature and humidity calculated. All data loggers indicated that experimental temperatures and humidity were maintained in both the incubators and in the colonies and that they were stable throughout the experiment (indicating that bees were not able to significantly modify the ambient colony temperature from that set by the incubator). The relative humidity in the 26°C incubator was 53 ± 5.5% and 56.5 ± 8.8% in the 33°C incubator. Bees were fed *ad libitum* with 50% w/v refined white sugar water solution and fresh-frozen, organic pollen every 2–3 days (Naturprodukter Raspowder Bipollen, Stockholm, Sweden). After 7 days in the incubator, all bees in each colony were marked with non-toxic paint (Färgpenna Lackstift, Biltema, Helsingborg, Sweden) to identify bees that had already completed, or were close to, adult development.

Between 11 and 20 days after the colonies were placed in the incubators, individual bees without prior colour markings were tagged with individual colour/number plate combinations that were glued to their thorax and subjected to testing within 4 days. The time each bee was exposed to the temperature treatment at the end of its development (**Table 1**) was explicitly included in our statistical models (see below) to control for the effects of exposure length. Note that each individual only experienced the temperature treatment for a maximum of 20 days at the end of their development, rather than for their entire developmental period – typically 25 days (Cnaani et al., 2000) – as such, they experienced simulated heatwave conditions rather than long periods of sustained high temperature. Based on an estimated 25 days of development time from egg to adult, bees were first exposed to their treatment temperatures from day 5 to day 14 (corresponding to approximately when bees hatch through to pre-pupation and the cessation of feeding) (Cnaani et al., 2000). A total of 60 workers from eight colonies were tested (with only a single worker being excluded from the behavioural analysis due to insufficient data) – 30 workers from four colonies at 33°C and 29 workers from four colonies at 26°C. Sample sizes for the morphological analysis vary because some specimens were damaged during the preparation process.

TABLE 1 | The number of days each bee spent at treatment temperatures before being marked and subjected to behavioural tests.

Bee ID	Temperature (°C)	Colony	Days to marking	Days to test
G48	26	One	14	15
G47	26	One	14	14
G46	26	One	14	15
G45	26	One	14	15
G44	26	One	14	14
G43	26	One	14	14
G42	26	One	14	14
B28	26	Two	14	14
B27	26	Two	14	14
B26	26	Two	14	14
B25	26	Two	14	14
B24	26	Two	11	13
B23	26	Two	11	13
B22	26	Two	11	13
W42	26	Three	19	20
W41	26	Three	19	20
W40	26	Three	19	20
W39	26	Three	19	20
W38	26	Three	19	20
W28	26	Three	11	16
W27	26	Three	11	13
W26	26	Three	11	14
Y24	26	Four	19	20
Y23	26	Four	19	20
Y22	26	Four	19	20
Y21	26	Four	19	20
Y20	26	Four	19	20
Y19	26	Four	11	14
Y18	26	Four	11	14
Y17	26	Four	11	15
Y33	32	Five	20	21
Y32	32	Five	20	21
Y31	32	Five	20	21
Y30	32	Five	20	20
Y28	32	Five	20	20
Y27	32	Five	20	21
Y26	32	Five	20	21
Y25	32	Five	20	21
G57	32	Six	20	21
G56	32	Six	20	21
G55	32	Six	15	16
G54	32	Six	15	19
G52	32	Six	15	16
G50	32	Six	15	16
G49	32	Six	15	16
B36	32	Seven	15	19
B35	32	Seven	15	19
B34	32	Seven	15	19
B33	32	Seven	15	16
B32	32	Seven	15	16
B31	32	Seven	15	19
B30	32	Seven	15	16
B29	32	Seven	15	19
W36	32	Eight	15	19
W35	32	Eight	15	19
W34	32	Eight	15	16
W33	32	Eight	15	16
W32	32	Eight	15	19
W31	32	Eight	15	16
W30	32	Eight	15	19

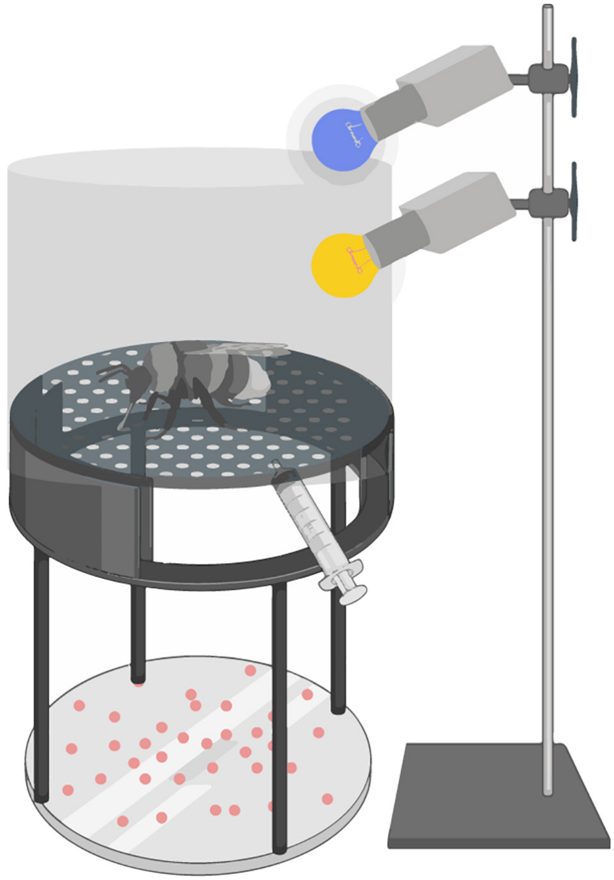

FIGURE 1 | Behavioural arena set-up. A schematic drawing of the experimental set-up used to investigate the effect of rearing temperature on the behavioural responses of bumblebees to different sensory stimuli. Bees were placed in the centre of the arena and presented with a light stimulus from above (UV or white light), an olfactory or mechanosensory stimulus delivered through a syringe from below (honey water odour or an air puff) or a drop of liquid (white sugar water or quinine) placed on the floor of the arena. Responses to these stimuli were recorded using a camera that filmed the set-up from above.

Selecting Treatment Temperatures

Considerable increases in bumblebee thermoregulatory behaviour have been observed when colonies were exposed to ambient temperatures of 26°C (Weidenmüller et al., 2002) and 27°C (Nasir et al., 2019). Other investigations have found that colonies function optimally below 30°C but temperatures in excess of this are detrimental to the number of workers produced in the first brood and to the lifespan of the colony (Nasir et al., 2019). Vogt (1986) considered colonies that experience temperatures of 32°C as heat stressed – ambient temperatures at and above 32°C elicit increased wing fanning, to cool the colony, with an associated increase in metabolic costs (Vogt, 1986). While 32°C has been reported as the preferred temperature for brood to be kept at Weidenmüller et al. (2002), brood temperature are typically 2°C warmer than the ambient air temperature inside the colony (Vogt, 1986; Weidenmüller et al., 2002). Therefore, an air temperature of 33°C inside the colony is likely to be beyond the optimal temperature for developing *B. terrestris*, with evidence of this being found in the modified wing shapes of adults which developed at 32°C (Gerard et al., 2018). Due to the above-described effects, 33°C was selected as the elevated temperature treatment.

Behavioural Experiments

Experimental Set-Up

Adult bees were fed *ad libitum* before the experiment and kept in darkness for the duration of the experiment. The experimental setup consisted of a circular transparent plastic arena with a plexiglass lid and metal mesh bottom (**Figure 1**) 9 cm in diameter and 14 cm in height. Individual bees were caught in the colony and placed in the experimental setup in the dark for 2 min acclimation before being sequentially exposed to seven stimuli with 2 min dark rest periods in between stimulus presentations. The behavioural tests were performed at room temperature, 23 ± 2°C. Bees were recorded under red light using a video camera (Sony FDR-AX33, Tokyo, Japan) positioned above the arena. The first response upon exposure to each stimulus was examined *via* frame-by-frame playback of the video recording using VLC Media Player (VideoLan, 2006) and classified as either *expected* or *unexpected* (for details, see section **Table 2**) by an investigator blind to the treatment group of each bee.

Stimuli

The stimuli were chosen to test the function of different sensory systems – vision, mechanosensation, olfaction and gustation – and were expected to elicit attraction, aversion or neutral responses from the bees (**Table 3**). The stimuli were always presented to the bees in the same sequence, the order of which can be found in **Table 3**. The attractive stimuli consisted of honey odour, white sugar water, brown sugar water and UV light, the aversive stimuli consisted of an air puff or quinine and the neutral stimulus consisted of white light. The brown sugar water was the solution that the supplier, Koppert, uses as a standard nutritional food source in commercial colonies. The response of the bees to the stimulus was recorded upon their exposure to it. In the case of the UV and white lights (**Supplementary Figure 2**), the bees were given 1 min to respond, in the case of the odour and air puff stimuli, the response of the bees was recorded in the duration of the delivery and in the case of the sugar solutions and quinine, a droplet of the solutions were placed on the floor of the experimental setup and a response was recorded once the bees had encountered (walked over) the solution (for details see **Table 3**).

List of Behaviours

Based on preliminary experiments and whether the stimulus was aversive, attractive or neutral, a list of expected and unexpected responses (an ethogram) was generated for each sensory stimulus (**Table 2**). These expected responses were considered to be reasonable initial reflex reactions from the bees on exposure to these stimuli. The unexpected responses were reactions considered functionally inappropriate on exposure to that stimulus.

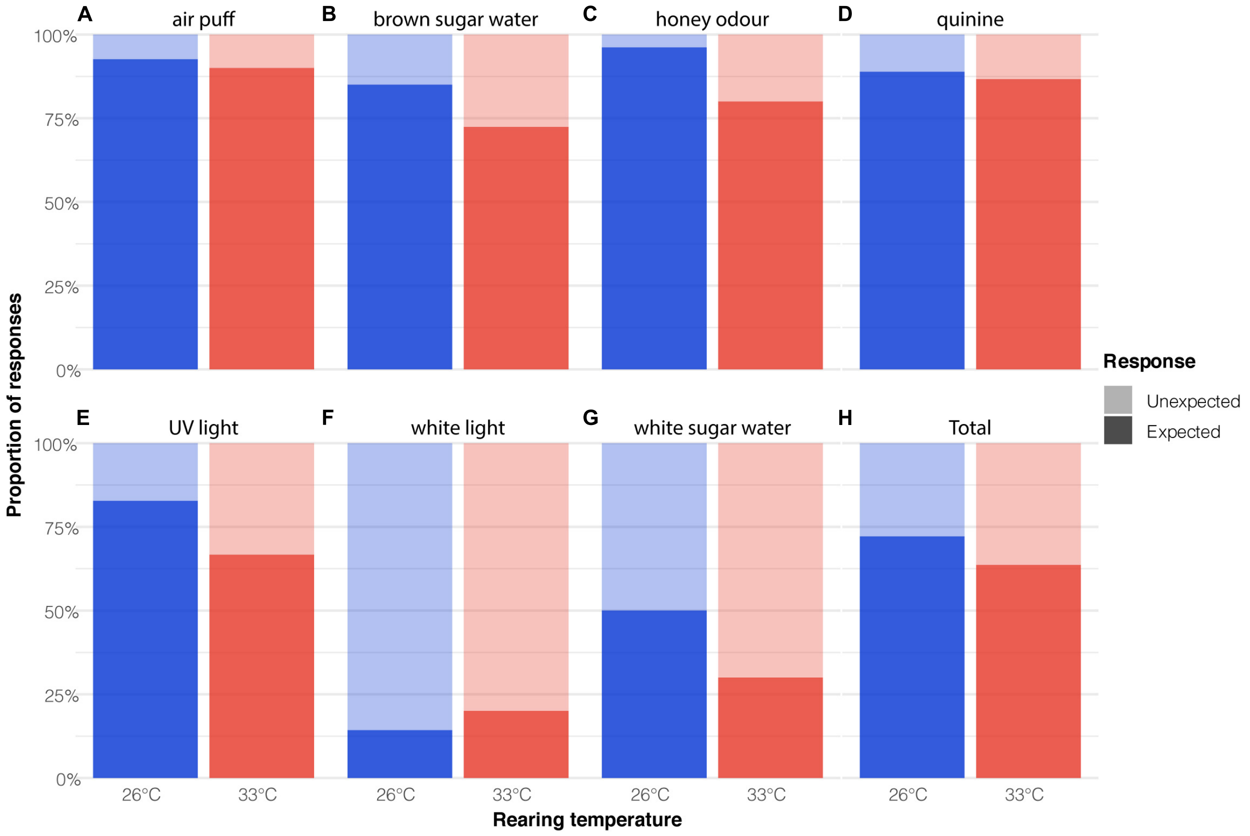

FIGURE 2 | The effect of developmental heatwaves on the behavioural response to sensory stimuli. The proportional of expected and unexpected behavioural responses (**Table 2**) of bees exposed to **(A)** air puff, **(B)** brown sugar water, **(C)** honey odour, **(D)** quinine, **(E)** UV light, **(F)** white light **(G)** white sugar water. **(H)** Total proportion of expected and unexpected behavioural responses for all presented stimuli. Sample size = 59.

TABLE 2 | Ethogram of correct and incorrect behavioural responses to various sensory stimuli.

Stimulus type	Expected responses	Unexpected responses
UV light	Flying, attempt to fly	No response, slow walking, fast walking, sitting still, climbing wall, grooming, biting mesh
Brown sugar odour	Antenna movement, proboscis extension, head toward the tube, stop on top of tube	No response, retraction, wiping face, biting mesh, attacking mesh
Quinine	Retract, wiping face/antenna	No response, climbing wall, drinking
White sugar water	Drinking, proboscis extension, antenna movement	No response, retract, wiping face
Brown sugar water	Drinking, proboscis extension, antenna movement	No response, retract, wiping face
Air puff	Buzzing, flying, laying on back, running, lifting leg, crunching, wiping face, climbing wall	No response, grooming
White light	Flying, attempt to fly	No response, slow walking, fast walking, sitting still, climbing wall, grooming, biting mesh, drinking sugar water

Morphological Measurements

Antennal length, forewing size and eye areas were selected as features to measure, because the functioning of these organs will affect how the bees interact with the different presented stimuli. Body size was measured as fresh mass to the nearest μg using a balance (Sartorius BP 310S, Göttingen, Germany). Bumblebees were euthanised using ethyl acetate and their mass was documented within 5 min of death followed by subsequent dissections. Antennae and proboscides were dissected from bumblebee workers, laid flat on 1 mm grid paper and photographed. The length of the right antenna from each individual was measured in ImageJ (Schneider et al., 2012). Right forewings were removed from each individual, photographed and their length and width was measured in ImageJ (Schneider et al., 2012). 3D volumetric scans of the head were acquired through X-ray microtomographic (micro-CT) scans (Taylor et al., 2019, 2020) at the TOMCAT beamline of the Swiss Light Source (beamtime number: 20191425). The left eyes were cropped using Drishti 2.6.4 (Limaye, 2012) and imported into Amira 6.2.0 (Thermo Fisher Scientific, Whaltham, United States). Eye surface area was determined by creating a patch of the left eye using the "Patchify Surface" function in Amira 6.2.0.

Statistics

All statistics were conducted using R v.4.0.2 (R Core Team, 2016). Binary response data were analysed using generalized mixed effects models with a binomial family from the lme4 package (Bates et al., 2015). A maximal model was constructed and simplified using likelihood ratio tests (LRT) to arrive at the minimum adequate model. The initial maximal model was fitted with main effects of temperature, time from treatment onset to being tagged, time from treatment onset to behavioural testing (equal to the total time spent in the treatment), stimulus type and body mass (**Table 4**). An interaction between temperature and stimulus type was also fitted along with a random intercept of bee ID nested in colony (**Table 4**). Significance of explanatory variables in the final model were analysed using type II ANOVAs from the car package (Fox and Weisberg, 2019). Subsequent pairwise comparisons were Tukey adjusted and estimated using the multcomp package (Hothorn et al., 2008). Morphological differences were assessed using a two-sample t-test.

RESULTS

There was no significant interaction between temperature and stimulus type (LRT, $X_6 = 4.04$, $p = 0.62$) nor was there a significant effect of fresh mass (LRT, $X_1 = 0.02$, $p = 0.89$). There was no significant effect of days taken to eclose or days to onset of behavioural testing (LRT, $X_1 = 3.27$, $p = 0.07$) There was a significant effect of both temperature (type II ANOVA, $X_{59,1} = 5.22$, $p < 0.03$) and stimulus type on the likelihood of bees responding in accordance with the *expected* criteria (type II ANOVA, $X_{59,6} = 0.03$, $p < 0.001$).

Post-hoc pairwise comparisons revealed that bees reared at 26°C made a greater number of expected responses compared with bees reared at 33°C (**Figure 2**, $Z = 2.29$, $p = 0.02$). Irrespective of temperature treatment, bees were more likely to respond in accordance with the expected criteria to UV light, honey water odour, quinine, an air puff and brown sugar water than they were to white sugar water and white light (**Figure 2**, $Z > 3.73$, $p < 0.01$).

There was no significant difference in the mean size of any of the measured morphological features (antennae length, eye area, forewing length, forewing width, proboscis length, body mass) between temperature treatments (**Figure 3**, T-test, $t_{22-60,17-58}$, < 1.85, $P > 0.17$).

TABLE 3 | Sensory stimuli used in the experiment.

Treatment	Exposure Time	Delivery mechanism
UV light (attractive/novel)	1 min	UV lamp
Honey odour (attractive/novel)	Until syringe was empty	60 ml syringe
Quinine (aversive/novel)	Until opportunity to react	One drop (Pasteur pipette)
White sugar water (attractive/familiar)	Until opportunity to react	One drop (Pasteur pipette)
Brown sugar water (attractive/novel)	Until opportunity to react	One drop (Pasteur pipette)
Air puff (aversive/novel)	One puff	Canned air (gas duster)
White light (neutral/novel)	1 min	Lamp

Sensory functions (vision, mechanosensation, olfaction and gustation) were tested using attractive (UV light, honey odour, white sugar water, brown sugar water) aversive (air puff, quinine), and neutral (white light) sensory stimuli that were either familiar or novel to the bees. Opportunity to react refers to the moment a bee was able to encounter the stimulus (e.g., when it was oriented directly toward the stimulus). Stimuli were presented to all bees in the order listed in the table.

DISCUSSION

We explored the functional consequences of elevated rearing temperatures during a period of development – such as those likely to be induced by heatwaves arising from global warming – on bumblebee behaviour by testing their initial responses to various novel and familiar sensory stimuli (**Table 3**). Bees that spent a portion of their development time at 33°C made 8% more unexpected responses than those reared at 26°C, a difference that would represent a significant negative effect on behaviour. Though these bees were naïve and tested in laboratory conditions, it is not implausible to posit that this could lead to many incorrect choices when extrapolated across the lifetime of a colony. Although maladaptive responses to relevant sensory stimuli could potentially be rectified by experience, previous work showing that honeybees raised at elevated temperatures have impaired memory (Tautz et al., 2003; Jones et al., 2005), suggests that this process may also be affected. The effect of elevated temperatures on the response to sensory stimuli was independent of the time the bees spent at that temperature. This suggests that elevated developmental temperatures can disrupt adult behaviour, independent of their duration – a shorter period of exposure to elevated temperatures is as detrimental as a longer period. This effect of elevated temperature on behavioural responses to sensory stimuli did not appear to be related to morphology; we found no differences between body masses or the size of important sensory structures such as antennae, eyes, wings or proboscides between the two treatment conditions (**Figure 2**).

Morphological changes, such as organ shape (Gerard et al., 2018), body size (Gerard et al., 2018) or levels of fluctuating asymmetry (Klingenberg, 2015) are often employed as indicators of environmental stress. We found that behavioural impairment occurred without a morphological correlate, indicating that the approach of using morphological differences to indicate environmental stress alone is not necessarily sufficient and other factors, such as behaviour, should be considered during such investigations. The elevated temperatures presented during the mid- and late stages of development in this study did not induce any morphological changes, unlike in Gerard et al. (2018), where a constantly elevated temperature throughout development caused changes to wing morphology. Since we exposed bumblebees in later stages of development to heatwave conditions, it is likely that body size and relative organ sizes were already developmentally fixed (Tian and Hines, 2018). A useful extension of this study would be to examine the effect of elevated

TABLE 4 | Structure of binomial family generalised linear mixed models.

	Response	Fixed effects	Random intercept
Maximal model	Binary (expected or unexpected)	Stimulus type * Temperature + Time from treatment onset to eclosion + Time from treatment onset to behavioural testing + Body mass	Individual nested within colony
Minimum adequate model	Binary (expected or unexpected)	Stimulus + Temperature	Individual nested within colony

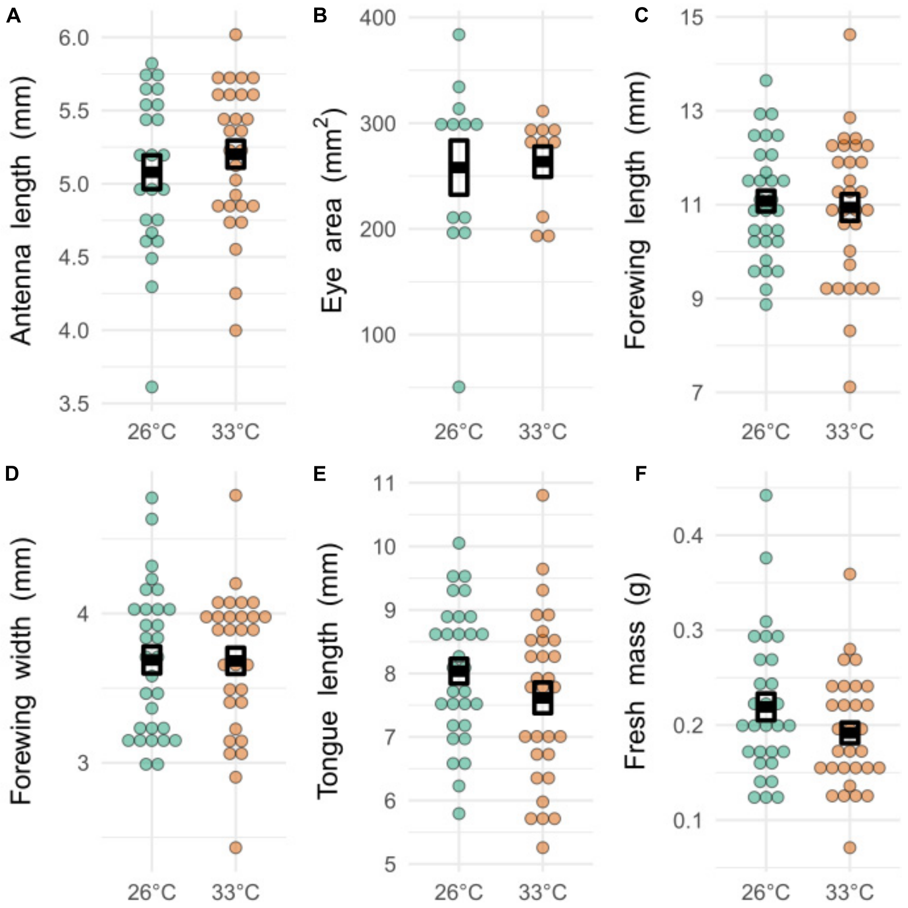

FIGURE 3 | Morphological measurements from bees exposed to developmental heatwaves of different temperatures. Each dot represents a single measurement from an individual bee. Dark boxes represent means ± S.E. There was no significant difference between the mean values of any of the morphological measurements. **(A)** Antenna length: $t_{51,44.4} = 0.83$, $p = 0.41$, size range = 3.61–6.02 mm. **(B)** Eye area: $t_{22,16.96} = 0.19$, $p = 0.85$, size range = 0.005–0.0384 mm^2. **(C)** Forewing length: $t_{60,54.64} = 0.38$, $p = 0.70$, size range = 7.12–14.6 mm. **(D)** Forewing width: $t_{60,57.96} = 0.04$, $p = 0.96$, size range = 2.43–4.79 mm. **(E)** Tongue length: $t_{60,55.51} = 1.35$, $p = 0.18$, size range = 5.26–10.8 mm. **(F)** Fresh mass: $t_{59,53.16} = 1.53$, $p = 0.13$, size range = 0.071–0.442g.

temperatures during early development and assess the extent of behavioural and morphological alteration.

The impairments in behaviour observed here, as a result of a heatwave-like event, likely have a neurological, rather than morphological basis. It is unclear if the impairment lies in sensory structures, ascending or descending neurons or in areas of the brain. Tautz et al. (2003) and Jones et al. (2005) concluded that decreased temperature during development caused subtle neurological changes that impaired short-term memory in honeybees. Further evidence for this can be found when examining mushroom bodies of honeybees reared outside of their optimum temperature (Groh et al., 2004). The number of microglomeruli (distinct synaptic complexes) fell as rearing temperature deviated away from the naturally held optimum. This effect was most pronounced within the olfactory input region, while the visual region of the mushroom body was less affected (Groh et al., 2004). Our data are consistent with these findings – a subtle neurological impairment could explain the poorer responses observed in bumblebees reared partly at 33°C. Further, short-term memory in honeybees (Tautz et al., 2003; Jones et al., 2005) was examined *via* the proboscis extension reflex, a common method for establishing learning and memory in bees (Giurfa and Sandoz, 2012). One of the responses of the bumblebees in this study was the extension of their proboscides (**Table 2**), indicating that the short-term memory issues reported in honeybees reared at abnormal temperatures might be mechanistically or neurologically similar to the unexpected behavioural responses we observe.

Although we generally observed an effect of elevated developmental temperature on the behavioural responses of bees, there was variation in the extent of the impairment depending on the sensory stimulus. For example, the proportion of expected responses to brown sugar water was similar in both temperature groups (85% at 33°C, 75% at 26°C), while the response differed more markedly in response to white sugar water (70% at 33°C, 50% at 26°C). It is unclear why there was a difference in the response to these two stimuli but it possibly relates to the fact that the brown sugar water had a "honey-like" odour while the white sugar water did not. There was also a difference in how the two temperature groups responded to the two light stimuli that were presented. The bees in this study were reared in the dark and had thus never seen light before, which may explain the higher proportion of unexpected responses to white light. Though, this does not explain the higher proportion of expected responses to UV light, which was equally novel to them. As the stimuli were always presented in the same order, it is also possible that the differences in the proportion of expected responses are related to the order of stimulus presentation. Nonetheless, this does not explain differences between the proportion of responses observed in the two temperature groups to each stimulus as both groups experienced the same order of presentation. Further experimentation and analysis would be required to ascertain why bees reared under elevated temperatures were better able to respond to some stimuli than others.

It is unclear at what temperature *B. terrestris* colonies begin to experience heat stress. The value appears to be somewhere between 26°C (Weidenmüller et al., 2002) and 32°C (Vogt, 1986). Whether the bees or the colonies were indeed under heat stress in this study is somewhat moot concerning what our results indicate – the elevated developmental temperatures were sufficient to induce an effect on adult behaviour. Heat stressed brood are an obvious and likely explanation behind our results, however, even if colonies were not under heat stress, the indication that sub-stressful elevated temperatures can cause behavioural impairment should be troubling to anyone paying attention to current trends in global climate.

Our data also do not indicate whether the elevated temperature experienced by the colony was the direct cause of the behavioural impairment we observed. Bumblebees can regulate the air and the brood temperatures independently (Vogt, 1986). We measured only the ambient temperature inside the colonies and did not directly measure the brood temperature; therefore it is possible that the brood were kept above or below the temperature set by the incubator, despite the air temperature within the colonies being consistently 33°C or 26°C. The temperature of the brood would need to be directly measured to assess the capacity of the bees to combat the overwhelming air temperature. If the brood are reared at temperatures different from ambient, it would indicate that there are indirect temperature effects on developing bees, likely a result of modified action by nurses. It is known that increased thermoregulatory behaviour lessens the attention that nurses provide to brood (Vogt, 1986). The observed behavioural impairments may therefore be caused directly *via* the temperature of the brood or indirectly *via* temperature causing disruption across the whole colony. Either proximate mechanism indicates that elevated temperatures over portions of development create behavioural impairments in adult bumblebees.

Our finding that bees experiencing periods of elevated temperature during development make sub-optimal responses to relevant sensory stimuli is more evidence of the dangers of climate change to heterothermic animals, which are susceptible despite their capacity to independently regulate their temperature. Bees in our experiment experienced elevated temperatures during their mid- and late developmental stages, mimicking heatwave periods, the risk of which are increasing across the northern hemisphere (Qiu and Yan, 2020) and increasing in frequency and magnitude across the globe (Perkins-Kirkpatrick and Lewis, 2020). Therefore, our data provide an indicator of how current and near-future climate change may affect bumblebees. Responding poorly to stimuli, relative to bees reared in more optimal temperatures, could come at a greater energetic cost and result in early worker death, which is a source of stress for a colony (Bryden et al., 2013). Furthermore, a reduced capacity to make appropriate responses to sensory stimuli will affect the speed at which correct decisions are made, impacting foraging efficiency and therefore colony fitness (Spaethe et al., 2001; Chittka et al., 2003; Kulahci et al., 2008).

Our findings add to an existing body of work that indicates that global warming will have a significant effect on pollinators (Shrestha et al., 2018), though few experiments have explicitly examined the effects of elevated rearing temperature on adult behaviour. The impairments presented here show that elevated rearing temperatures affect innate behavioural responses to sensory stimuli. Our experiment tested basic behavioural responses but the results indicate that elevated rearing temperatures are likely to affect more complex behaviours, as well as learning and memory (Tautz et al., 2003; Jones et al., 2005). Further investigations are necessary to understand if these behavioural impairments are mitigated by age, the effects of increased learning and to uncover the mechanisms by which impairments occur.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MG, ZJ, CR, and EB designed the study. CP, ZJ, VW, ZM, MG, and CR collected the data. AM acquired data and provided technical micro-CT expertise. CP performed the analysis and drafted the manuscript with EB. All authors critically revised the manuscript and gave approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.776830/full#supplementary-material

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Continuous Variation in an Aposematic Pattern Affects Background Contrast, but Is Not Associated With Differences in Microhabitat Use

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Variation in aposematic signals was once predicted to be rare, yet in recent years it has become increasingly well documented. Despite increases in the frequency with which polytypism and polymorphism have been suggested to occur, population-wide variance is rarely quantified. We comprehensively sampled a subpopulation of the poison frog *Oophaga sylvatica*, a species which is polytypic across its distribution and also shows considerable within-population polymorphism. On one hand, color pattern polymorphism could be the result of multifarious selection acting to balance different signaling functions and leading to the evolution of discrete sub-morphs which occupy different fitness peaks. Alternatively, variance could simply be due to relaxed selection, where variation would be predicted to be continuous. We used visual modeling of conspecific and heterospecific observers to quantify the extent of within population phenotypic variation and assess whether this variation produced distinct signals. We found that, despite considerable color pattern variation, variance could not be partitioned into distinct groups, but rather all viewers would be likely to perceive variation as continuous. Similarly, we found no evidence that frog color pattern contrast was either enhanced or diminished in the frogs' chosen microhabitats compared to alternative patches in which conspecifics were observed. Within population phenotypic variance therefore does not seem to be indicative of strong selection toward multiple signaling strategies, but rather pattern divergence has likely arisen due to weak purifying selection, or neutral processes, on a signal that is highly salient to both conspecifics and predators.

Keywords: aposematism, crypsis, poison frog, *Oophaga sylvatica*, visual modeling

INTRODUCTION

Aposematic (warning) signals evolve to convey important information related to prey defenses to potential predators (Stevens and Ruxton, 2012; Caro and Ruxton, 2019). Predators evolve, or learn, to associate certain prey characteristics with chemical or physical defenses and subsequently avoid prey bearing these signals (Stevens and Ruxton, 2012; Caro and Ruxton, 2019). By the simplest functional interpretation of aposematism, warning signals should be readily recognizable, reliable, and memorable to facilitate rapid associative learning (Mappes et al., 2005; Stevens and Ruxton, 2012). Positive frequency dependent selection is, therefore, expected to drive further homogenization of signal characteristics with common local phenotypes being avoided more consistently than rare phenotypes (Mappes et al., 2005; Chouteau and Angers, 2011; Chouteau et al., 2016).

In nature, however, aposematic signals can vary considerably between individuals of a single species, both within and between populations (Briolat et al., 2019). Considerable attention has been paid to how warning coloration evolves and functions (Caro and Ruxton, 2019), yet basic questions related to polymorphism and polytypism remain. Does variation serve an adaptive function such as local specialization to distinct environmental conditions, or is it merely permitted due to a relaxation in selection where neutral processes can lead to signal divergence? Once variation has arisen in allopatry, it may then be reinforced through mechanisms related to local predation pressure, assortative mating or intraspecific agonistic interactions, or a combination of several factors, which can further drive divergence between genetically and physically isolated populations (Gray and McKinnon, 2007; Briolat et al., 2019). Variation can, however, also arise in sympatric populations without physical barriers to gene flow (Rojas, 2017; Briolat et al., 2019). The mechanisms underlying the evolution and persistence of this variance are, however, less well understood, and in many cases it is still unclear whether such variation has evolved for an adaptive function.

The characteristics of animal color patterns are shaped by the simultaneous influence of multiple selection pressures, leading to potential trade-offs rather than the optimization of single functions (Endler and Mappes, 2004; Stevens, 2007; Stevens and Ruxton, 2012; Briolat et al., 2019). For example, individual components of aposematic signals can be locally adapted for saliency when viewed against particular microhabitats, serve to incorporate additional defensive signals, or contain traits used in sexual selection. Based on its primary function, each element could theoretically have its own distinct optimal form which influences fitness under specific sources of selection. Polymorphism can evolve due to adaptive benefits, whereby different forms arise as a by-product of unique adaptive processes (Briolat et al., 2019). Alternatively, instead of being fostered by selection, polymorphism may simply be permitted where purifying selection is weak, or optimal signal efficacy is difficult to achieve, leading to variation arising *via* neutral processes (Briolat et al., 2019). With such distinct plausible mechanisms at play, it is perhaps not unsurprising we have a weak overall understanding of why intra-population variance in aposematic signals exists.

Some of the most salient examples of phenotypic variation are found in the Neotropical poison frogs (Dendrobatidae), which exhibit notable examples of both discrete and continuous variation in color and pattern, found both in sympatry (polymorphism) and allopatry (polytypism) (Summers et al., 2003; Maan and Cummings, 2012; Rojas, 2017). Color patterns have evolved both for defensive and communicative functions, and in many cases under the influence of multiple selection pressures (Summers et al., 1999; Saporito et al., 2007; Maan and Cummings, 2008, 2009, 2012; Wollenberg et al., 2008; Tazzyman and Iwasa, 2010; Crothers et al., 2011; Cummings and Crothers, 2013; Rudh, 2013; Crothers and Cummings, 2015; Dreher et al., 2015). Consequently, signals with multiple components or functions have arisen due to context specific interactions between sources of selection and/or neutral processes (Wollenberg et al., 2008; Tazzyman and Iwasa, 2010; Crothers et al., 2011; Crothers and Cummings, 2013, 2015; Cummings and Crothers, 2013; Barnett et al., 2018). For example, aposematic signals have been co-opted and exaggerated by intraspecific communication (Maan and Cummings, 2008, 2009), can blend together when viewed from a distance to act as camouflage (Barnett et al., 2018), and can disrupt an observer's ability to track escape movements (Hämäläinen et al., 2015).

In poison frogs, discrete color forms have evolved in geographically isolated populations as founder effects and honest signaling of local resource availability have been reinforced through sexual imprinting, selective mate choice, and predator learning (Summers et al., 1999, 2003; Darst et al., 2006; Reynolds and Fitzpatrick, 2007; Maan and Cummings, 2008, 2009, 2012; Wang and Shaffer, 2008; Tazzyman and Iwasa, 2010; Crothers et al., 2011; Richards-Zawacki and Cummings, 2011; Crothers and Cummings, 2013; Hegna et al., 2013; Yang et al., 2019). For example, in *Oophaga pumilio*, where dietary derived toxicity is low, selection for camouflage has outweighed selection for conspicuous signaling, and more cryptic colors and behaviors have evolved (Maan and Cummings, 2012; Rudh et al., 2012; Rudh, 2013). Within color morphs of *O. pumilio*, continuous variation in color saturation and brightness have been associated with intraspecific communication, with brighter individuals more likely to win physical contests and attract mates (Crothers et al., 2011; Crothers and Cummings, 2013, 2015; Dreher et al., 2017). Moreover, in *O. pumilio* certain phenotypes and behaviors are correlated with microhabitats which enhance signal efficacy (Pröhl and Ostrowski, 2011; Willink et al., 2014), although they do not appear to be behaviorally manipulating saliency on fine scales (Dugas et al., 2020). In another polymorphic and polytypic poison frog species, *Dendrobates tinctorius*, sympatric color variation can also correlate with specialization in specific microhabitats, or behavioral syndromes that optimize signal saliency within their chosen microhabitats (Rojas et al., 2014a,b; Rojas, 2017).

The poison frog *Oophaga sylvatica* shows polytypic variation across its range as well as remarkable within-populational polymorphism (Roland et al., 2017). Limited insights into color pattern evolution can be gleaned from population-level genetic assessments, which indicate well-defined clades broadly split between north and south, but also reveal large geographic regions with high levels of genetic admixture, including within polymorphic populations (Roland et al., 2017). Polymorphic populations are proposed to either be the result of admixture between phenotypically distinct adjacent populations in secondary contact, or due to a range extension from a polymorphic population into adjacent populations (Roland et al., 2017). Either scenario suggests that color patterns may not be under strong selection within the northern clade populations, and/or that coloration does not play a significant role in the geographic structure of phenotypically divergent *O. sylvatica* populations across at least substantial portions of its distribution.

We explored the role of phenotypic variation in a highly variable population of *O. sylvatica* found in northwestern Ecuador. These frogs display high contrast dorsal patterns that are highly distinct from their leaf litter substrates (Yeager and Barnett, 2020). However, as early as in the formal description of *O. sylvatica* it has been noted that human observers could easily confuse frogs from a similar red/black morph with the forest floor (Funkhouser, 1956), suggesting a potential balance between camouflage and conspicuous signaling.

We used computational visual models, representing the vision of diverse predator classes and conspecifics, to study the perception of phenotypic variation by sources of natural and sexual selection. We first addressed whether frog variation was continuous, or if it could be grouped into visually distinct clusters, and then quantified which components of the phenotype (chromatic (hue), achromatic (brightness), or pattern contrast) best defined phenotypic variation. Next, we investigated whether variance between the aposematic signals of individual frogs correlated with the microhabitats in which they were found to either maximize or minimize visual contrast. We expected that regardless of whether variation is continuous or discrete, if it has evolved to facilitate distinct behavioral, defensive, or reproductive strategies then it should be associated with differences in saliency based on microhabitat use (Rojas et al., 2014a,b; Rojas, 2017; Briolat et al., 2019; Barnett et al., 2021a). Conversely, variation may be permitted under relaxed selection, and attributable to non-adaptive processes such as drift. In such instances we would expect variation to be continuous and not associated with signaling differences within their occupied microhabitats. Deciphering how this variation arises and is maintained within a single population has important implications for our understanding of how predators generalize behaviors across variable aposematic signals, how multiple selection pressures interact, how the evolution of discrete polymorphism may arise from a monomorphic ancestor, and the evolutionary implications of polymorphism (Gray and McKinnon, 2007; Briolat et al., 2019).

MATERIALS AND METHODS

Data Collection

In March 2020, we photographed 35 *Oophaga sylvatica* (Perla morph) at the private forest reserve "Bosque Protector la Perla" near La Concordia, Ecuador. This represented a comprehensive survey of every individual observed in the area by a team of five experienced observers, over 2 days. The Perla morph is approximately 26 mm in length and is predominantly black with a red pattern that varies from small spots to larger irregular blotches, and to whole patches of homogenous color (**Figure 1**). Previous work suggests that the frogs' colors have very high internal and external contrast, and that UV reflectance is minimal both from the frogs and their natural leaf litter background (Yeager and Barnett, 2020). Frogs were spotted using exhaustive visual surveys. We cannot ensure that frog orientation was not altered due to the presence of the observers, however, the background is isotropic, and we used the location where the frog was first spotted as the microhabitat context in which potential predators would process frogs' visual signals.

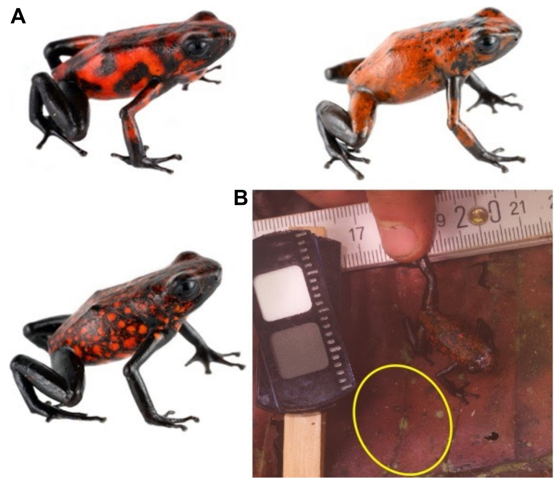

FIGURE 1 | Study system. **(A)** Populations of *O. sylvatica* in northern Ecuador are characterized by red and black color patterns. Significant variation can be found within a single population, including the population sampled here. **(B)** Standardized photographs were taken where subsequent analyses featured chromatic, achromatic, and pattern contrasts between the entire frogs' dorsum when compared against patches of the microhabitat (yellow circle) and random patches of habitat. Frog photos **(A)** courtesy of J. Culebras (Photo Wildlife Tours).

We photographed each frog within the microhabitat where it was first observed (**Figure 1B**), following methods detailed in Yeager and Barnett (2020, 2021). In brief, each image was taken using a quartz converted UV sensitive Canon EOS 7D camera combined with a metal body NIKKOR EL 80 mm lens. As UV reflectance is negligible in this population ($n = 4$ tested in Yeager and Barnett, 2020) we did not include UV wavelengths and only utilized images in the human visible range (400 – 700 nm). The camera was mounted on a tripod and the lens was fitted with a Baader UV-IR blocking filter that allowed transmission from 420 to 680 nm. All photographs were taken under natural ambient lighting, each image included a 10% and a 77% neutral reflectance standard, and we saved all photographs in RAW format.

Image Processing and Visual Modeling

We used the MICA toolbox (Troscianko and Stevens, 2015) to standardize and scale the images in ImageJ v1.52k (Schneider et al., 2012). From each image, we selected regions of interest (ROIs) from within the red and black patches of the frog, around the frog's whole body, and around a patch of adjacent substrate of a size equal to, or slightly greater than, that of the frog (**Figure 1**). We then assessed color and pattern using three well characterized computational models of visual perception representing important sources of natural selection (predatory birds and snakes) and sexual selection (conspecific frogs).

For our model of bird vision, we used the tetrachromatic visual system of the Eurasian blue tit (*Cyanistes caeruleus*, Paridae). The blue tit has four single cones (λ_{max}: UV = 372 nm, SWS = 413 nm, MWS = 508 nm, and LWS = 573 nm) that populate the retina with a ratio of 1:2:2:3, and one double cone (λ_{max}: D = 565 nm) (Hart et al., 2000; Hart, 2001). As UV was negligible, we excluded these wavelength (<400 nm) to produce a tetrachromatic VIS-sensitive model which covered 400–700 nm (Yeager and Barnett, 2020, 2021). The snake model used the trichromatic vision of the coachwhip (*Masticophis flagellum*, Colubridae) and included three single cones (λ_{max}: UV = 362 nm, MWS = 458 nm, and LWS = 561 nm) with a ratio of 17:2:1 (Macedonia et al., 2009; Maan and Cummings, 2012). To represent the frogs' own visual system, we used the trichromatic vision of the closely related *O. pumilio* (Dendrobatidae). The *O. pumilio* visual model included three single cones (λ_{max}: SWS = 466 nm, MWS = 489 nm, and LWS = 561 nm) with a cone ratio of 4:3:1 (Siddiqi et al., 2004; Maan and Cummings, 2012).

We calculated chromatic (ΔS) and achromatic (ΔL) contrast between the red and black regions of each frog (internal contrast), and between the mean of the whole frog and the mean of each background (external contrast), using the log-linear receptor noise limited model implemented through the MICA toolbox (Vorobyev et al., 1998; Vorobyev and Osorio, 1998; Troscianko and Stevens, 2015). Chromatic contrast was calculated using the responses of the single cones and we calculated achromatic contrast from the response of the D cone in the bird model and from the LWS cones in the snake and frog models. As specific predator species which could influence frog coloration are not known for *O. sylvatica*, and to keep our results comparable to our previous studies (Yeager and Barnett, 2020, 2021; Barnett et al., 2021b) all Weber fractions were set at 0.05. The model computes visual discrimination in a manner equivalent to "just noticeable differences" (JNDs) where higher values indicate greater ease of color discrimination. A score of 1 is the absolute discrimination threshold defined by intrinsic noise within the photoreceptor and a score of 3 is a realistic discrimination threshold under natural lighting conditions (Vorobyev and Osorio, 1998).

Pattern energy was calculated over each of the whole frog ROIs and each of the background ROIs using Fast Fourier bandpass filtering (granularity analysis) from the achromatic channel of each visual model in the MICA toolbox. We measured pattern energy as the standard deviation of the pixel values for five filter bands that doubled in spatial frequency at each step from 16 px (0.5 mm) to 256 px (8.0 mm). To compare pattern energy distributions between the frogs and their backgrounds we calculated the area between pattern energy curves using a piecewise linear function (function *approxfun*) from base R v4.0.5 (R Core Team, 2021). Hereafter, the area between frog and background pattern energy curves is referred to as "pattern contrast," higher values indicate that the frog's pattern was more distinct from the background.

Quantifying Intra-Populational Variation

To examine whether frog color pattern variation was continuous or divided into visually distinct sub-morphs we used *k*-means clustering and factor analysis in R v4.0.5 (Barnett et al., 2021a; R Core Team, 2021). For each of the frogs, we used the internal chromatic (ΔS) and achromatic (ΔL) contrast as well as the pattern energy at each of the five spatial scales (0.5, 1.0, 2.0, 4.0, and 8.0 mm). In the *k*-means clustering, we used the gap statistic (function *clusGap*) from R package *cluster* (Maechler et al., 2021), to estimate the optimal number of clusters (Tibshirani et al., 2001). We set the maximum number of clusters to 10 and ran 5000 Monte Carlo bootstrap samples to compute our reference distribution. We then interpreted the smallest local maximum in gap score as the optimal number of clusters (Tibshirani et al., 2001; Maechler et al., 2021). Next, we used factor analysis with two factors and varimax rotation (function *factanal*) from base R v4.0.5 (R Core Team, 2021) to investigate which parameters of the color pattern contributed the most to intra-populational variation (Barnett et al., 2021a). We repeated these analyses separately for the bird, snake, and frog visual models. Two frogs were removed from the snake analysis due to image calibration issues.

Background Contrast

We next asked whether frog variation affected contrast against the background. As the majority of the frogs' variation was found in the distribution of pattern (see *Factor 1* in Results: Intra-populational variation), and as the red and black are highly distinct from the background (Yeager and Barnett, 2020; **Figure 2** and **Supplementary Figures 1–3**), we focused on how pattern variation affects background pattern matching and distance-dependent color blending by comparing the whole frog ROIs to their local backgrounds. We compared the mean hue, mean luminance, and pattern energy distribution of each frog to that of the microhabitat where it was first encountered (external chromatic, achromatic, and pattern contrast), and then ran a series of linear models comparing each form of external contrast to Factor 1 from our factor analysis (function *lm*) using base R v4.0.5 (R Core Team, 2021). The suitable approximation of model assumptions was checked by plotting the distribution of the model residuals, and the significance of the dependent variables was estimated against a null model by the F statistic. A significant relationship would suggest that frog variation is associated with differences in background matching, whereas a non-significant relationship would suggest that any differences in external contrast were affected by background heterogeneity, independent from frog variation.

Background Choice

To test whether frogs were associated with microhabitats that either minimized or maximized the contrast of their own color and pattern characteristics we compared mean achromatic, mean chromatic, and pattern contrast between each frogs' local microhabitat and the alternate microhabitats where the other 34

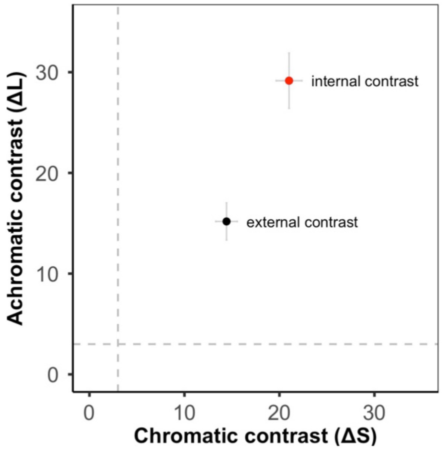

FIGURE 2 | Color contrast from the bird visual model (means ± SE, gray dashed lines show the discrimination threshold equivalent to 3 JND). There was high achromatic and chromatic contrast both between the red and black regions of the frogs (internal contrast – red circle) and between the whole frog and its local substrate (external contrast – black circle).

individuals were observed. We calculated the median contrast of the 34 alternate backgrounds for each frog and compared local contrast to alternate contrast using a series of paired-sample Wilcoxon tests in base R v4.0.5 (R Core Team, 2021). For each visual model, we ran separate tests for achromatic, chromatic, and pattern contrast. A significant result would suggest that frogs are associated with specific microhabitats.

RESULTS

Intra-Populational Variation

Color patterns were overall found to be highly contrasting, both in terms of within-frog contrast (e.g., red versus black patches) and between the frogs and their microhabitats (**Figure 2** and **Supplementary Figures 1–3**). In the *k*-means clustering, we found that for each visual model (bird, snake, and frog) the optimal number of clusters was one, suggesting that frog variation was continuous and not divided into discrete sub-morphs (**Figure 3** and **Supplementary Figures 4**, **5**). Factor analysis revealed that for each visual model two factors were sufficient to explain >75% of frog variance (**Table 1**). Factor 1, which explained >55% of variance for each model, weighted heavily toward pattern across all size classes. Factor 2, which explained <32% of the variance in each model was more variable. In the bird and snake models Factor 2 (bird = 20.2% variance, snake = 31.5% variance) mostly corresponded to internal chromatic and achromatic contrast, whereas in the frog model Factor 2 (19.6% variance) predominantly corresponded to low spatial frequency patterning.

TABLE 1 | Percent variance explained by factor analysis for each visual model.

Variance explained (%)	Bird	Snake	Frog
Factor 1	55.7	56.4	55.8
Factor 2	20.2	31.5	19.6
Cumulative variance	**75.8**	**87.9**	**75.4**

In each visual model the majority of variance is explained by Factor 1 which corresponds to pattern energy across all spatial scales. Bold values significant ($p < 0.05$).

Background Contrast

When using the bird and snake visual models, we found that there was no significant relationship between Factor 1 (~pattern) and either external achromatic or chromatic contrast. However, there was a significant positive relationship between Factor 1 and external pattern contrast, where frogs with higher Factor 1 scores were more distinct from their local backgrounds (**Figure 4**; **Table 2**; **Supplementary Figures 6**, **7**; **Supplementary Table 1**).

In the frog visual model, we found no significant relationship between Factor 1 and achromatic contrast, but there was a

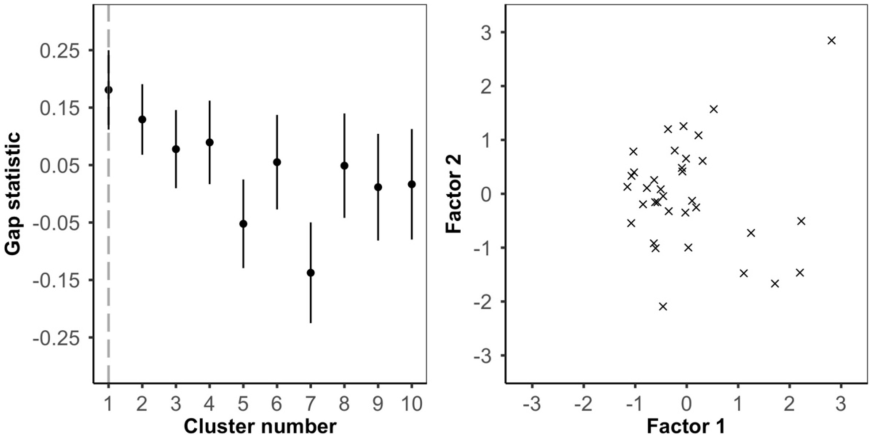

FIGURE 3 | Analysis of intrapopulation variance using the bird visual model. **(left)** *k*-means clustering. Gap statistics for clusters 1 to 10 show that one cluster is optimal and frog color pattern variance is continuous. **(right)** Factor analysis. Plotting frogs along factor 1 (~pattern) and factor 2 (~internal color contrast) shows no clear or distinct clusters.

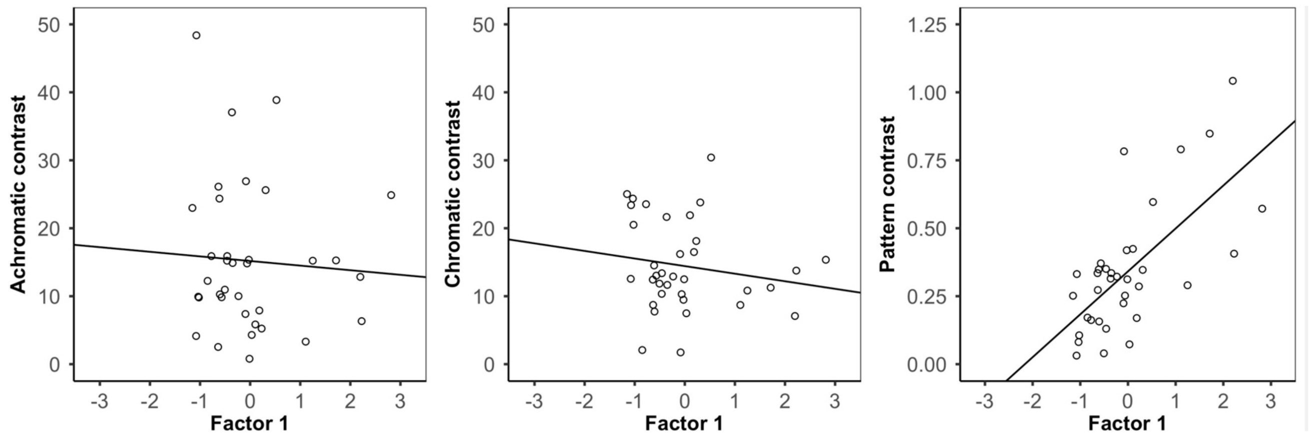

FIGURE 4 | Analysis of background contrast from the bird visual model. Correlation between Factor 1 (˜pattern variation) score and the achromatic **(left)**, chromatic **(middle)**, and pattern **(right)** contrast of each frog and its local background. There was no relationship between frog variation and either achromatic or chromatic contrast, but there was a significant relationship between frog pattern and pattern contrast where frogs with higher Factor 1 scores had greater pattern contrast against their local backgrounds.

significant relationship between Factor 1 and both chromatic and pattern contrast. Frogs with higher Factor 1 scores were more distinct from their local backgrounds in both chromatic contrast and patterning, although chromatic contrast remained well above the discrimination threshold in both conditions (**Table 2**).

Effects of Occupied Microhabitats on Frog Signals

Frogs were most commonly found in leaf litter (n = 27) or green vegetation (n = 8) substrates. We found no significant effect of background type (local vs. alternative) on frog contrast against the background in the bird and frog visual models (**Table 3**). Similarly, in the snake model we found no significant effect of background type on achromatic and pattern contrast, but there was a marginally significant effect for chromatic contrast (V = 170, p = 0.048; **Table 3**).

TABLE 2 | Statistical results from the linear models comparing achromatic, chromatic, and pattern contrast against their local microhabitat substrate and Factor 1 from the factor analysis, which explained >55% of total frog variance.

	Bird	Snake	Frog
Achromatic contrast	Adj. R^2 = −0.026 $F_{1,33}$ = 0.13, p = 0.723	Adj. R^2 = −0.028 $F_{1,33}$ = 0.11, p = 0.737	Adj. R^2 = −0.028 $F_{1,33}$ = 0.08, p = 0.781
Chromatic contrast	Adj. R_2 = −0.001 $F_{1,33}$ = 0.94, p = 0.340	Adj. R^2 = −0.008 $F_{1,33}$ = 0.75, p = 0.392	Adj. R^2 = 0.103 **$F_{1,33}$ = 4.89,** **p = 0.034**
Pattern contrast	Adj. R^2 = 0.434 **$F_{1,33}$ = 27.05,** **p < 0.001**	Adj. R^2 = 0.600 **$F_{1,33}$ = 48.91,** **p < 0.001**	**Adj. R^2 = 0.447** **$F_{1,33}$ = 28.48,** **p < 0.001**

Significant relationships suggest that frog variation affects contrast against the background. Conversely, non-significant relationships suggest that differences in contrast to the background are independent of frog variation and result from background heterogeneity. Bold values significant (p < 0.05).

DISCUSSION

The evolution and maintenance of intraspecific variation in aposematic signals continues to draw considerable interest (Rojas, 2017; Briolat et al., 2019). Although in *O. sylvatica* the extent of color pattern variation found within populations is not as striking as that observed between populations (Roland et al., 2017), resolving basic questions related to the degree and organization of phenotypic divergence is an essential step in beginning to understand broader scales of both polymorphism and polytypism in aposematic species (Briolat et al., 2019). A central prediction of within-population polymorphism is that if variance exists, it should be correlated with differences in signal saliency to serve distinct adaptive roles, such as in separate microhabitats within heterogeneous landscapes (Gray and McKinnon, 2007), or where a single color pattern serves divergent anti-predator and social functions (Cummings and Crothers, 2013).

Using a population-wide sampling effort, we sought to understand fine-scale phenotypic variance. We found intra-population color pattern variation in the Perla population

TABLE 3 | Statistical results from the paired-sampled Wilcoxon tests comparing achromatic, chromatic, and pattern contrast between local and alternate background types.

	Bird	Snake	Frog
Achromatic contrast	V = 268, p = 0.451	V = 208, p = 0.201	V = 237, p = 0.207
Chromatic contrast	V = 233, p = 0.184	**V = 170, p = 0.048**	V = 220, p = 0.128
Pattern contrast	V = 340, p = 0.692	V = 317, p = 0.525	V = 337, p = 0.728

A significant relationship suggests that frogs are associated with backgrounds that either increase or decrease contrast more than the median alternative background. Bold values significant (p < 0.05).

of *O. sylvatica* to be continuous, rather than discrete, and largely driven by pattern elements, rather than chromatic or achromatic contrast. Moreover, although individuals differed in pattern distinctness from the background, we found no strong evidence that different individuals were associated with particular microhabitats, such as if they were selecting signaling environments that would either enhance or reduce signal contrast. Taken together, our data suggest that frogs exhibit highly variable patterns which still maintain consistent chromatic and achromatic contrast. However, although seemingly highly variable, the frogs' aposematic signals do appear constrained to a small range of colors such that any variance is likely still perceived as within the same aposematic grouping. Our data are, therefore, consistent with a single fitness peak (or perhaps a broad plateau), with variation arising as a product of weak purifying selection, honest signaling, and/or neutral drift on some elements of the color pattern.

In aposematic signals both color and pattern have been found to convey important information to potential predators (Stevens and Ruxton, 2012; Caro and Ruxton, 2019). Prey bearing patterns comprised of multiple highly contrasting elements have higher survival than prey with single colors (Preißler and Pröhl, 2017). However, in predator education, hue and brightness appear to play a more critical role than the particular arrangement of the pattern (Aronsson and Gamberale-Stille, 2008; Kazemi et al., 2014; Sherratt et al., 2015). In *O. pumilio*, for example, color and pattern are both involved in aversion learning and when controlling for color, larger spotted patterns have been found to be more salient and more likely to be avoided than smaller spotted or plain patterns (Hegna et al., 2011; Qvarnström et al., 2014; Preißler and Pröhl, 2017; Barnett et al., 2021b). However, under natural conditions, both color and pattern can be highly divergent without necessarily increasing predation risk (Hegna et al., 2011; Richards-Zawacki et al., 2013).

The arrangement of pattern elements can also allow seemingly conspicuous signals to incorporate aspects of camouflage, for example, dual signaling as a function of observer viewing distance or by context dependent disruptive coloring (Marshall, 2000; Tullberg et al., 2005; Barnett and Cuthill, 2014; Honma et al., 2015). Distance-dependent signaling takes advantage of limitations in observer visual perception such that when viewed from a distance a high contrast pattern blends together to match the background (Marshall, 2000; Tullberg et al., 2005; Barnett and Cuthill, 2014; Caro et al., 2016). Although the contrast between the average color of the frogs and the background (external contrast) was lower than that found within the frogs' pattern (internal contrast), external chromatic and achromatic contrast was still high. We therefore found no strong evidence that this population of *O. sylvatica* are utilizing distance-dependent pattern blending. Alternatively, in disruptive camouflage, high contrast patterns can interfere with the visual assessment of shapes and edges (Stevens et al., 2006; Stevens and Merilaita, 2009). We did not explicitly test for disruptive coloring but unlike predictions for aposematism, disruptive camouflage can be more effective when sympatric individuals vary in the expression of pattern characteristics (Troscianko et al., 2021), and future studies could explore this alternative. With this in mind, it is important to note that to test for differences in microhabitat use, we only characterized the local patch of substrate immediately surrounding the frogs. However, we emphasize these were the very patches where frogs were initially detected, representing ecologically relevant contexts. Yet when the whole complexity of the visual environment is considered, these frogs may be more difficult to detect under certain conditions than currently recognized. Future experiments are needed to explicitly test the role of color pattern variation in combining aposematic signals with alternative anti-predatory strategies such as disruptive coloration.

Although we found no direct evidence for an adaptive benefit of variation, within-population pattern heterogeneity could potentially play a significant, yet indirect functional role in polymorphism and polytypism. As aposematic signals are often co-opted *via* sexual selection for mate choice and intra-sexual conflict, patterning may convey information important for intraspecific communication, although research into this possibility has so far predominantly focused on color rather than pattern (Crothers and Cummings, 2013, 2015). The observed variance in pattern could, for example, assist in identifying territory intruders or serve as an honest signal in mate choice or for mediating agonistic interactions (Maan and Cummings, 2008, 2009; Crothers and Cummings, 2013, 2015). In *O. pumilio* future preferences for color-based assortative mating are imprinted during parental care, and to date evidence is limited to coloration because the populations sampled lack pattern elements (Yang et al., 2019). However, were *O. sylvatica* tadpoles to imprint on elements of female phenotypes which include pattern as well as color during maternal tropic egg provisioning, the mother's phenotype could have downstream influences in female mate choice and/or male/male aggression. Albeit indirect, intra-population variance could shape the color pattern evolution in *O. sylvatica* in a mechanism similar to coupled drift proposed in *O. pumilio* by Tazzyman and Iwasa (2010). In coupled drift models female preference (though certainly also potentially applicable to male/male aggression) is linked with color patterns, where preferences diverge according to neutral processes and then reinforce color pattern divergence (Tazzyman and Iwasa, 2010; Gehara et al., 2013). If *O. sylvatica* populations diverge in a method such as coupled drift, then high intra-population variance should enhance between-population divergence, especially those populations which are small or are found in fragmented forest patches.

It has long been predicted that sympatric variance in aposematic signals should be limited, and that which does occur should largely be adaptive (Mappes et al., 2005; Briolat et al., 2019). Recent research adds to a growing body of evidence that this is not universally true, and that relaxed or weak selection can produce or permit significant levels of sympatric intraspecific variation (Richards-Zawacki et al., 2013; Briolat et al., 2019; Bliard et al., 2020). We found that variation within the Perla population of *O. sylvatica* was not divided into visually distinct categories, and that variation was not associated with particular microhabitat characteristics. Therefore, it is unlikely that signal variance has evolved due to strong selection producing locally adapted phenotypes across a variable fitness landscape, but rather variable patterns remain as purifying selection from predators is weak. These findings highlight how seemingly salient characteristics of animal color may at first evolve through neutral processes, which could have implications for our understanding of the evolution of polymorphism, polytypism, and the evolutionary processes which can ultimately lead to speciation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.5061/dryad.hmgqnk9j0.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because animals were only photographed and had no other manipulations. Permits were granted by the Ministerio del Ambiente, Ecuador, for research permission (Permit Number: 014-2019-IC-FLO-DNB/MA).

AUTHOR CONTRIBUTIONS

JY and JB conceived the manuscript, conducted the analyses, and wrote the manuscript, JY collected the data. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.803996/full#supplementary-material

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Animal Coloration in the Anthropocene

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Natural habitats are increasingly affected by anthropogenically driven environmental changes resulting from habitat destruction, chemical and light pollution, and climate change. Organisms inhabiting such habitats are faced with novel disturbances that can alter their modes of signaling. Coloration is one such sensory modality whose production, perception and function is being affected by human-induced disturbances. Animals that acquire pigment derivatives through diet are adversely impacted by the introduction of chemical pollutants into their environments as well as by general loss of natural habitat due to urbanization or logging leading to declines in pigment sources. Those species that do manage to produce color-based signals and displays may face disruptions to their signaling medium in the form of light pollution and turbidity. Furthermore, forest fragmentation and the resulting breaks in canopy cover can expose animals to predation due to the influx of light into previously dark environments. Global climate warming has been decreasing snow cover in arctic regions, causing birds and mammals that undergo seasonal molts to appear conspicuous against a snowless background. Ectotherms that rely on color for thermoregulation are under pressure to change their appearances. Rapid changes in habitat type through severe fire events or coral bleaching also challenge animals to match their backgrounds. Through this review, we aim to describe the wide-ranging impacts of anthropogenic environmental changes on visual ecology and suggest directions for the use of coloration both as an indicator of ecological change and as a tool for conservation.

Keywords: coloration, visual environment, climate change, landscape change, conservation

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INTRODUCTION

The recent intersection of multiple global crises—climate change and public health—has brought the aesthetic enjoyment of "nature" to the forefront of collective human consciousness. As a species that tends to place the visual above other senses of perception, our knowledge systems are often centered around visual observations of natural phenomena and natural life, color in particular. As such, changes in our visual environments are often the first to be noticed and studied. Natural environments have been shaped by human societies for millennia (Sullivan et al., 2017), but recent decades have seen an acceleration of habitat loss and destruction due to changing land-use and climate. The rate of these changes outpaces natural rates of adaptation (Heinrichs et al., 2016; Greenspoon and Spencer, 2021). As these systems face threats from climate change, habitat loss and pollution, visual ecologists must reckon with the ramifications for the production, perception, evolution, and application of coloration. The introduction of novel stressors and the amplification of pre-existing ones beyond levels that animals can behaviorally ameliorate presents a range of consequences for the evolutionary and ecological dynamics of these populations. While visual ecologists and biologists have thus far been engaged in questions around the mechanisms of color production and perception, the field has yet to provide a synthetic understanding of the effects of anthropogenic stressors on these mechanisms. Conversely, conservation science has yet to incorporate cues from visual ecology as way to monitor populations at risk.

Over the course of this review, we attempt to lay out the documented effects of various anthropogenic stressors on different aspects of visual ecology. The production of color, either through *de novo* synthesis or dietary acquisition, is impacted from multiple directions by environmental pollution and habitat loss (Peneaux et al., 2021). The fragmentation or loss of a habitat also leads to a fragmentation of the dietary resources essential for the sequestering of pigmentary compounds such as carotenoids. In order for color phenotypes to function properly, the color patterns must be perceived under optimal lighting conditions, whether in ambient air or water. Rapid changes to the photic environment due to environmental pollution and landscape change disrupt the perception and processing of signals that are often crucial to species' recognition and mate choice. Furthermore, color patterns used to achieve crypticity or thermoregulation can be rendered ineffective, and even counterproductive, due to accelerated changes to climate patterns and the seasonal composition of habitats. Additionally, epidermal color traits present great potential as non-invasive yet powerful indicators of environmental change, while the colorscape of an ecosystem, measured through the color composition and variation within and across species in the community presents an interesting alternative metric to report on the health of ecosystems. Finally, we outline present uses of visual information from the natural world for ecological monitoring and possibilities for the future incorporation of visual ecology for conservation.

COLOR PRODUCTION

Central to the study of animal coloration is the presence of pigment sources in the animal's habitat, whether as prey or plant material. This dependence of coloration on environmental conditions means that the color traits are often honest indicators of individual quality (Hamilton and Zuk, 1982; Naguib and Nemitz, 2007). Only individuals with good access to the necessary nutrients or the ability to overcome stresses can express costly phenotypes. Color in animals may be presented through either pigment deposition in the epidermis or through nanostructures such as those found in bird feathers and lepidopteran wings. Of the many pigment classes, the two most used, especially in birds, are melanin and carotenoids, each of which have different mechanisms of production. Carotenoids are primarily acquired through diet and are costly and energy intensive to produce, thereby indicative of the ability of individuals to compete and forage for the necessary dietary components (McGraw, 2006a). Melanin (eumelanin and phaeomelanin) is synthesized endogenously in melanocytes and may be limited by oxidative stress, hormones, and amino acid precursors (McGraw, 2006b). Pteridines are another class of pigments that are not as well studied as carotenoids or melanin but are increasingly regarded as indicators of individual quality (Stuart-Fox et al., 2021). Although structural coloration is primarily dependent on the incidence of light in the environment, some aspects of structural colors may be condition-dependent and constrained by protein availability in the diet (Prum, 2006). Anthropogenic landscape and climate change have introduced novel environmental constraints ranging from a direct loss of nutrient sources due to habitat loss to the effects of temperature change on endocrinology and biochemical pathways essential to pigment production.

Carotenoids

Carotenoid-based ornamental traits have been established as condition-dependent and therefore honestly indicative of individual quality (Hill, 1995, 2006) based on experimental and correlational studies (Weaver et al., 2018). The term "individual quality" is an index of body condition, used primarily in the context of female mate choice (Weaver et al., 2017) and sexual selection for male ornamental traits (Møller, 1991), which include highly plastic color signals. Several studies that experimentally manipulate the availability of carotenoid sources in the diet have demonstrated this plasticity and subsequent link between the habitat and quality (Peneaux et al., 2021; Stuart-Fox et al., 2021). This link may be disrupted when the carotenoid-based coloration is constrained by the environment in two ways—(a) through lack of availability of carotenoid sources such as insects, fruits and young shoots in the habitat and (b) constraints on other non-carotenoid macro-nutrients in the habitat, such as proteins and fats that are necessary for carotenoid absorption and expression through signals. As landscapes and habitats decline in quality, carotenoid-based coloration may start to be construed as an indicator of habitat condition rather than individual quality (Lifshitz and St Clair, 2016; Weaver et al., 2018; Peneaux et al., 2021).

The concept of using carotenoids as biological indicators of environmental changes is not a novel one (Hill, 1995). There is extensive literature on the relationship between the amount of carotenoid or carotenoid precursors available in the environment and the signal quality of animals, primarily avian species, which use carotenoid-based coloration. While a considerable proportion of the literature in this field is dedicated to experimentally inducing variation in carotenoid availability through captive breeding and diet manipulation to show the direct link between carotenoids in the environment and the level of display as ornaments in birds (McGraw et al., 2005; McGraw and Parker, 2006; Shawkey et al., 2006), some studies have relied on field observations of nesting in the wild. Free-ranging birds with wider growth bands on their tails (dependent on better overall nutrition) exhibit more saturated colorful plumage (dependent on carotenoid availability) (Hill and Montgomerie, 1994; Senar et al., 2003). The American kestrel (*Falco sparverius*) sequesters carotenoid-based yellow coloration in patches around the eyes, above the bill and on the tarsi (**Figure 1**). These patches of color are on the skin, and not on plumage and therefore can be directly linked to pigment deposition in the epidermis and not optic structures as seen in feathers. Bortolotti et al. (2000) present data on the genetic vs. environmental contributions to variation in carotenoid coloration in kestrels, collected through cameras set up in nest boxes to record prey variability and through spectrophotometry to quantify carotenoid levels. They found that the abundance of voles in territories was negatively associated with plasma carotenoid concentration in the predatory birds. Those that consumed relatively higher proportions of voles over other prey types, such as grasshoppers, frogs, dragonflies, or small birds, had lower concentrations of carotenoids, suggesting that environmental limits on the diversity of prey can lead to compromised color-based signaling as well as poorer health and quality. In a second study (Bortolotti et al., 2003) the authors exposed juvenile and adult kestrels to polychlorinated biphenyls (PCBs) through diet and measured plasma carotenoid concentrations and plumage coloration before and during the breeding period. Their results showed that PCBs disrupted both color deposition and plasma carotenoid levels during the breeding period. In male kestrels that were fed PCBs, plumage coloration became significantly duller. PCB exposed females did not lose carotenoid-based color during the breeding session, as expected and as seen in control females. Female kestrels fed the control diet lost their carotenoid coloration due to the routine allocation of carotenoids to eggs. The disruption of this mechanism by PCBs highlights the many ways organic pollutants can affect reproductive fitness. Another study by Fernie et al. (2001) showed that PCB contaminated females experienced delays in egg laying. In yet another case of carotenoid-based signaling reflective of individual health, Kristiansen et al. (2006) studied the coloration of bills and eye-rings in the great black-backed gull (*Larus marinus*). They found that adult male gulls in better condition (measured as residuals from the regression between body size and body weight) showed bills and eye-rings with higher color saturation while female gulls with higher color intensity laid larger eggs and had greater clutch sizes.

Carotenoid based displays may be further environmentally constrained by the energetic costs of pigment production. Acquisition of carotenoids is dependent on both the availability of pigments as well as the availability of sufficient caloric content in the environment (Hill, 2000). Diet places energetic constraints on the biochemical pathways of pigment production as well as on the supplementary compounds such as lipoproteins that are just as essential to pigment synthesis and deposition (Tyczkowski and Hamilton, 1986). There is increasing evidence linking protein limitations in urban landscapes to declines in insect communities, a key feature of the avian diet (Tallamy and Shriver, 2021). Among the many multi-dimensional changes in climate and land-use patterns, urbanization and the resulting built environments may be the most far-reaching in this regard. Built environment is marked by a large-scale rapid transition from natural habitat to anthropogenic structures, accompanied by increased pollution, higher human population densities and significant increases in oxidative stress levels (Hutton and McGraw, 2016). As birds are one of the few classes of animals that continue to inhabit urban spaces after the transition from natural vegetation to built-up spaces, they may serve as a good indicator of rapid environmental change through the highly plastic carotenoid-based color patterns they use for conspecific signaling (Peneaux et al., 2021).

Melanin

Although melanin-based coloration has often been thought of as being primarily under genetic control with little influence from environmental factors (Griffith et al., 2006), inter-individual variation in melanin-based patches in the Eurasian kestrel (*Falco tinnunculus*) points to a different story. Fargallo et al. (2007) examined gray plumage in kestrels as an indicator of variation in environmental conditions during the early developmental stages of male nestlings. Through data collected from nest boxes over 3 years, they found that the production of gray color patches in male nestlings was correlated with prey richness in the environment and could not be attributed to genetically determined maternal quality allocated into eggs. Their results, combined with established knowledge about the biochemical pathway of melanogenesis and the higher cost of brown (phaeomelanin) vs. gray/black (eumelanin) pigmentation (Jawor and Breitwisch, 2003), demonstrate the dietary constraints on melanin production in kestrels. The incidence of gray color patches in male nestlings was higher in years with greater vole abundance. As melanin pigmentation in male kestrels may be attributed to quality signaling, either toward females for mate selection, or to advertise sex to parents for differential allocation of resources, large scale climate or land use changes could lead to fluctuations in prey supply and subsequently the quality of kestrel nestlings. New data (Fay et al., 2020) on the synchrony between vole population cycles and kestrel breeding patterns sets this dynamic in perspective as a possible case study for the effects of climate change on dietary sources of melanin pigmentation.

Structural Coloration

Sexually selected structural color ornaments are considered honest signals of quality due to their diet-driven plasticity. Protein is especially limiting for structural coloration of plumage and skin as well as tissue and feather formation through keratin and collagen. Certain colors commonly used for signals in birds are often produced through a combination of pigments and structural coloration (Prum and Torres, 2003). It is this association between pigment and optic structures that make macronutrients like proteins and fats important limiting factors in addition to carotenoid intake. Indeed, protein intake, or the lack thereof, has been found to affect other non-carotenoid based visual signals as well. Male blue tit nestlings with lower plasma protein concentrations exhibit tail feathers in a more vivid blue/UV (Peters et al., 2007). Lower levels of proteins circulating in the bloodstream are indicative of these proteins being allocated to the formation of nanostructures in feathers instead. In another study that investigated the effects of cholesterol level variation in zebra finch (*Taeniopygia guttata*) ornamental coloration, researchers found that bill coloration faded significantly in finches whose diets had less cholesterol (McGraw and Parker, 2006). Similarly, the iconic iridescent patch in male Anna's hummingbirds (*Calypte anna*) is constrained by

FIGURE 1 | Birds whose pigmentation is affected by environmental pollution. **(A)** The American kestrel (Falco sparverius) in Florida, by Andrew Morrfew. Male kestrels that were fed PCBs in the laboratory setting showed duller plumage coloration **(B)** House finch (Haemorhous mexicanus) in Baja California, by Ron Knight. The house finch exhibits a decrease in red plumage coloration with exposure to herbicides. **(C)** Eurasian kestrel (Falco tinnunculus) in the Annamalai Hills, by T.R. Shankar Raman. The melanin-based plumage color has been shown to fluctuate in response to diet diversity (Fargallo et al., 2007). **(D)** Great tit (Parus major) in Lancashire by Francis Franklin. The yellow breast coloration has been shown to grow duller with exposure to metal pollutants, while melanin-based black stripes grow brighter.

protein: hummingbirds that were fed higher protein levels grew brighter crown feathers (Meadows et al., 2012).

Pollutants and Color Production

In addition to being impacted by dietary constraints, integumentary pigmentation and structurally derived colors are also affected by anthropogenic pollution. These pollutants can range from pharmaceuticals, to pesticides, to industry-related compounds, to metals (Lifshitz and St Clair, 2016). As previously established here, color displays that act as honest indicators of quality can be heavily constrained by diet and nutritional deficiencies in their respective environments. Broadly, polluting compounds impact animal coloration through multiple pathways: by inhibiting or disrupting the acquisition of carotenoid precursors, by replacing similar compounds in key biochemical pathways and by causing general declines in overall health and condition (Pacyna et al., 2018). The abundance of literature (**Table 1**) on the effects of pollutants on plumage coloration in birds suggests that carotenoid-based coloration could be used as an indicator of pollution. Organic pollutants such as DDT (dichlorodiphenyl trichloride), PCB and PAH (polycylic aromatic hydrocarbons) have been shown to reduce hue and saturation in birds as varied as gulls and harriers (Pérez et al., 2010; García-Heras et al., 2017). Similarly, metallic pollution as a byproduct of industrial smelter plants has led to significant decreases in carotenoid-based color deposition. As a visual indicator that can be measured from a distance, pigmentary color display in birds is a far less intrusive method of assessing the impacts of anthropogenic pollution than sampling blood and tissue, especially in endangered or vulnerable species whose populations cannot afford to be sampled (Yang et al., 2021).

PERCEPTION AND SIGNAL VISIBILITY

Visual modes of communication require that the signal passes to the receiver without undue disruption. Animals rely on ambient light for the visual processing of color signals—it can modulate the level of contrast between patches of color and thus the efficacy of the signal itself (Endler, 1980; Rosenthal and Stuart-Fox, 2012). For instance, for terrestrial animals that use warning coloration, the medium of ambient air is often subject to natural variation due to weather patterns and vegetation (Rosenthal, 2007). Anthropogenic changes in climate and habitat structure as well as the introduction of artificial light can alter the visual signaling environment beyond the normal threshold of variation (Lim et al., 2008). Forests are one such example of a habitat type whose lighting conditions are characterized by complex interplay between vegetation structure, weather, and time of day (Endler, 1993). While natural variation in ambient light in forests over the course of a day can be offset by minor behavioral adjustments, more dramatic changes brought about by logging and land clearing can result in animals being unable to

TABLE 1 | The effects of anthropogenic pollutants on plumage coloration in birds.

Anthropogenic factor	Independent variable	Taxa	Color trait	Effect on coloration/signaling	References
Organic pollutants					
Polycyclic aromatic hydrocarbons (PAH)	Dietary supplementation of fuel oil (ml) (from Prestige oil spill)	Yellow legged gulls (*Larus michahellis*)	Red bill spot (for sexual signaling)	Decrease in size and color intensity of red bill spot	Pérez et al., 2010
Herbicide (paraquat dichloride)	Paraquat concentration (g/L)	House finch (*Haemorhous mexicanus*)	Red plumage	Decrease in plumage hue (carotenoid based)	Giraudeau et al., 2015a
Organochlorine compounds	PCB and DDT concentration in plasma	Harriers (*Circus maurus*)	Plumage	Lower carotenoid hue and saturation in chicks and adults with higher levels of DDT; nestlings with DDT had yellower plumage than those without	García-Heras et al., 2017
Organochlorine compounds	Dietary PCB concentration	American kestrels (*Falco sparverius*)	Plumage, tarsi and eye-rings	PCB fed male kestrels showed duller plumage colors	Bortolotti et al., 2003
Herbicide (Diquat dibromide)	Diquat dosage in drinking water	Red-legged partridges (*Alectoris rufa*)	Beak spot and eye rings	Diquat fed adult male partridges showed paler beak and eye rings	Alonso-Alvarez and Galván, 2011
Metals					
Metal pollution from vehicular traffic	Distance from major road traffic; Feather metal concentration	Great tit (*Parus major*)	Breast feathers	Birds found farther away from the polluted site had higher carotenoid chroma; higher feather metal scores associated with lower UV chroma	Grunst et al., 2020
Metal pollution	Mercury (Hg) concentration in feathers	Great tit (*Parus major*)	Yellow breast patch	Decrease in brightness of yellow breast patch with Hg concentrations	Giraudeau et al., 2015b
Metal pollution	Cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) concentration in feathers	Great tit (*Parus major)*	Black breast stripes	Increase in size of melanin-based black breast stripe in birds collected closer to pollution sites.	Dauwe and Eens, 2008
Metal pollution	Heavy metal concentration in feathers (Al, As, Cd, Cr, Cu, Fe, Hg, Pb, Sb, Se, Zn, and V)	Tree swallows (*Tachycineta bicolor*)	Plumage	Declines in plumage brightness	Lifshitz and St. Clair, 2019
Metal pollution	Lead (Pb) concentration in feathers	Feral Pigeons (*Columbus livia*)	Plumage	Birds exposed to lead had lower iridescent feather brightness.	Chatelain et al., 2016

communicate as effectively as before (Delhey and Peters, 2016). The presence of atmospheric aerosols due to anthropogenic emissions has been found to have decreased global clear sky visibility (Wang et al., 2009), with potential ramifications for taxa, such as sandhoppers, that rely on skylight radiance for orientation and navigation (Ciofini et al., 2021). The impacts of this decrease in irradiance on animal coloration have yet to be examined. Transitional light regimes, such as those seen at dawn and dusk, have also experienced changes due to light pollution (Spitschan et al., 2016). However, the impacts of these changes on the visual ecologies of non-human animals have yet to be investigated comprehensively. Similarly, elevated turbidity in aquatic systems due to eutrophication, sediment run-off, increased storm intensity from climate change and bottom dredging causes drastic changes in irradiance (van der Sluijs et al., 2011), thereby disrupting the medium of transmission and perception of visual signals.

The effects of water turbidity have been well documented in cichlids and guppies (Chapman et al., 2009; Camargo-dos-Santos et al., 2021). Seehausen et al. (1997, 2008) have ascertained, through lab experiments and field data, that changes in ambient light in Lake Victoria due to eutrophication have led to the hybridization of several differently colored cichlid species. As female cichlids prefer to mate with conspicuously colored males (Seehausen et al., 2008), the perception of these conspicuous colors is crucial to the maintenance of mating systems. Male nuptial coloration, often presented as bright reds or blues to offer a striking contrast against yellow light, is impacted by turbidity in two ways: (a) the lack of photosynthetically useful light causes declines in dietary sources of carotenoids and (b) the costliness of bright colors and the decline in ambient light causes males to develop duller colors over generations (Seehausen et al., 1997). Turbidity reduces the overall efficacy of color-based signals, followed by a loss or decline in male nuptial colors, and finally a decrease in overall species diversity and phenotypic diversity due to the loss of sensory barriers against hybridization. As sexual selection contributes significantly to species and phenotypic diversification and the maintenance of diversity in cichlids and guppies (Selz et al., 2014), a breakdown of these selective forces due to environmental conditions could lead to declines in the genetic diversity and by extension, perhaps to resilience of a population to further challenges.

Butterflies that rely on lighting environment for the efficacy of their defensive coloration mechanisms are particularly dependent on forest cover for survival. As they employ a combination of pigmentation and structural coloration to achieve a fine balance between color for thermoregulation and as an anti-predator strategy, significant changes in both climate and land use patterns mark them as especially vulnerable. In a wide-ranging comparative study of the effects of deforestation on coloration in Amazonian butterflies, Spaniol et al. (2020) show how in recent years, butterflies have experienced a reduction in diversity of defensive coloration strategies. Primary growth forests, especially in the tropics (Adams et al., 2014) offer a greater range of visual environments that are associated with specific color phenotypes. Species that inhabit low-light environments, i.e., primary growth forests with dense canopies, are more susceptible to population declines following disturbance events that convert them to higher-light environments (Patten and Smith-Patten, 2012). Through their sampling of butterflies in primary forests and early succession stage areas, Spaniol et al. (2020) find that butterflies that rely on dull colors and patterns (measured as saturation and hue) for camouflage continue to persist in recently disturbed forest sites, whereas conspicuously colored butterflies show declines. Brighter colored species may be exposed to greater predation risk due to the opening up of habitats from disturbance. This exposure is sometimes combined with the introduction of novel, invasive predators to the systems that have not undergone the learning processes that make conspicuous color patterns effective as warning signals (Ciuti et al., 2012). Overall, deforestation and the creation of smaller, more disturbed fragments of forest appear to select for lower color diversity as well as species diversity in neotropical butterflies (Spaniol et al., 2019, 2020). Butterflies are a particularly charismatic taxonomic group whose colors and patterns represent a large range of specialized adaptations closely associated with their habitats. As these habitats face extensive deforestation and changes to the light environment and predator composition, butterflies may be used as model taxa for the monitoring of forest composition and degradation.

Artificial light at night is a unique feature of modern human society that affects many aspects of animal behavior (Gaston et al., 2013). The advent of new lighting technologies such as the energy-efficient LED bulb to offset the energetic costs of older incandescent light bulbs has introduced a wider range of artificial spectra to nocturnal animals (Owens and Lewis, 2018; Desouhant et al., 2019). Fireflies and glow-worms are examples of nocturnal insects that use the darkness of the nighttime as a background against which to emit sexual signals. Elgert et al. (2021) investigated the effects of artificial light on sexual signaling in the glow-worm (*Lampyris noctiluca*) and found that female glow-worms ceased glowing when exposed to artificial light at night for an extended period of time. Briolat and others have shown how artificial light affects coloration too, specifically, using elephant hawkmoths (*Deilephila elpenor*) and the impact of different types of artificial lighting conditions on their pollination and anti-predator behavior (Briolat et al., 2021). As highly efficient and effective nocturnal pollinators, hawkmoths rely on their night vision systems to fly directly toward specific flowers. The authors tested the effect of artificial lights on the ability of moths to discriminate flower colors at night and found that broadband amber light, most commonly emitted from amber LED lights, inhibited color discrimination. With regard to anti-predator behaviors, they also found that narrow band light sources, such as orange LED, disrupt the moths' ability to perceive and discriminate the color of backgrounds against which they choose to finally rest at night to avoid detection by diurnal avian predators the next day.

FUNCTIONAL ASPECTS OF COLORATION

Rapid environmental change can be expected to impact the functionality of color patterns as much as it does their acquisition and perception. One of the most striking effects of climate change on the adaptive function of coloration is seen in arctic mammals and birds that undergo seasonal color change to achieve crypsis against their changing backgrounds. As the duration of annual snow cover shortens with warmer temperatures each year (Post et al., 2009), arctic animals are faced with mismatching their backgrounds, leading to increased predation risk (Pedersen et al., 2017). Similarly, ectotherms that rely on coloration for thermoregulation must contend with warmer temperatures by either evolving lighter colored bodies or shifting their ranges to cooler and shadier regions. Changes to the landscape, in the form of forest fragmentation or wildfires, can compound on the effects of climate change and severely affect the distribution of morphotypes within a polymorphic population.

Climate Change in Mammals

Climate change has diverse effects on animal coloration (**Figure 2**). Some animals that inhabit upper temperate and polar regions or migrate between them undergo seasonal color change. These animals, primarily birds and mammals that use coat or plumage color polyphenism to camouflage against snow, are directly compromised by seasonal mismatches between daylength and modern changes in temperature and precipitation (Forrest and Miller-Rushing, 2010).

Photoperiod is the primary driver of seasonal polyphenism in arctic mammals that undergo molting to match the backgrounds of their changing landscape (Zimova et al., 2018). Although photoperiod itself is not affected by climate change, the discrepancy between seasonal transitions and photoperiod is particularly important as a driver of increased mortality due to camouflage mismatch between coat color and habitat substrates that change with season (Zimova et al., 2020b). Snowshoe hares undergo seasonal molts to match their background, between a uniform brown coat in summer and a uniform white in winter, with intermediate phases of brown and white patches during the seasonal transitions in autumn and spring. In a series of studies on phenotypic plasticity of snow-shoe hares (*Lepus americanus*) in response to decreased snow cover and mismatches with coat color, researchers used data collected over 3

FIGURE 2 | Animals whose colors are impacted by climate change. **(A)** The Orange sulfur butterfly (*Colias eurytheme*) in Nogales, Arizona by Alan Schmierer. *C. eurytheme* relies on wing color to reach optimal temperatures for flying and foraging. **(B)** Snowshoe hare (*Lepus americanus*) in Denali National Park by Tim Rains. Snowshoe hares are increasingly experiencing camouflage mismatch with decreases in snow cover due to climate change. **(C)** Least weasel (*Mustela nivalis*) in Bialowieza Forest by Stormbringer76. Weasels in their winter pelage are also experiencing camouflage mismatch with decreasing snow cover and period. **(D)** Monarch butterfly (*Danaus plexippus*) in Aston Township, Pennsylvania by Derek Ramsey. Monarch wing color darkness is important for flight endurance during migration.

years on snowpack and color polyphenism to illustrate the extent of seasonal mismatch and associated mortality (Mills et al., 2013; Zimova et al., 2016, 2020a). A large portion of mortality is directly attributed to predation, making their predator interactions a primary selective pressure for local adaptations. Through their long-term study, the authors established a lack of or limited phenotypic plasticity in coat color phenology: onset of seasonal molt appeared to be fixed and determined by photoperiod rather than temperature and snow cover (Zimova et al., 2014). Increased color contrast between the hares' coat color and their substrate led to increased mortality. Moreover, hares have not adjusted their behavioral defenses against predation to compensate for the decline in crypticity either (Zimova et al., 2014). Population and climate pattern modeling of mortality rates show that climate driven phenological mismatches are capable of driving severe population declines.

In another example, Atmeh et al. (2018) demonstrated the effects of seasonal camouflage mismatch on winter survival rates in the common weasel (*Mustela nivalis*). In the Białowieża Forest in Poland, two morphs of weasel—*M. n. nivalis* and *M. n. vulgaris*—occur sympatrically. While there are few differences in their summer coat coloration, the former undergoes a winter molt to a white coat, but the latter retains its brown coat year-round. Visually mediated predation by raptors and other larger mammals is often the largest mortality factor for weasels, making their ability to achieve crypsis against their seasonally changing background of utmost importance to survival (Zub et al., 2008). The investigators used a combination of weasel model-predator interactions and live-trapping of weasels over a decade to assess the population ratios of the two color morphs. In their field experiments, they used artificial weasel models that corresponded to the two morphs to determine predation risk induced by camouflage mismatch. They demonstrated that camouflage, or the lack thereof, was the most significant factor influencing detection by predators. They also found a negative but non-significant association between the detection rate and percentage snow cover. Through live trapping, they found that the proportion of *M. n. nivalis,* the morph that molts to white in winter, actually decreased parallel to the decrease in the number of days of snow cover in the forest. The authors thereby predicted that the two morphs will suffer different rates of mortality, with *M. n. nivalis* experiencing higher mortality than *M. n. vulgaris* with decreasing snow cover, perhaps leading to the fixation of the latter morph in the population over time.

Climate Change in Insects

Ectotherms that rely on body color for thermal regulation are particularly affected by changing global temperatures. Darker colored animals have an advantage over lighter colored animals in cooler climates as they are more efficient at increasing their body temperatures above that of the ambient atmosphere (Clusella-Trullas and Nielsen, 2020). In warmer climates, however, lighter colored ectotherms can remain active in the day for longer. This constraint on color and thermoregulatory abilities has led to seasonal polyphenisms in several insect species, particularly well documented in the

North American sulfur butterflies of the genus *Colias* (Watt, 1968, 1969; Hoffman, 1978; Kingsolver, 1983a,b; Kingsolver and Buckley, 2015). Summer morphs of both species display orange or yellow colors on their hindwings, due to pteridine pigments, while spring and fall morphs show a darker underside of their hindwings, due to the replacement of pteridines with melanin. In their normal resting position, the hindwings are folded upward, exposing their undersides to the absorption of solar radiation. The lighter color of the summer morph minimizes overheating, enabling it to stay active longer in the day. The butterflies are triggered by a photoperiodic change from the longer summer months to shorter winter months to undergo morphological changes to their wings. Watt (1968, 1969) studied the mechanism of polyphenism in *C. eurytheme* and the effect of temperature on butterfly activity patterns, specifically, daily flying time. He demonstrated that the darker butterflies reached high body temperatures needed for flying longer in the day even under lower ambient temperatures, entailing an adaptive advantage according to season and photoperiod. Hoffman (1978) also investigated the correspondence between photoperiod and hindwing darkening through laboratory experiments and field observations in Central California by exposing larval instars of *C. eurytheme* at each stage to different photoperiods and ambient temperatures. He found that seasonal polyphenism is controlled by photoperiod during larval development, and that temperature during this time has no significant effect on seasonal hindwing darkening. Kingsolver and Buckley (2015) investigated melanin variation in alpine and sub-alpine populations of *Colias meadii* to estimate the impacts of climate change on butterfly fitness (measured as net reproductive rate) and thermoregulatory traits (wing solar absorptivity, measured as degree of melanism on ventral hindwings). They then used the results of experiments conducted previously (Kingsolver, 1983a,b; Buckley and Kingsolver, 2012) and historic climate data from 1955 to 2010 to construct models that predict the direction of selection and the evolutionary responses to climate change. Their models predicted selection for decreased wing melanin as temperatures rise, but the degree of selection remained weak, suggesting that evolutionary responses to climate change may not match the rate of change of environmental conditions.

Monarch butterflies, whose migration cycles are the source of much concern in recent years (Zylstra et al., 2021), depend on wing coloration for their flight ability. Indeed, the link between butterfly wing color and flight ability has been established through studies by of *Pieris* butterflies in North America (Kingsolver and Wiernasz, 1991; Kingsolver, 1995). The two types of pigments responsible for the iconic wing colors of monarchs (**Figure 2**) are pteridine and melanin. The ability of monarchs to successfully navigate their migratory path is dependent on the thermoregulatory capacity of their wings, which is a function of wing surface coloration. Wild-caught monarchs with redder or darker shades of orange flew longer distances in the laboratory setting (Davis et al., 2012). Another study by Davis (2009) found that migrating butterflies tended to be redder than individuals in the overwintering or breeding stages. These results build on a study (Hanley et al., 2013) that found wild monarchs captured from the Gulf coast (warmer temperatures) were more melanized than monarchs from the Great lakes (colder temperatures). Monarchs that fly longer distances, i.e., those sampled at the Gulf coast, exhibit darker colored wings in order to absorb more solar energy. While monarch migration has been put forth as a complex trait to be used to study the impacts of climate change on population dynamics (Green, 2021), the strong link between wing color and flight performance during migration may allow us to use of wing color traits as a biological indicator of environmental change.

Zeuss et al. (2014) extended the relationship between coloration and thermoregulation to entire clades by conducting a phylogenetic comparison of butterflies and dragonflies' color lightness and thermal environments to demonstrate a correlation between the two. Their models showed that ventral wing surfaces became lighter with increasing temperatures in European butterflies. The authors also found that dragonfly assemblages across Europe became lighter colored over the last century. They attribute this directional change to climate warming forcing a skew in thermoregulatory coloration. They also predict range shifts in darker colored insects toward cooler and shadier regions.

As insect clades experience shifts toward color lightness or darkness due to broad changes in climate patterns, the plant assemblages they interact with are also expected to shift to adapt to an altered sensory and thermal landscape too (Shrestha et al., 2018). Flowers that generally rely on color to signal to pollinators must reach a trade-off between thermoregulation through pigmentary absorption of solar radiation and colors that are visible to their specific insect pollinator's vision systems (Lacey et al., 2010). With increasing temperatures and altered precipitation patterns, an imbalance in this trade-off can arise (Koski et al., 2020). Although anthocyanin-based colors are a strong influence on plant-pollinator interaction, anthocyanin also functions as a photosynthetic and photoprotective pigment (Gould, 2004). The reproductive structures of flowers are heat-sensitive and rely on pigments to regulate internal temperatures (van der Kooi et al., 2019). Using historical data from herbarium specimens and historic climate data (temperature, precipitation, and vapor pressure deficit), Sullivan and Koski (2021) assessed changes in floral pigmentation over 124 years in 12 North American polymorphic species that spanned 10 genera. They scored herbarium species as pigmented (red, pink, blue, or purple), unpigmented (white) and mixed. Color polymorphic species that experienced larger increases in aridity showed an increase in frequency of pigmented specimens over the timeframe. On the other hand, species that underwent larger increases in temperature in their respective environments showed a decline in the number of pigmented specimens. As warmer temperatures are often accompanied by and even exacerbate the effects of aridity in regions like western and southwestern North America, this creates conflicting selective forces for anthocyanin pigmentation. We may then expect to see new distributions of pigmented and unpigmented morphs, based on novel microclimates and niches created by climate change.

Landscape Change

The interaction of habitat fragmentation and climate change as separate but interlinked creates new environments that force species to either adapt rapidly or perish. While climate change may cause directional selection for traits that ameliorate thermal and drought related stresses, landscape change narrows the breadth of adaptations possible by reducing the potential for range expansion (**Figure 3**). Deforestation and intense fire events that have increased in frequency in recent years (Cattau et al., 2020) are two such habitat modifying forces that severely affect the species composition of ecosystems as well as intraspecific phenotypic variation. The Eastern red-backed salamander (*Plethodon cinereus*) has been used by Cosentino et al. (2017) to study the combined effects of forest fragmentation and climate change on morphotype distribution. The loss of or fragmentation of forests leads to greater edge effects such as drier and warmer soil conditions (Murcia, 1995; Laurance, 2004). These effects are exacerbated by warmer temperatures caused by climate change. Selection for climate ameliorating traits may then be either intensified by the loss of habitat or dampened by the regeneration of forest patches. Coupled with warmer temperatures owing to climate change, forest fragments represent a shortage of thermal refuges (Monasterio et al., 2009). As a terrestrial amphibian that requires moist and cool conditions, the red-backed salamander is particularly vulnerable to changes in temperature and moisture. *Plethodon cinereus* occurs as two discrete, genetically based color morphs that are differentially distributed in the forests of eastern North America. The unstriped (black) individuals are linked to warmer and drier microclimates than striped individuals. Cosentino et al. (2017) used historical data from 1880 to 2013 as well as data they collected between 2013 and 2015 on salamander morph frequencies, climate variables and forest cover. They modeled the interactive effects of mean annual temperature and forest cover on the proportion of striped individuals and found that years with higher temperatures saw a greater proportion of striped individuals in areas with higher forest cover. The proportion of striped morphs was also lower in areas that had experienced forest loss due to urbanization and agriculture, owing to the loss of thermal refuges. Their results support the suggestion that landscape factors can either exacerbate or ameliorate the effects of climate change. This association between genetically based color morphs and microclimates also lays down the potential for sympatric speciation with further warming and forest fragmentation. The red-backed salamander may rely on the persistence of multiple color morphs through, frequency dependent selection (Fitzpatrick et al., 2009) to survive predation, the greatest contributor to salamander mortality (Grant et al., 2018). Each of these morphs has also been shown to exhibit different anti-predator behaviors (rates of tail autotomy) that may be genetically correlated with coloration (Venesky and Anthony, 2007). Sympatric speciation along the basis of color could perhaps put each resulting species at greater risk of extinction without the buffering

FIGURE 3 | Animals whose coloration are impacted by landscape change. **(A)** Pygmy grasshopper (*Tetrix subulata*) by Hedwig Storch. Melanic morphs of the pygmy grasshopper are favored over non-melanic morphs after a fire event. **(B)** Western fence lizard (*Sceloporus occidentalis*) in Joshua Tree National Park by Hannah Schwalbe. The western fence lizard has been shown to perch on burnt twigs after a fire event to match its background better. **(C)** Eastern red-backed salamander (*Plethodon cinereus*) in Mississauga, Ontario by Ryan Hodnett. The striped morph of the eastern red-backed salamander relies on cooler and more moist microclimates that are declining due to forest fragmentation and climate change. **(D)** Humbug dacyllus (*Dascyllus aruanus*) in Lembeh, Indonesia by Rickard Zerpe. The dascyllus is one species of reef-dwelling fish that is experiencing greater predation rates due to coral bleaching.

effects of polymorphism and frequency dependent selection (Forsman et al., 2008).

Fire

Fire melanism is a well-documented trait occurring in animals that inhabit fire-dominant ecosystems (Karlsson et al., 2008). In regions that experience a fire season, habitat succession and the associated changes in visual environments are major drivers of polymorphisms and polyphenisms (Hocking, 1964). The stability of these succession cycles may be threatened by changes in climate and land use patterns that have led to a marked increase in frequency and intensity of fires in these regions (Steel et al., 2015). Color polymorphic animals that inhabit ecosystems shaped by fire often have melanistic and non-melanistic morphs. Rocha et al. (2015) used coloration, specifically, the frequency of darker individuals and darker species to assess the impact of fire disturbance events on the composition of Brazilian grassland insect assemblages. The authors sampled insects from five orders and categorized individuals into 52 morphotypes. Measuring color phenotype distribution through color density (high density meant brighter tones, low density meant darker tones), their results showed higher frequencies of darker colors in burned areas and higher frequencies of lighter colors in unburned areas. Rowell (1971) describes three possible mechanisms by which melanic morphs may be distributed in a fire shaped landscape—(a) natural selection for dark morphs on a dark substrate, (b) phenotypic plasticity in response to fire and associated darkened substrates, and (c) behaviorally different dispersal patterns. In order to establish a causal link between fire events and increased predation on lighter phenotypes in burned, darkened substrates, one would need direct evidence of altered predator-prey interactions.

Karpestam et al. (2012, 2013) do just this with the pygmy grasshopper (*Tetrix subulata*) in human predation trials. Over the course of two studies, the first a capture-mark-recapture study of natural populations of *T. subulata* in the wild in southwest Sweden, and the second a controlled human detection experiment, they examined differences in rates of detection for images of the three color morphs of grasshoppers against a range of naturally occurring backgrounds. In order to validate the use of humans as "predators" for their detection study, they compared the rate of detection of grasshopper images on computer screens to rates of capture of live individuals from their previous study (Karpestam et al., 2012) and to morph frequencies in the wild. They found that while no singular color morph was cryptic in all visual backgrounds, the detection rate of each of the three morphs was different across backgrounds that represented the various stages of habitat succession following a fire event. The authors established the context dependency of crypsis in a color polymorphic species and the idea that visual predation and background matching may contribute to the differential distribution of dark and light phenotypes in a post-fire environment. This association between adaptive color polymorphism and habitat heterogeneity provides a marker for the conservation of habitat complexity and the prevention of fragmentation.

The chaparral ecosystem of California is characterized by a fire season that is crucial to the community ecology and assemblages of that region. Over the course of a seasonal cycle, the landscape undergoes significant changes to its visual characteristics, ranging from dark and soot covered immediately post fire to verdant in the winter. Background matching and selective perching behavior in the western fence lizard (*Sceloporus occidentalis*) have been studied as adaptations to fire (Lillywhite et al., 1977). The lizards are dark colored and have been found, through field sampling after the Laguna fire of 1970 and laboratory-based choice tests, to selectively perch on darker colored stalks after a fire. As the successive stages of post-fire recovery ensue, the lizards perch on different substrates, such as dark colored rocks, to match their background. While the efficacy of their background matching has not been tested with predation trials, the authors hold that predation may be the selective force driving the maintenance of this adaptation.

Marine

The wide-ranging impacts of coral bleaching caused by climate change have been well documented, but the exact causes of declines in coral associated fish species have yet to be comprehensively laid out with empirical evidence. Coker et al. (2009) have attempted to address this knowledge gap with their study on the predation of reef fishes after a coral bleaching event. Coral reefs are a crucial feature of benthic ecosystems, providing complex habitat structure that mediates and maintains biotic interactions. The disappearance of colors from reefs due to bleaching events effectively removes the background against which reef-dwelling fish have evolved complex colors and patterns for a range of functions. The authors found that two species of Indo-Pacific damselfish (*Pomacentrus moluccensis* and *Dascyllus aruanus*) experienced greater rates of predation due to mismatches between the bleached coral background and body coloration.

Hemingson et al. (2022) recently extended this link between coral bleaching events and reef fish body coloration by developing and using a novel measure of color at the community level. Using reef community composition data collected over 27 years from the Great Barrier Reef, they looked at broad changes in the color composition of the cryptobenthic reef-dwelling fish community after mass bleaching events. Immediately following a bleaching event in 1998, the focal reef community saw a decline in color area (a quantitative measure of fish abundance derived from non-metric multidimensional scaling) of over 30%. The development of community-level measures of coloration is a crucial step toward connecting visual ecology and conservation biology. The authors of the study have also particularly used the human visual system (RGB colorspace) to quantify coloration and to draw attention to the aesthetic function of coral reefs in garnering interest in marine conservation.

Large scale changes to the marine landscape can include increased levels of anthropogenic noise pollution. While the bulk of literature addressing the ecological consequences of noise from ports and ships examines their effects on acoustic channels of communication and signaling, Carter et al. (2020) showed that noise may have far reaching effects on carapace color change in the shore crab (*Carcinus maenas*). They tested the effects of ship noise on changes to carapace luminance in juvenile shore crabs over molts using playback experiments over 8 weeks. Crabs exposed to ship noise were found to have changed luminance significantly less than those exposed to control ambient noise. Luminance is often the most elementary measure of background and thus lends itself as a good indicator trait for anthropogenic stresses. Ship noise also resulted in crabs being less camouflaged to predator vision as modeled digitally. As camouflage and other visual defenses are the first layer of protection against predators, crabs must also adopt secondary defense mechanisms to employ once spotted by a predator. The authors also examined the effects of loud ship noise on juvenile crabs' ability to respond to an artificial predator. Individuals exposed to the ship noise treatment were found to react to predation less often, and retreat into refuges slower than those exposed to ambient control noise. While the study did not look at the physiological mechanisms behind the effects of loud noise on luminance and color change, the authors posit that stress induced by noise may affect differential investment of resources (Sokolova et al., 2012) as well as the endocrinology of individuals. Color change and luminance change are tied to molting, which is regulated by hormones that are also associated with stress (Chung et al., 1999; Chung and Zmora, 2008). Luminance change during a molt is an energetically costly process to undergo and the energy budget is strained further by the increased metabolic rate associated with noise-induced stress (Wale et al., 2013).

APPLICATION

Thus far in the review, we have presented the impacts of anthropogenic landscape and climate change on the production, perception, and functionality of color patterns in a range of taxa. In demonstrating the inhibitory effects of environmental pollutants on the acquisition of pigments and pigmentary precursors, we suggest the use of ornamental color traits as non-invasive indicators of environmental change. The disruption of color signals and communication in terrestrial and aquatic systems by forest fragmentation, artificial light, and turbidity raises implications for the conservation of these systems. These anthropogenic modifications to the environment are particularly important as novel stimuli that can severely affect species and populations with low genetic or phenotypic variation and low levels of plasticity to accommodate these changes. The close link between the functionality of adaptive color phenotypes and climate and landscape factors especially highlights the need for conservation policies and practices that incorporate coloration and visual ecology.

Environmental Indicators

Coloration can be an indicator of environmental change. For example, ocean color remote sensing (OCRS) monitors ocean phytoplankton at potentially very large geographic scales. Started in 1998, the advantages of OCRS are access to biogeochemical proxies in remote areas, coverage several times each day, and the capability of separating chlorophyll concentrations and irradiance. Since it is satellite driven, OCRS can be obscured by cloud cover, sea ice or turbidity of river water that limit coverage (Babin et al., 2015). Nonetheless, ocean coloration can give us insights into the role of phytoplankton in marine biogeochemistry, the global carbon cycle, and responses of marine systems to climate change. It is a means by which we can monitor algal blooms, sediment plumes, and coastal eutrophication (Groom et al., 2019).

At a smaller scale, coral bleaching is a sensitive measure of the effects of changing sea temperatures because coral polyps expel zooanthellae which produce reactive oxygen species with increasing temperatures, and these are toxic to corals. Other triggers of bleaching are solar irradiance, changes in salinity, pollution, and ocean acidification (Hoegh-Guldberg, 1999). Rapid bleaching in response to environmental stress and the impact it has on the public make it an effective rallying point for addressing rising sea temperatures.

Normalized difference vegetation index is a method of measuring reflectance of the planet in the red and near-infrared bands (NIR) as viewed by satellite (Carlson and Ripley, 1997). Live plants absorb solar radiation but leaves re-emit it in the near-infrared because wavelengths > 700 nm are too small to be involved in molecule synthesis within the plant. Thus, normalized difference vegetation index is the ratio of (NIR − red)/(NIR + red) such that positive values indicate dense vegetation cover and hence intact undisturbed forests. This tool is also compromised by cloud cover, soil reflectance and atmospheric effects, especially water vapor.

Coloration as an Instrument for Conservation

There are numerous anecdotes and limited evidence that coloration in nature attracts people to it and promotes conservation sympathy (Caro et al., 2017). Temperate deciduous regions that witness autumnal color changes in foliage color are sources of seasonal tourism (Hall et al., 2011). In North America, people speak fondly of the colors of autumnal deciduous leaves in New England, the mid-west and in Canada and vacations are structured around these events. "Leaf peeping" as a tourist activity has seen a recent resurgence with lockdowns due to the COVID-19 pandemic forcing people to rediscover natural spaces in their neighborhoods (McMahon, 2020). Similarly, spring wildflower super-blooms (**Figure 4**) in California deserts and vernal meadows attract tourists each year. Accumulating evidence on the effects of natural spaces on human health shows that ecosystems with higher species diversity are likely to provide greater eco-system services in urban areas (Aerts et al., 2018). As our review demonstrates the link between biodiversity and color diversity, human health benefits may present a more compelling angle from which to push for the protection of natural spaces.

The Woodland Trust in United Kingdom advertises membership using brightly colored spring flowers.[1] Examples

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[1] www.woodlandtrust.org.uk

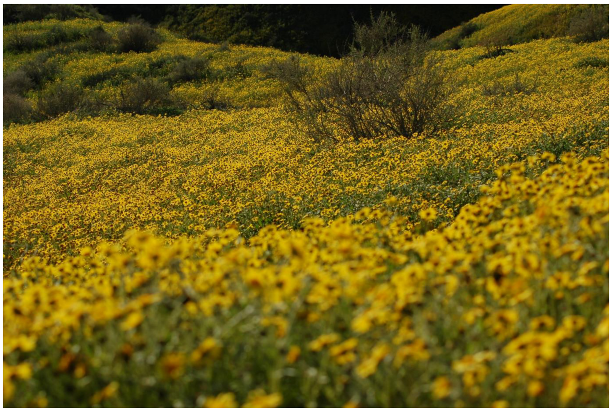

FIGURE 4 | Wildflower superbloom in Southern California, March 2020 (Photo by Tim Caro).

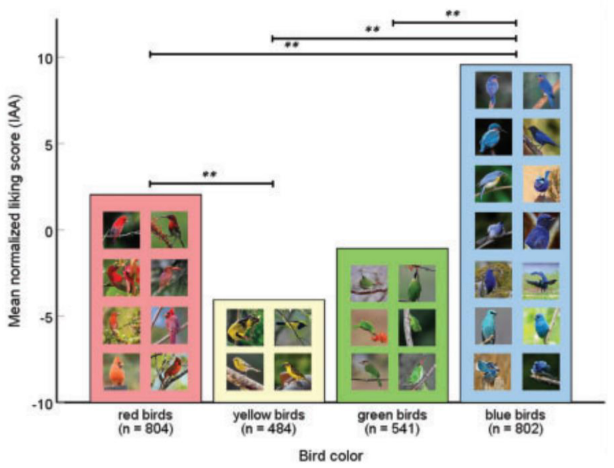

FIGURE 5 | Human bird color preferences ($\eta^2 = 0.028$). **The mean difference is significant at the 0.01 level. Adapted from Thömmes and Hayn-Leichsenring (2021).

of colorful animals being used in conservation are numerous and include poison dart frogs, toucans, parrots, and birds of paradise. For instance, penguin species with brighter red or yellow coloration feature more in publications (Stokes, 2007). In an experimental study, 19–29 year-old Czech citizens preferred lighter colored passerines, especially blue and green birds, although they preferred patterned birds if they had been manipulated to a gray scale (Lišková et al., 2015). Thömmes and Hayn-Leichsenring (2021) discovered that the birds with red color patches received more "likes" on Instagram than those with yellow patches while blue received more than any other color (**Figure 5**). At an institutional level, many nations prefer colorful species as their national birds (**Table 2** and **Figure 6**).

Some systematic research has explored the ability of coloration in animals to generate willingness to pay for conservation activities. Prokop and Fančovičová (2013) manipulated pictures of conspicuous aposematic taxa and showed them to 10–20 year-old children and young adults in Slovakia. They found that conspicuous pictures elicited a greater willingness to protect the same species rendered cryptic. Curtin and Papworth (2020) found that British people from across a wide span of age-groups preferred imaginary animals that were multi-colored as opposed to those with fewer colors and that this went some way to predicting donations made to single species charities. These studies have implications for choosing flagship species that advertise nature and as educational tools, symbolize a nation's natural heritage, promote the profile of an NGO, raise money, or help set up a reserve (**Figure 7**; Caro, 2010). But they carry a warning because colorful species may be more likely to be traded than duller species. Frynta et al. (2010) asked respondents to rank parrot species according to perceived beauty. They preferred species that were colorful, large, and long-tailed. Perceived beauty was positively correlated with size of the worldwide zoo population but not IUCN listing indicating zoos target beautiful but not endangered parrots for breeding.

AVENUES FOR FUTURE RESEARCH

Here, we briefly outline a series of future research possibilities on coloration and conservation as they relate to production, perception, function and application. Regarding the production of color, we see that the establishment of baseline levels of color diversity and saturation in populations is important for monitoring trends and trajectories of the evolutionary ecology of taxa. Using color as a qualitative measure of the health of an ecosystem presents a relatively non-invasive avenue for investigating the effects of large-scale landscape changes on phenotypic diversity, and by extension, the general biodiversity of a system. As this review and others (Lifshitz and St Clair, 2016; Peneaux et al., 2021) have established, environmental stressors such as metallic pollutants and herbicides have traceable impacts on the acquisition and deposition of pigments such as carotenoids and melanins in a range of avian species. The investigation of the effects of these stressors on other color pigments such as pteridines, psittacofulvins, and ommochromes presents another valuable path for future research. This would also widen the range of taxa capable of being monitored on the basis of coloration.

With regard to color perception in anthropogenically altered habitats, it is necessary to establish large scale quantitative measures of color traits along gradients of environmental change. These metrics may be constructed through systematic meta-analyses of each eco-system type to create standards against which climate change and landscape change can be assessed. By determining linkages between the phenotypic plasticity of color traits to genetic and epigenetic variation, we may more accurately evaluate the mechanisms by which color traits are affected by anthropogenic changes to the environment. Finally, color based measures of environmental changes may present more visually appealing ways of engaging with the general public when communicating climate and conservation science.

TABLE 2 | National birds of Latin America. Bold indicates colorful species.

Nation	Bird	Coloration
Guatemala	Resplendent quetzal (*Pharomachrus mocinno*)	**Green, red, white**
Honduras	Scarlet macaw (*Ara macao*)	**Red, yellow, blue**
Nicaragua and El Salvador	Turquoise-browed motmot (*Eumomota superciliosa*)	**Turquoise, green, orange, black, white**
Belize	Keel-billed toucan (*Ramphastos sulfuratus*)	**Green, orange, blue, yellow, black, scarlet**
Cuba	Cuban trogon (*Priotelus temnurus*)	**Black, gray, red, metallic blue**
Puerto Rico	Puerto Rican spindalis (*Spindalis portoricensis*)	**Black, white, orange, olive green**
Haiti	Hispaniolan trogon (*Priotelus roseigaster*)	**Green, blue, red, white, gray**
Peru	Andean cock-of-the-rock (*Rupicola peruvianus*)	**Orange, brown**
Venezuela	Venezuelan troupial (*Icterus icterus*)	**Black, orange, white**
Not colorful		
Mexico	Golden eagle (*Aquila chrysaetos*)	Brown
Costa Rica	Clay-colored thrush (*Turdus grayi*)	Two shades of brown
Panama	Harpy eagle (*Harpia harpyja*)	White, dark gray
Dominican Republic	Palmchat (*Dulus dominicus*)	Brown, ochre
Columbia, Ecuador, Bolivia, Chile	Andean condor (*Vultur gryphus*)	Gray-brown
Guyana	Hoatzin (*Opisthocomus hoazin*)	Brown, gray, speckled white
Brazil	Rufous-bellied thrush (*Turdus rufiventris*)	Gray, ochre
Paraguay	Bare-throated bellbird (*Procnias nudicollis*)	White, black face
Argentina	Rufous hornero (*Furnarius rufus*)	Light rufous
Uruguay	Southern lapwing (*Vanellus chilensis*)	Black, white, gray-brown

Bold indicates colorful species.

FIGURE 6 | National birds of South American countries. Clockwise from the left: **(A)** Scarlet Macaw (*Ara macao*), the national bird of Honduras, by Bernard Dupont in Puerto Jimenez, Costa Rica. **(B)** Keel-billed toucan (*Ramphastos sulfuratus*), the national bird of Belize, by Lauri Vain at Macaw Mountain Bird Park and Nature Reserve, Honduras. **(C)** Turquoise browed motmot (*Eumomota superciliosa*), the national bird of Nicaragua and El Salvador, by Asa Berndtsson in Costa Rica. **(D)** Andean cock-of-the-rock (*Rupicola peruviana*), the national bird of Peru, by Jerry Thompson at San Diego Zoo, United States. **(E)** Resplendent Quetzal (*Pharomachrus mocinno*), the national bird of Guatemala, by Cephas in Monteverde, Costa Rica. **(F)** Venezuelan Troupial (*Icterus icterus*), the national bird of Venezuela by Betty Wills at Bonaire, Venezuela.

FIGURE 7 | Charismatic and colorful flagship species from around the world used in conservation and restoration campaigns. Clockwise from top left: **(A)** *Acinonyx jubatus* (Cheetah in Sabi Sands by James Temple), one example of colorful and charismatic but endangered species used to head conservation campaigns and used often to promote eco-tourism and mammal conservation research in the southern African countries. **(B)** *Enteroctopus dofleini* (GFNMS—Giant Pacific Octopus—NOAA). Marine conservation organizations and the Monterey Bay and Seattle aquaria along the west coast of North America have used the Pacific giant octopus in promotional campaigns and merchandize to raise funds for their projects. **(C)** *Amphiprion ocellaris* (Clown anemonefish in Papua New Guinea by Nick Hobgood). The clown anemonefish (Amphiprion ocellaris) is instantly recognizable as a representative of tropical coral reefs and a recent study by Boudin et al. (2020) shows the effectiveness of the clownfish as a flagship to encourage conservation practices in young children, particularly in the context of the popular film Finding Nemo. **(D)** *Oophaga histrionica* (Harlequin poison frog by Mauricio Rivera Correa), often targeted by poachers for the illegal pet trade due to their striking color patterns. **(E)** *Panthera tigris* (Tiger drinking water, Mudumalai National Park by Timothy A. Gonsalves), the iconic symbol of Indian wildlife conservation, with the establishment of protected areas such as national parks and tiger reserves based around tiger populations. **(F)** *Calypte anna* (Anna's hummingbird, UC Berkeley Botanic Garden by Becky Matsubara), with its striking patch of iridescence, is used by University of California Master Gardeners to encourage California residents to convert monoculture residential lawns to native wildlife supporting gardens.

CONCLUSION

Reviews that bridge the link between visual ecology and human induced environmental changes are limited at present (Delhey and Peters, 2016; Peneaux et al., 2021; López-Idiáquez et al., 2022). While sensory ecology research relies heavily on the observation of changes in the environment—for example, seasonal camouflage and associated color change, seasonal polyphenism in butterflies—anthropogenic changes to the environments are only recently being factored into coloration studies. Delhey and Peters (2016) addresses the documented effects of changes in the visual environment on visual ecology, while we have attempted to cover the impacts of broader anthropogenic changes in climate and land-use. Peneaux et al. (2021) lays out an argument for carotenoid-based coloration in avian species to be used as an indicator of environmental pollution for monitoring the health of wild populations. While the bulk of literature studying the effects of environmental pollution on pigment production and deposition is skewed toward avian species, we attempt to make a case for using color as an indicator of pollution. The effects of anthropogenic noise on acoustic communication and signaling have been well-documented, with recent reviews by Chhaya et al. (2021) and Duquette et al. (2021) comprehensively covering the implications of anthropogenic noise and acoustic ecology for conservation.

In this review we have tried to unpick the relationship between coloration and anthropogenic change to show that coloration in nature as viewed in 1900 will not be the same as coloration viewed in 2100. Changes will be brought about by alterations in pigment production and structural coloration influenced by dietary changes, themselves mediated by landscape change. Changes in air or water transmission media will create new selection pressures on signal coloration usually making species less colorful. Changes in climate and landscape will alter the strength of selection by effecting coloration-background mismatch in color changing organisms and polymorphic species. More optimistically, coloration as an indicator of landscape change, and at a large scale has the potential to attract people's interest in nature and promote funding and effort toward the conservation agenda. Nature's palette is always changing due to alteration in natural and sexual selection pressures but now we can expect some rapid changes ahead.

AUTHOR CONTRIBUTIONS

MK collected the literature and drafted the manuscript. TC conceived of the study, revised, and edited drafts. Both authors contributed to the final manuscript.

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Aeroscapes and the Sensory Ecology of Olfaction in a Tropical Dry Forest

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Aeroscapes—dynamic patterns of air speed and direction—form a critical component of landscape ecology by shaping numerous animal behaviors, including movement, foraging, and social and/or reproductive interactions. Aeroecology is particularly critical for sensory ecology: air is the medium through which many sensory signals and cues propagate, inherently linking sensory perception to variables such as air speed and turbulence. Yet, aeroscapes are seldom explicitly considered in studies of sensory ecology and evolution. A key first step towards this goal is to describe the aeroscapes of habitats. Here, we quantify the variation in air movement in two successional stages (early and late) of a tropical dry forest in Costa Rica. We recorded air speeds every 10 seconds at five different heights simultaneously. Average air speeds and turbulence increased with height above the ground, generally peaked midday, and were higher overall at the early successional forest site. These patterns of lower air speed and turbulence at ground level and overnight have important implications for olfactory foraging niches, as chemotaxis is most reliable when air movement is low and steady. We discuss our results in the context of possible selective pressures and observed variation in the foraging ecology, behaviors, and associated morphologies of resident vertebrates, with a focus on mammals. However, these data also have relevance to researchers studying socioecology, invertebrate biology, plant evolution, community ecology and more. Further investigation into how animals use different forest types, canopy heights and partition activities across different times of day will further inform our understanding of how landscape and sensory ecology are interrelated. Finally, we emphasize the timeliness of monitoring aeroecology as global wind patterns shift with climate change and human disturbance alters forest structure, which may have important downstream consequences for biological conservation.

Keywords: aeroecology, olfactory ecology, tropical dry forest (bosque seco tropical), air speed, sensory landscape, sensory evolution

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INTRODUCTION

Air is a dynamic and ever-changing medium, and aeroscapes (defined as patterns in air speed and direction; Vogel, 1996) are an integral part of terrestrial ecosystems. Efforts to integrate aeroscapes into the study of organismal behavior and ecology (collectively termed aeroecology; Kunz et al., 2008; Diehl, 2013) have revealed that animals react adaptively to variables such as air speed and turbulence (Frick et al., 2013; Diehl et al., 2017). These same factors are expected to have pronounced effects on the propagation and uptake of sensory information (Finelli et al., 2000; Muller-Schwarze, 2006). For example, greater air speeds will disperse the odors of plants and animals farther, but the resulting turbulence is likely to disrupt the spatial distribution of odor plumes, challenging the ability of organisms to navigate toward the odor source (i.e., anemotaxis; Murlis, 1997; Conover, 2007; Bingman and Moore, 2017). This tradeoff in signal propagation and efficacy is well-studied in insects, which optimize their flight paths in response to air speed while tracking odor plumes (Aluja et al., 1993; Cardé and Willis, 2008; Hennessy et al., 2020). At the same time, many mammals possess relatively complex olfactory systems, and they, too, are sensitive to variations in the aeroscape (Moulton, 1967; Svensson et al., 2014). For example, air speed is known to affect the olfactory orientation and behavior of carnivorans—red foxes, striped skunks, raccoons, polar bears, domestic dogs (Ruzicka and Conover, 2011, 2012; Togunov et al., 2017; Jinn et al., 2020)—as well as primates, such as ring-tailed lemurs (Cunningham et al., 2021). Still, there has been little effort to explore the spatiotemporal factors that govern a given aeroscape, or the effects of this variability on the aero-sensory ecology of mammals, especially in forest ecosystems.

Forests are complex habitats in the vertical and horizontal planes (Ennos, 1997), and this level of heterogeneity is reflected in the form of highly variable aeroscapes (Baynton, 1969; Heydel et al., 2014). For example, the understory is essentially sheltered from the winds affecting the upper canopy, which can create striking vertical disparities in air speeds (Aoki et al., 1978; McCay, 2003). Scant air movement in the understory is expected to favor efficient anemotaxis, but the magnitude of vertical variation in an aeroscape can be offset temporally—e.g., at night, when air is cooler and moving at diminished speed—or spatially as a function of standing forest biomass (Murlis et al., 2000; McCay, 2003). The essential limitation is that these factors are rarely measured simultaneously or folded into our understanding of mammalian aero-sensory ecology and evolution.

To contribute toward building this literature, we studied spatiotemporal variation in the aeroscape of a lowland tropical dry forest in Costa Rica. We recorded variation in air speed and turbulence as a function of: (1) vertical position and (2) diel periodicity. We measured each of these variables at two sites, chosen to reflect two habitat types—early and late successional forest. Our goal is to better understand how aeroscapes vary within a forest, and how this variation might mediate the distribution of odorant molecules through the aeroscape. This information is essential for understanding the selective pressures that have shaped the olfactory anatomy and behaviors of resident animals, as well as plant reproductive strategies.

MATERIALS AND METHODS

Study Site

We collected data in Sector Santa Rosa of the Área de Conservación Guanacaste, northwestern Costa Rica. The site is a tropical dry forest with two distinct seasons: a dry season from December through May and a wet season from June through November (Campos, 2018; Janzen and Hallwachs, 2020; Melin et al., 2020). Sector Santa Rosa forests are primarily secondary, stemming from restoration and reforestation efforts that began in the 1970s (Janzen and Hallwachs, 2020), and forest composition and structure varies between early and late successional stages. Canopy height ranges from 6 to 15 m depending on successional stage; the canopy is typically taller in areas of later succession (Kalacska et al., 2004; Powers et al., 2009).

Data Collection

We used cup anemometers (WL-11; Scarlet Tech, Taipei, Taiwan) to collect air speed data from May to June 2021. The instruments have a sensitivity range of 0.6–50 m/s, a resolution of 0.1 m/s, and an accuracy of ±2%, per manufacturer specifications. We built and erected two scaffold towers: one in a late successional forest (10.838617, −85.614283; 1,086 m a.s.l.), and the other in an early successional forest (10.839383, −85.616383; 906 m a.s.l.). To each tower, we affixed five anemometers at heights of 0.5, 3.5, 5.5, 7.5, and 10 m (**Figure 1**). The devices were set to data-logging mode, recording average and maximum air speed in 10-s intervals.

Data Analysis

We obtained the mean, median, and standard deviation of air speed for: (1) each sampling height and (2) time of day. We calculated air turbulence as standard deviation of air speed/mean air speed (McCay, 2003). The sensitivity threshold (0.6 m/s) of our anemometer risks a systematic bias against low air speeds, especially in the understory. Accordingly, we imputed values below this limit by using survival analyses, a method developed for the health sciences but adopted for analyzing environmental data with detection limits (Helsel, 2004). Using the survival (Therneau, 2021) and NADA (Lee, 2020) packages in R (v. 4.1.0., R Core Team, 2021), we constructed Kaplan–Meier estimates for data recorded at each site independently for each height and time of day, considering air speeds ≤0.6 m/s to be left-censored.

To detect significant differences in air speeds and turbulence as a function of vertical height and time of day, we conducted Cox proportional hazard modeling via the survival package in R. As these hazard models are designed for right-censored data, we transformed our data by using the "flipping" method of Helsel (2004), which subtracts all observed values from a constant to convert left-censored data to right-censored. Since the same column of wind is recorded simultaneously at all 5 observed heights for a given site, there is a high degree of collinearity between the height and time of day predictor variables, therefore we modeled each separately and present two models. To address our first aim, we modeled air speed as a function of vertical position, based on height from the ground ("Height") in two different habitat types. To address our second aim, we modeled air speed as a function of diel periodicity ("Time of Day") in two different habitat types. For both models, the outcome variable was the Kaplan–Meier estimate of average wind speed, and an interaction term of study site and height, or study site and time of day, was included as a predictor variable. For the Height model, the continuous variable distance

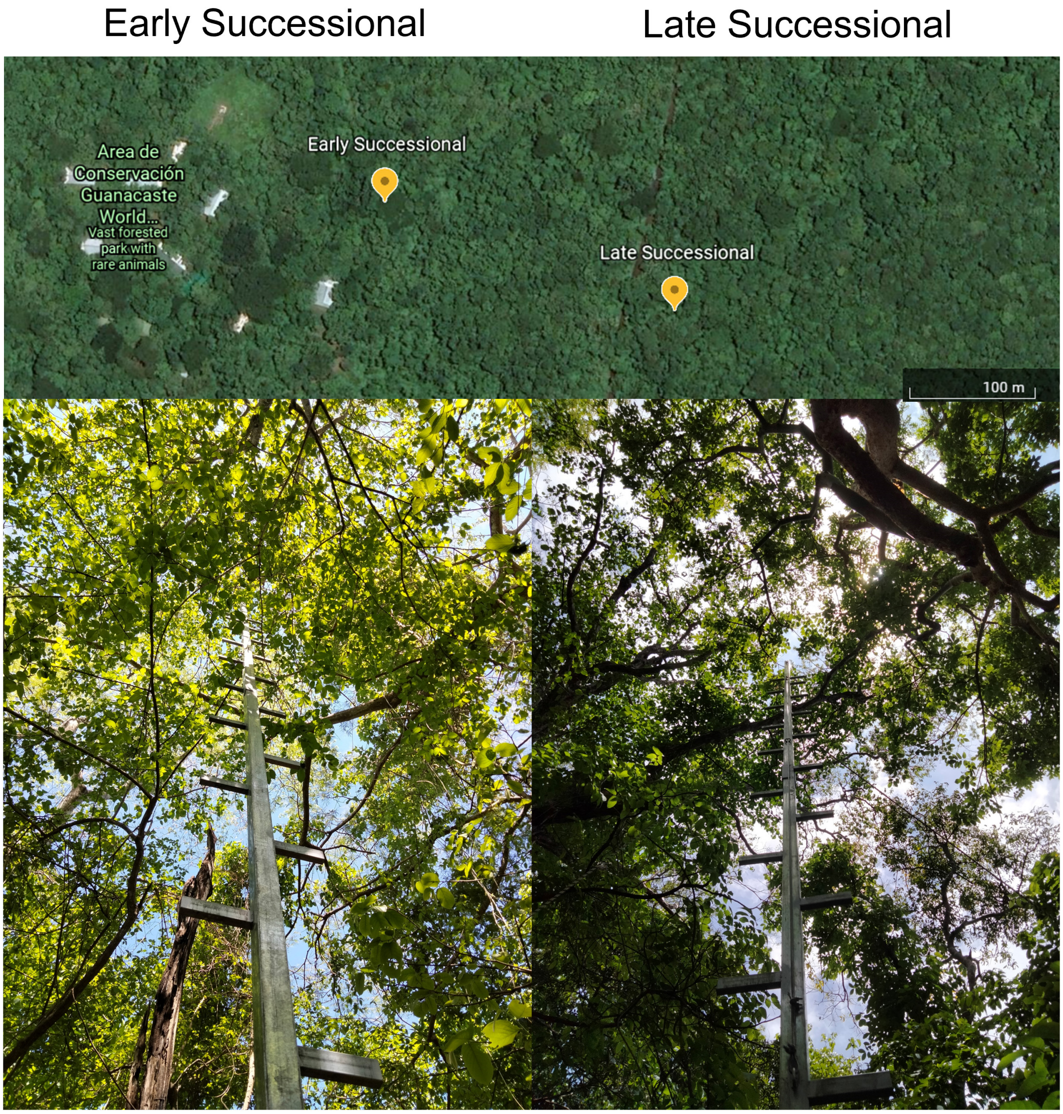

FIGURE 1 | Scaffold system used to fix five anemometers per tower at heights of 0.5, 3.5, 5.5, 7.5, and 10 m. The anemometers were moved between our early successional forest site and our late successional forest site at 2 week intervals.

from the ground (in meters) was used, while for the Time of Day model, because of the circular nature of temporal data, we employed a sinusoidal model with separate cos and sin terms (Simmons, 1990; Cazelles et al., 2008). Code for all analyses can be found at https://github.com/allegradepasquale/wind_speed_project.git.

RESULTS

Vertical Variation

Air speeds increased as a positive function of vertical height, and mean air speeds differed between the study sites, with greater mean speeds recorded in the early successional forest (**Figure 2** and **Table 1**). The interaction term of study site and height was also significant (**Table 1**). Variation in air turbulence followed a similar pattern: mean air turbulence scores varied as a positive function of vertical height and were greater in the early successional forest (**Figure 2**). Site differences in our measure of air turbulence were pronounced near ground level (0.5 m), but values tended to converge with increasing vertical height (**Figure 2**).

Temporal (Diel) Variation

Air speed varied significantly with time of day: winds were stronger from late morning to early afternoon and were lowest overnight (**Figures 3**, **4**). As with our analyses of height, the main effect of study site, as well as the interaction between study site and time of day, were also significant (**Figure 4**

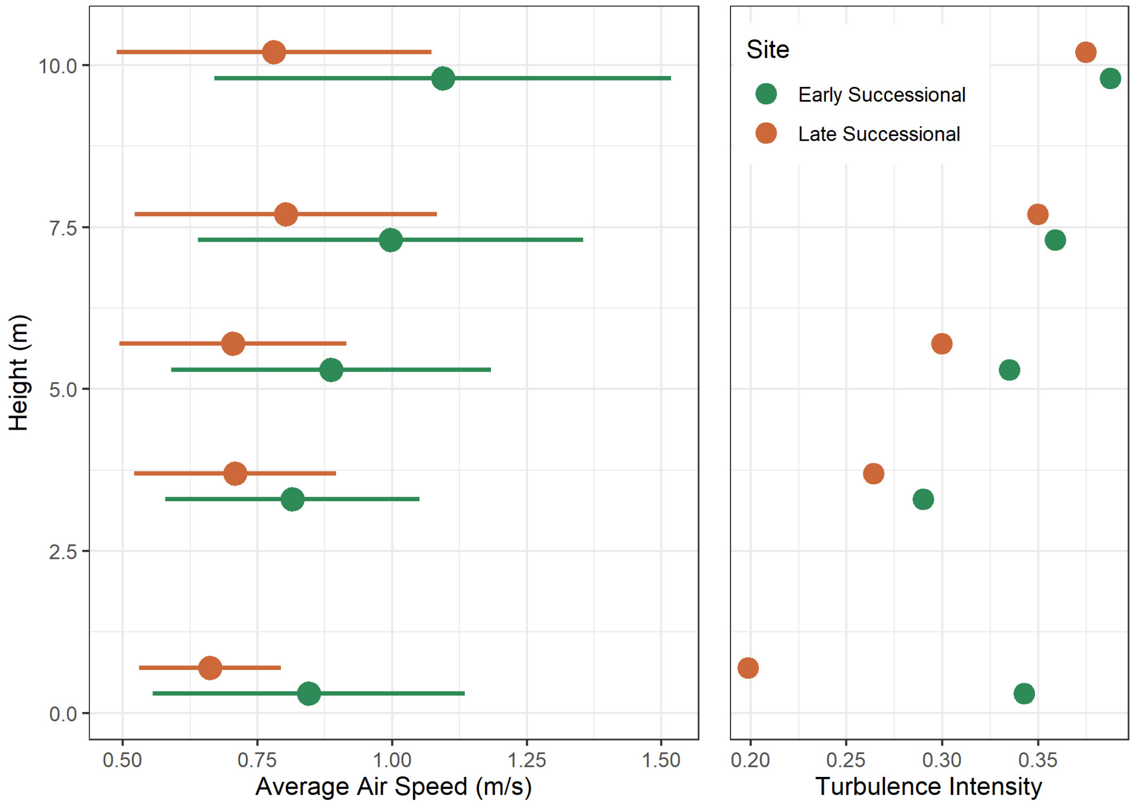

FIGURE 2 | Average air speeds (±standard deviation) and turbulence score at different heights from the ground for our early successional and late successional forest sites, generated from Kaplan–Meier estimates.

TABLE 1 | Results of cox proportional hazard models for mean air speed by vertical position and time of day (i.e., diel cycle).

Model	Coefficient	Hazard ratio	Std. error	*Z*-Score	Lower 95%	Upper 95%	*P*-value
Height model							
Height	0.078143	1.0812778	0.000611	127.74	1.08	1.0826	<2e-16*
Site	−1.07235	0.3422013	0.006444	−166.3	0.3379	0.3466	<2e-16*
Height:Site	0.002447	1.0024507	0.001013	2.415	1.0005	1.0044	0.0158*
Time of day model							
Site	−1.077947	0.340294	0.003573	−301.70	0.3379	0.3427	<2e-16*
Time of day (sin function)	−0.277369	0.757775	0.002983	−92.97	0.7534	0.7622	<2e-16*
Time of day (cos function)	−0.477793	0.620151	0.003117	−153.28	0.6164	0.6240	<2e-16*
Time of day (sin function):Site	−0.068203	0.934071	0.004753	−14.35	0.9254	0.9428	<2e-16*
Time of day (cos function):Site	−0.233284	0.791928	0.004983	−46.82	0.7842	0.7997	<2e-16*

Asterisks represent statistical significance ($p < 0.05$).

and **Table 1**). Turbulence scores were highest at midday and lowest overnight.

DISCUSSION

Air movement is a dynamic aspect of landscape ecology that shapes the way animals experience the world. In this study, we quantified air movement in a tropical dry forest as a function of vertical position and diel periodicity in two forest types. We found that air speed and turbulence increased with height, peaked midday, and were lower in the late successional forest. Taken together, our findings suggest spatiotemporal predictability in the aeroscape of a tropical forest and motivate a discussion of how animals adapt to and exploit these patterns.

Vertical Variation in Aeroscapes

We detected a steep increase in air speeds as a function of vertical height, a result that replicates findings from other forest ecosystems (Baynton, 1969; Oliver and Mayhead, 1974; Aoki et al., 1978; Kruijt et al., 2000; McCay, 2003). The prevailing

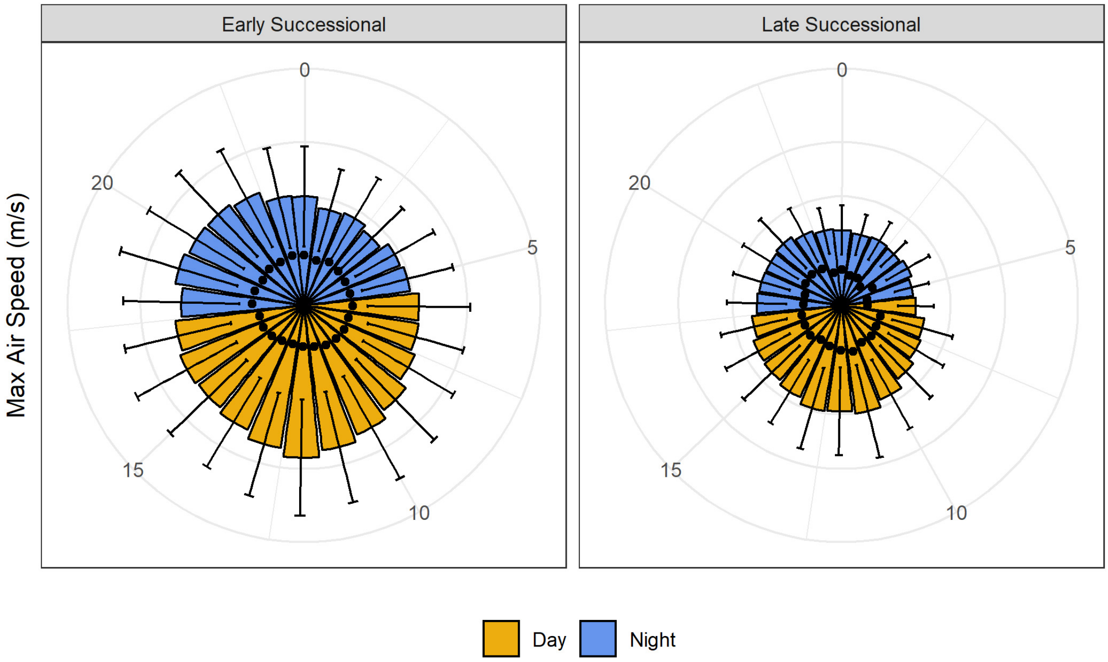

FIGURE 3 | Maximum air speeds in meters per second at our early and late successional sites over the diel cycle. Black dots represent turbulence scores.

explanation for this pattern is that air movement is impeded near the ground by understory vegetation, tree trunks, and surface topography; however, the implications of such a gradient for olfactory ecology are underexplored. Fruits, for example, emit odors (generally lightweight, ephemeral volatile and semivolatile organic compounds) that are easily dispersed by air movement (Rodríguez et al., 2013; Nevo et al., 2018, 2020), although excessive air speeds can over-disperse odor compounds and disrupt anemotaxis (Svensson et al., 2014). Still, it follows that the vertical position of fruit will determine the probability and efficiency of long-distance odor detection and foraging by seed-dispersing mutualists (Santana et al., 2021). Anemotaxis toward food resources is well-studied among invertebrates (Zjacic and Scholz, 2022) and increasingly so among mammals. For example, experiments with bats have elicited klinotaxis in response to fruit odors (Thies et al., 1998; Korine and Kalko, 2005; Leiser-Miller et al., 2020; Brokaw et al., 2021; Brokaw and Smotherman, 2021), and a field experiment by Fleming et al. (1977) found that bats will deviate from their flyways by as much as 50 m to acquire fruits mounted to 1.5-m poles set up moments before sunset. Further, experiments with coatis and ring-tailed lemurs have shown that they can detect fruit from distances up to 20 m (Hirsch, 2010; Cunningham et al., 2021). These findings suggest that aero-sensory ecology via stimulus response can complement and extend the critical importance of spatial memory to the localization of foods (Janson, 1998; Janmaat et al., 2014; Dahmani et al., 2018). Looking forward, it stands to reason that our understanding of cognitive ecology will only be strengthened as we incorporate and integrate the systematic study of aeroscapes and other sensory landscapes into research frameworks.

Many animals use olfactory signals to communicate with one another for reproduction, dominance, and territory defense, as well as to discriminate conspecifics from heterospecifics. In mammals, these occur mainly through the deposition of scent marks (Johnson, 1973; Irwin et al., 2004; Kollikowski et al., 2019). Our results suggest that olfactory signals may remain more localized when deposited nearer to the ground than higher in the canopy. It is noteworthy, then, that olfactory communication appears to be particularly prevalent in terrestrial mammals, which have olfactory receptor gene repertoires that have undergone three times as much gene duplication than those of volant, arboreal, and aquatic mammals (Hughes et al., 2018). At the same time, low air speeds will limit long-distance dispersal of odors. It is possible that species and habitat-specific optima for olfactory signal height exist and may vary across taxa. For example, Ethiopian wolves, gray wolves, and pine martens use raised-leg urination, a behavior thought to reinforce territorial boundaries by dispersal of urinary odor plumes. Urine deposition above, rather than on, the ground may improve scent dispersal (Peters and Mech, 1975; Macdonald, 1980; Pulliainen, 1981; Alberts, 1992; Sillero-Zubiri and Macdonald, 1998). Turning to another mammalian radiation, many primate species that have evolved glands dedicated for scent deposition, including ring-tailed lemurs, mandrills, drills, and sifakas, are largely terrestrial (Delbarco-Trillo et al., 2011; Drea, 2015; Vaglio et al., 2016). Systematic study of olfactory signaling and receiving behaviors, along with investigation of co-occurring anatomical and genetic variation, in taxa occupying different vertical niches will allow this hypothesis to be tested. In general, the intersection of sensory and aeroecology holds

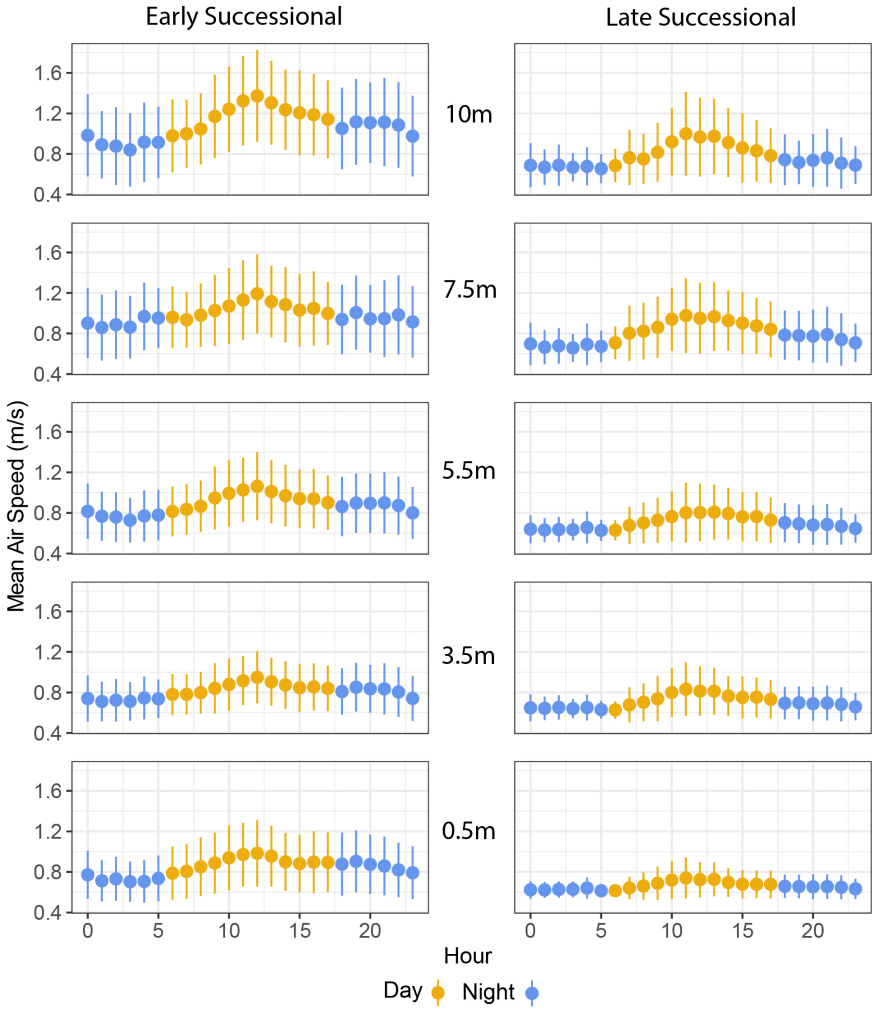

FIGURE 4 | Mean air speeds at two forest sites as a function vertical height and diel periodicity. The *x*-axis begins at 00:00 h (midnight) and ends at 23:00 h (11:00 pm).

untapped potential for better understanding the wide variation in animal sensory traits.

Temporal (Diel) Variation in Aeroscapes

We detected pronounced variation across the 24-h day, with peak and nadir air movements at midday and overnight, respectively. This pattern is almost certainly due to ambient temperature flux because warmer air moves faster and is less stable (Pleijel et al., 1996; McCay, 2003; Monteith and Unsworth, 2013; but c.f. Baynton, 1968, 1969; Rapp and Silman, 2012 for other patterns). Following similar arguments to those concerning vertical variation, nocturnal aeroscapes may be preferential for animals that detect and localize scents. Many nocturnal animals rely more on olfaction than vision, but the reasons are usually couched in the language of constraint—there is scant light at night, so vision is limited (Barton et al., 1995; Balkenius et al., 2006; Borges, 2018; Niimura et al., 2018). Underappreciated, however, is the idea that olfaction is more effective at night due to the relative stillness of air, especially in the understory, where air speeds and turbulence tend to be lowest (Murlis, 1997; Muller-Schwarze, 2006). Lack of wind produces a high signal-to-noise ratio for a given odor plume, resulting in a stronger olfactory signal. This nocturnal environment may thus select for olfactory-driven ecologies and social interactions, perhaps even in the absence of selection driven by the loss of visual ability (Drea et al., 2019).

Spatiotemporal Interactions

Vertical and diel variation interact to create diverse aeroscapes, ranging from the windiest and most turbulent environment of the upper canopy at midday, to the comparative stillness of the understory at night. Such interactions create opportunities for convergence. We found that aeroscapes in the daytime understory are comparable to those in the canopy at night, which raises the possibility of convergent olfactory signals and sensitivities in the animals that occupy these distinct niches

(Barton, 2006; Valenta et al., 2013; Brokaw and Smotherman, 2020; Nevo and Ayasse, 2020). Interestingly, the olfactory ecology of nocturnal terrestrial frugivores may depend in part on wind-mediated fruit falls during daytime, suggesting vertical day-night integration of aeroecologies (Augspurger and Franson, 1987; Borah and Beckman, 2021). The upshot is that daytime canopy conditions are suboptimal for anemotaxis in the service of frugivory and seed dispersal, which may explain why so-called "bird-fruits" in the upper canopy emit little scent (Gautier-Hion et al., 1985; Howe, 1986; Lomáscolo et al., 2010; Valenta et al., 2018; Valenta and Nevo, 2020).

Some angiosperm plants, including *Ficus* and *Nicotiana*, exhibit diurnal rhythms in fruit and flower chemistry (Raguso et al., 2003; Borges et al., 2011; Burdon et al., 2015; Ripperger et al., 2019; Balducci et al., 2020) timed to maximize their availability and attractiveness for pollinators and seed dispersers. These diurnal rhythms may have evolved in response to diurnal patterns in air movement, such that compounds produced during the day may be heavier and more robust against turbulent conditions than those produced at night, which may be lighter and more easily propagated under calmer conditions (Alberts, 1992; Muller-Schwarze, 2006). Flowers, which are generally more delicate and ephemeral than fruits, are often produced early in the morning, which may reflect a compromise between protection from wind-damage and availability to vision-mediated pollinators such as bees and birds (Herrera, 1990; Bloch et al., 2017). Setting aside the aeroecology of frugivory and pollination, aeroscapes are also essential to another aspect of plant reproductive biology: wind dispersal (Kennedy, 1978; Friedman and Barrett, 2008). Flowering and the eventual abscission of wind-dispersed seeds is greatest at midday, when air movement is highest, which suggests some level of aerosensation (Bohrer et al., 2008; Wright et al., 2008; Caplat et al., 2012). Such hypotheses invite future testing.

Intraforest Variation

We recorded higher air speeds and greater turbulence in the early successional habitat compared to the later one, possibly reflecting the lower levels of standing biomass. This pattern is expected for habitats with greater levels of anthropogenic disturbance that have caused vegetative loss (Raynor, 1971; Muller-Schwarze, 2006; Klein et al., 2021). Such results have potentially important implications. For example, increased air movement may affect the relative colonization of wind-dispersed versus animal-dispersed plants in disturbed areas (Cadenasso and Pickett, 2001; Cubiña and Aide, 2006; Nathan et al., 2008), altering community dynamics, habitat suitability to frugivores, and reforestation efforts (Janzen, 1988; Vieira and Scariot, 2006; de la Peña-Domene et al., 2018; Camargo et al., 2020). Greater air movement and turbulence are also expected to negatively affect animals that rely on odor plumes (Shukla et al., 1990; Zhang et al., 1996; Gandu et al., 2004). Consequences include impediments to animal foraging and communication, as discussed above, as well as effects on predator-prey networks.

Sensory detection of predators often involves scent, and many species are particularly attuned to the odors of their relevant predators (Weldon, 1990; Kats and Dill, 1998; Apfelbach et al., 2005). Rats are particularly sensitive to 2,4,5-trimethylthiazoline, a component of red fox anal gland secretions (Laska et al., 2005) and mice, rats, and stoats, for example, have been shown to avoid carnivore and apex predator odors (Ferrero et al., 2011; Garvey et al., 2016). Indeed, higher wind speeds have been found to impede predator detection by mule deer (Bowyer et al., 2001) and other mammal species (Cherry and Barton, 2017). The sensory impact of air movement can be further compounded by spillover to the other senses: wind creates acoustic and visual noise, which may reduce detection of stimuli by other senses, further impeding predator detection (Hayes and Huntly, 2005; Carr and Lima, 2010; Francis et al., 2012). Overall, future work could usefully address the diverse ways that anthropogenically modified aeroscapes affect the aero-sensory ecology and habitat use of resident flora and fauna (Damschen et al., 2008). As these influences could ultimately affect species distributions (Bowyer and Kie, 2009; Breitbach et al., 2012), we urge the incorporation of aeroscapes into existing conservation and evolutionary frameworks (e.g., assessment of "edge effects" and "landscapes of fear") and general inclusion into future studies of anthropogenic disturbance and deforestation/reforestation dynamics (Laundre et al., 2010).

Limitations and Future Directions

Our results offer insight into the variability of air movement within a heterogeneous landscape, although some caution must be noted. First, due to equipment limitations, we were unable to sample early and late successional forest sites simultaneously, and instead sampled them sequentially. This serial approach raises the possibility that temporal differences in climate, not habitat heterogeneity, were driving the difference that we observed between sampling locations. Further, due to the logistical challenges of erecting scaffolds in a protected habitat, we were unable to sample replicates of early and late successional forest conditions. The extent to which our two study locations exemplify such habitat conditions is therefore uncertain, and could usefully be explored in greater detail in future studies sampling multiple locations per forest type. Lastly, the detection limits of our anemometers prohibited direct measurement of the slowest air speeds. While prohibitively expensive for our study design, solid-state anemometers have the advantage of greater measurement range. Future studies may also benefit from incorporating variation in wind direction, as this also has important implications for olfactory-based orientation (Kennedy, 1978; Togunov et al., 2017; Jinn et al., 2020).

Our study contributes to the emerging field of aeroecology by quantifying variation in the aeroscape of a lowland tropical dry forest and drawing attention to the implications for sensory signal propagation, plant reproduction, and mammalian sensory evolution. This topic is timely as shifting air movement patterns due to anthropogenic climate change may disrupt aeroecological interactions, with the potential far-reaching effects on animal behavior, population dynamics, and species distributions (Usbeck et al., 2010; McInnes et al., 2011; Young et al., 2011; Lewis et al., 2015). Understanding the impacts of aeroscapes on sensory ecology and evolution will be key for predicting how animals will respond to changing environments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AD contributed to the study design, led data collection and analysis, and wrote the manuscript. JH co-led data analysis alongside AD and contributed to the writing of the manuscript. CG contributed to the data collection, study design, and writing of the manuscript. ND contributed to the study design, data analysis, and writing of the manuscript. AM contributed to the study design, data collection and analysis, and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Remote Sensing of Floral Resources for Pollinators – New Horizons From Satellites to Drones

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Insect pollinators are affected by the spatio-temporal distribution of floral resources, which are dynamic across time and space, and also influenced heavily by anthropogenic activities. There is a need for spatial data describing the time-varying spatial distribution of flowers, which can be used within behavioral and ecological studies. However, this information is challenging to obtain. Traditional field techniques for mapping flowers are often laborious and limited to relatively small areas, making it difficult to assess how floral resources are perceived by pollinators to guide their behaviors. Conversely, remote sensing of plant traits is a relatively mature technique now, and such technologies have delivered valuable data for identifying and measuring non-floral dynamics in plant systems, particularly leaves, stems and woody biomass in a wide range of ecosystems from local to global scales. However, monitoring the spatial and temporal dynamics of plant floral resources has been notably scarce in remote sensing studies. Recently, lightweight drone technology has been adopted by the ecological community, offering a capability for flexible deployment in the field, and delivery of centimetric resolution data, providing a clear opportunity for capturing fine-grained information on floral resources at key times of the flowering season. In this review, we answer three key questions of relevance to pollination science – can remote sensing deliver information on (a) how isolated are floral resources? (b) What resources are available within a flower patch? And (c) how do floral patches change over time? We explain how such information has potential to deepen ecological understanding of the distribution of floral resources that feed pollinators and the parameters that determine their navigational and foraging choices based on the sensory information they extract at different spatial scales. We provide examples of how such data can be used to generate new insights into pollinator behaviors in distinct landscape types and their resilience to environmental change.

Keywords: remote sensing, flower, insects, pollination, behaviour, foraging, drone, satellite

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INTRODUCTION

Pollination of flowering plants by insects is essential to the functioning of natural and agricultural ecosystems and to the global food supply (most recently reviewed by Khalifa et al., 2021; Montoya et al., 2021). These pollination services depend in fundamental ways on the spatio-temporal distribution of floral resources, which are dynamic across time and space. They are also shaped by anthropogenic landscape fragmentation and climate change with a potential to reduce or eliminate relationships within intricate ecosystems that are balanced by both the species composition and abundance of flowering plants (e.g., Biesmeijer et al., 2006; Memmott et al., 2007; Montero-Castaño and Vila, 2012; Goulson et al., 2015; Wenzel et al., 2020). Alterations in the availability of floral resources and the macro- and micronutrients they provide may have important effects on the population strength, health, activity and navigation patterns of pollinators across a range of spatial scales – from the plant to the flower patch and wider landscape. Consequently, the landscape context is critical to understanding pollination behaviors and their effects on pollinator populations (e.g., Winfree et al., 2007; Betts et al., 2019; Pamminger et al., 2019; Timberlake et al., 2021; Nicholls et al., 2022). It is timely to assess how spatial data describing the time-varying spatial distribution of flowers can be obtained by integrating remote sensing technology more firmly into behavioral and ecological studies. We review here how new technology and approaches can be applied to overcome challenges in obtaining accurate description of landscape structures and the dynamic distribution of floral resources that impacts pollinator abundance, activity and movements.

Vision guides most pollinators' behavioral repertoires, as shown by long-standing research of vision and visually guided behaviors in bees, flies and other insect pollinators (for reviews see Menzel et al., 1997; Egelhaaf and Kern, 2002; Srinivasan, 2011; Hempel de Ibarra et al., 2014; Behnia and Desplan, 2015; Kelber and Somanathan, 2019). For demonstrating causality, the environmental conditions, typically inside the laboratory, are often manipulated to reduce complexity and to control the presentation of visual stimuli or landmarks. This has helped to characterize rules of movements at different spatial scales, unraveling how insects navigate (e.g., Collett and Collett, 2002; Collett et al., 2006; Knaden and Graham, 2016; Webb, 2019). However, it is still difficult to carry out behavioral experiments over larger spatial scales, in field environments or heterogeneous landscapes, partly because most pollinators are flying insects and small in body size. There are limited options for tracking their flight trajectories and these do not resolve small-scale movements and behavioral choices during a natural foraging trip. The other challenge is to design behavioral studies and identifying suitable study areas based on easily quantifiable landscape parameters, such as the distribution of foraging locations and floral densities. Remote sensing offers opportunities to analyze floral resources *in situ* along a range of spatial and temporal scales (**Table 1**). This information could enhance our understanding of how and at what scales the distribution of floral resources in the environment affects pollinator movements. For ecological questions such information also allows scientists to evaluate how fitness and abundance of pollinators is affected in different landscapes.

TABLE 1 | Definitions for "coarse," "medium," and "fine" spatial resolutions and revisit periods used in this paper.

	Spatial resolution (m)	Revisit period (days)
Coarse	10+	30+
Medium	1–10	11–29
Fine	<1	<10

Terrestrial remote sensing approaches, where the Earth's ecosystems are monitored by sensors on board satellites (e.g., Xie et al., 2008), airplanes (e.g., Lan et al., 2009), unpiloted aircrafts also known as drones (Anderson and Gaston, 2013) and ground-based platforms have transformed scientific understanding of the dynamics in global processes, particularly the world's vegetated ecosystems. Remote sensing works by capturing changes in the spectral signatures or structural traits of vegetation (at different grains, depending on the sensor and platform used) so that their physiological, biochemical and spatial properties can be analyzed. When using remote sensing to characterize flowering vegetation, researchers have obtained can information in the form of multispectral images, reflectance spectra, or point cloud data (Willcox et al., 2018; Krishnasamy et al., 2019). Traditional field techniques involving manual floral counts using quadrats or transects are often laborious, time-consuming and only measure floral features detectable or predefined by the observers' eyes Furthermore, in an analysis of methodologies employed by 159 studies, Szigeti et al. (2016) found that ecologists use a wide range of manual sampling methods but those are often not reported in sufficient detail, leading to a lack of standardization of sampling methods within the field. Additionally, due to the time and labor costs associated with manual sampling, the authors conclude that many floral samples capture only a fraction of the areas relevant to pollination and therefore may not be truly representative of the range of floral resources that can be accessed by pollinators. Remote sensing approaches can increase both the spatial and temporal efficiency of floral sampling as it allows for flowers or flower patches to be surveyed over areas covering several hundreds of meters (drones) to several thousands of kilometers (satellites) in only a few hours, facilitating coverage of large portions, if not all of, pollinators foraging and/or migration ranges. However, it is critical to differentiate between resources that are available to pollinators in the environment, which flowers pollinators actually exploit and how pollinators use distinct floral resources. Behavioral, physiological or foraging data on pollinators must be paired with remote sensing approaches that examine various aspects of floral resources that lie within an insect's flight range. In conclusion, selecting the sensors and imaging platforms most appropriate for capturing the spatio-temporal dynamics of floral resources depends on the ecological and behavioral questions of interest.

This review focuses on the little explored nexus of remote sensing and pollination studies, concentrating on a discussion of the various ways that remote sensing data can be used to characterize floral resources in the field for integration into pollination studies. We explore how different remote sensing platforms can be used to address three critical questions relevant to pollination science which are:

- How isolated are floral resources?
- What resources are available within a flower patch?
- How do floral patches change over time?

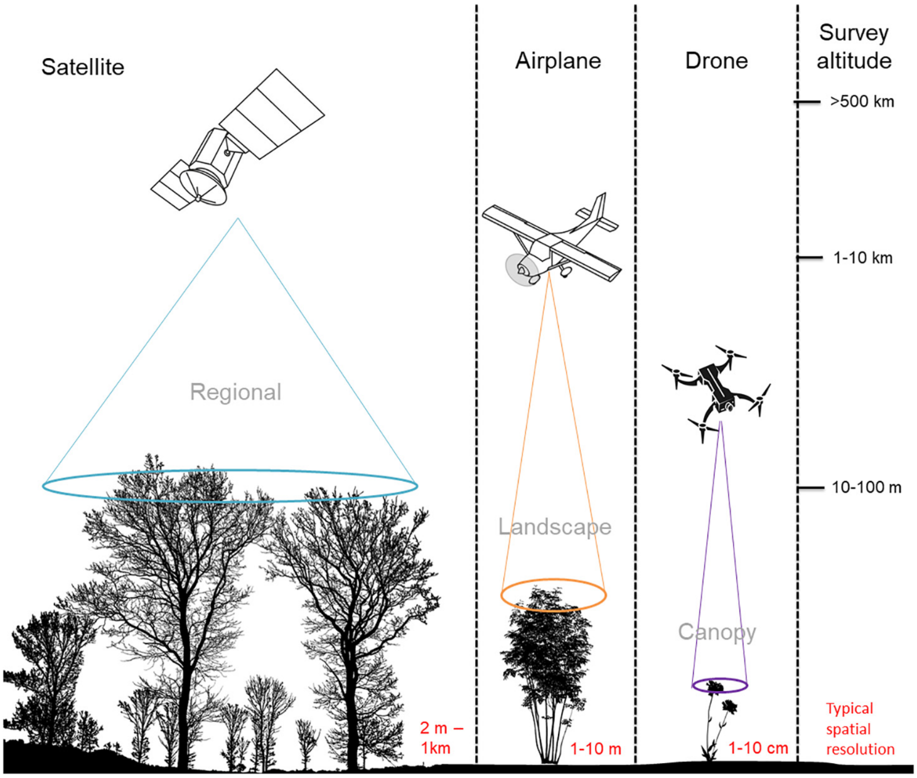


FIGURE 1 | The approximate operating altitudes of satellite-, airplane-, and drone-based imaging platforms. The corresponding fields of view used to capture vegetation from these different altitudes illustrate the extent of the landscape that can be viewed from a single image by sensors onboard each imaging platform.

Background: Remote Sensing Approaches for Landscape Ecological Assessment

The spatio-temporal distribution of flowering vegetation can be measured using a variety of sensors carried by satellites, airplanes and drones. To frame the technical methods presented in the rest of this paper, we provide some technical specifications of platform survey heights and resultant spatial data grains (**Figure 1**); temporal vs. spatial trade-offs (**Figure 2**) and details of combined platform-sensor capabilities with examples (**Table 2**). Sensors carried by these platforms may be passive, and measure sunlight reflected from the Earth or may be active, producing their own source of light that is subsequently reflected back and measured. Passive satellite systems such as those used on the NASA Landsat (30 m spatial resolution in the visible, near-infrared, and short-wave infrared portions of the light spectrum, i.e., VIS, NIR, SWIR—for a list of abbreviations and terminology used throughout this paper see **Table 3**) and Sentinel-2 (10–20 m spatial resolution in the VIS, NIR, SWIR) missions can capture landscape features at coarse and moderate spatial resolutions from a few spectral bands using multispectral sensors. However, commercial satellites such as Worldview-3 (1.24 m VIS and NIR, 3.7 m SWIR) are capable of generating fine spatial resolution datasets, allowing for more detailed explorations of vegetation features, albeit at relatively high financial cost. Additionally, hyperspectral datasets that sample the light spectrum across hundreds of narrow band-width channels can be generated from research satellites (e.g., Hyperion, Gaofen-5) and from hyperspectral sensors mounted on drones, but the mechanisms to understand how the complex hyperspectral metrics measured over moderate resolution pixels relate to ecological phenomena need further exploration (Roberts et al., 2011). Yet some efforts are ongoing to explore these complexities and find new information sources (e.g., 'Spectranomics' approach where spectral diversity might provide a proxy for ecosystem diversity – explored by Asner and Martin, 2016). In the context of vegetation studies, multispectral and hyperspectral data obtained from sensors mounted on satellites, airplanes and drones are often processed using vegetation indices (VIs), which are the reflectance ratios of two or more spectral bands (**Table 4**). Probably the most widely used VI is the normalized difference vegetation index (NDVI; Gao et al., 2020). NDVI ratios reflectance in the near infra-red (NIR) to the red portion of the visible spectrum because healthy plants tend to absorb red light and reflect it strongly from their spongy mesophyll layer in the NIR. This reflectance ratio can be used in a simple way to determine the probability that a given pixel represents vegetation on the ground, allowing researchers to build up a map of the spatial distribution of vegetation over a given area. It can also be used in a more complex sense to serve as a proxy for other vegetation parameters where the

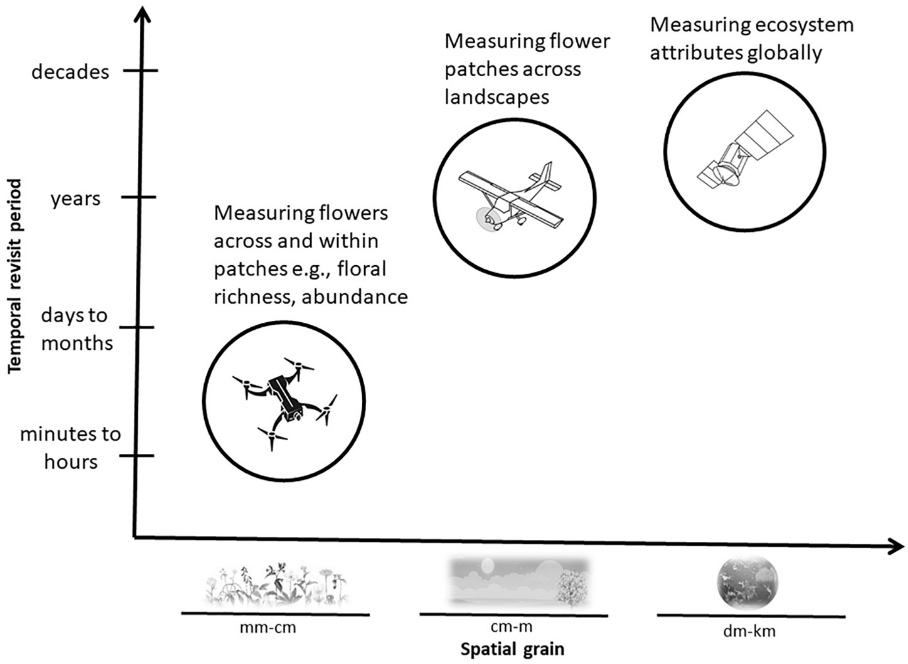


FIGURE 2 | Satellites, airplanes, and drones usually carry sensors that measure flowering vegetation at distinct temporal revisit periods and spatial grains. Typical vegetation features that can be efficiently measured by each of these remote sensing platforms are shown in relation to the temporal and spatial capabilities of each system.

TABLE 2 | Examples of the types of data acquired by satellite, airplane, and drone-based imaging platforms, their approximate range of spatial resolutions (at typical flight heights), extents, temporal resolutions and costs to the user.

Platform	Spectral data	Structural data	Typical range of spatial resolution	Maximum extent	Maximum temporal resolution	Cost
Satellite	-Multispectral -Hyperspectral (mostly from commercial satellites)	LiDAR Radar systems*	0.3 m–1 km**	Up to tens of thousands of km	2 days to 1 week+	-Data cost: Free (most research satellites), \$ 1- 40 + /km^{2} (commercial satellites)
Airplane	-Multispectral -RGB photographs -Hyperspectral	LiDAR	∼1 m	Up to a few thousand km	Unlimited	-Cost of chartering airplane: ∼ \$1,000- \$10,000/h -Sensor cost: ∼\$1,000–\$50,000
Drone	-Multispectral -RGB photographs -Hyperspectral	LiDAR Structure from motion photogrammetry	∼0.01 m	Up to ∼ 10 km	Unlimited	-Drone cost: ∼\$50-\$10,000 with inbuilt camera -Sensor cost: ∼ \$100 (Go-Pro)- \$50,000 (hyperspectral sensor)
Ground-based sensor	-Multispectral -RGB photographs -Hyperspectral	Terrestrial Laser Scanner (TLS) Structure from motion photogrammetry	∼0.01 m	Less than 1 km	Unlimited	-Sensor mount cost: ∼\$10 (stationary tripod)- \$1,000 (moving platform) -Sensor cost: ∼ \$100 (Go-Pro)- \$50,000 (hyperspectral sensor)

***Commercial satellites often have sensors with finer spatial resolutions than research satellites. *We limit discussion in this paper to LiDAR because radar systems deliver different structural information which is more complex to analyze.*

magnitude of NDVI is linked to vegetation features such as canopy structural parameters e.g., leaf area index (Roberts et al., 2011; Gao et al., 2020). Note that there are a great number of vegetation indices, all slightly varying in their algorithms, but many widely used for similar purposes (Elvidge and Chen, 1995; Viña et al., 2011; **Table 4**). Most published research using VIs to examine flowering vegetation come from agriculture (e.g., Fang et al., 2016; Wan et al., 2018; Yang et al., 2022) or conservation (e.g., Hunt and Williams, 2006; Tvostik et al., 2019) and use VIs to quantify flower number or patch size of the same floral species and do not differentiate between distinct flower types within the same patch. Such studies have reported differing accuracies depending on the flower type and VI used, for example, Wan et al. (2018) compared the accuracies of ten different VIs to quantify oilseed rape crops, and reported classification accuracies ranging between 0.61 and 0.91. There are

TABLE 3 | Abbreviations and terminology.

Abbreviations	
RGB	Red, green, blue
VI	Vegetation index
UV, VIS, NIR, SWIR	Ultraviolet spectrum: 300–400, visible spectrum: 400–700 nm, near-infrared spectrum: 780–1,400 nm, short-wave infrared spectrum: 1,400–3,000
NDVI	Normalized difference vegetation index
LiDAR	Light detection and ranging
TLS	Terrestrial laser scanner
FOV	Field of view of a camera
Terminology	
Multispectral imagery	Imagery produced using many different portions of the light spectrum, usually 3-10 distinct spectral bands. Bands are broader than those used in hyperspectral imagery, typically over 20 nm.
Hyperspectral imagery	Imagery produced using sensors which typically use hundreds of contiguous (overlapping), narrow spectral bands with the capability to produce a continuous spectrum. These usually operate across the visible, near-infra-red and short-wave-infrared regions of the spectrum. As hyperspectral sensors are heavier than multispectral sensors, they are usually deployed on platforms with high payload capacities.
Point cloud	A set of data points in space representing an object. Each point contains a set of Cartesian coordinates (X, Y, Z).
Structure from Motion (SfM)	A method for generating 3D point clouds describing object structures, based on overlapping 2D photographs.
photogrammetry	Photographs captured from distinct viewing angles are analysed using computer vision algorithms and processed via bundle adjustment to deliver information about the structural qualities of surface objects.
Discrete LiDAR	A remote sensing system where a laser emits a pulse of light that hits 3D objects and records only a few (typically 3–5) light returns from each laser pulse.
Waveform LiDAR	A remote sensing system where a laser emits a pulse of light that hits 3D objects and records a profile of light returns over time.

fewer studies examining floral diversity using vegetation indices, and these studies are tasked with maximizing the reflectance of different floral types from each other and against the background. While distinguishing floral species based on reflectance spectra is possible both manually and by using classification algorithms, this task is complicated when flowers are spectrally similar. Therefore, object-based analyses on RGB imagery are often employed for detection of individual floral species based on different floral features such as size and shape, although these methods can sometimes be computationally intensive, involving machine learning. Despite their relatively high computational demands, such studies have typically reported accuracies in distinguishing floral types ranging between around 0.70 to over 0.90 (Gogul and Kumar, 2017; Cibuk et al., 2019 Islam et al., 2020; Togacar et al., 2020) depending on the features extracted and the algorithms employed.

The structures of vegetation can be resolved through light detection and ranging (LiDAR) sensors mounted on ground-based ("terrestrial laser scanning," TLS), drone and airplane platforms. While LiDAR doesn't provide information on floral distribution (since most LiDARs record only structural rather than spectral parameters), it can be used to resolve landscape elevation and plant structures, allowing researchers to understand the spatial and volumetric distribution of floral patches when integrated with optical and infra-red products from different systems (Sellars and Jolls, 2007; Hosoi and Omasa, 2009; Wu et al., 2019). LiDAR emits pulses of light and captures backscatter from vegetation stored in point clouds to measure the height of objects within landscapes, with capabilities for describing the 2.5-D or 3-D representations of their structures. Discrete LiDAR systems provide information about the top of canopies and the underlying terrain allowing basic metrics of canopy height to be determined, but missing the detail of what lies between the tree tops and the ground (hence, "2.5-D"). Conversely, TLS can deliver 3D information by scanning canopies from the ground up; whilst waveform LiDAR can resolve full volumetric canopy structure by measuring how the emitted light interacts with vegetation between the treetops and the ground. There are now waveform LiDARs in the air (Hancock et al., 2017; Coops et al., 2021) and on the International Space Station (NASA GEDI mission). Additionally, LiDAR data can be used within models of radiative transfer to simulate processes of light penetration which can provide information about the amount of light reaching forest understories (Salas, 2020). New innovations in multispectral LiDAR might provide novel opportunities for pollination scientists because this potentially allows for both spectral and structural data to be captured.

At the landscape scale, data obtained from satellites can be used to analyze patches of floral resources at coarse- and moderate- grain, such that each pixel represents light reflected over an area of several dozen to hundreds of meters on the ground (e.g., Hofmann et al., 2011; Leong and Roderick, 2015; Beduschi et al., 2018). Compared to other remote sensing platforms, the use of satellite data is a relatively mature technique for assessing vegetation and is often used in landscape studies to map vegetation and flower patches. Although aerial imagery captured from airplanes can provide spatial information at moderate-grain, the use of this platform for mapping vegetation is comparatively rare in the pollination literature, likely due to the high financial and logistical costs associated with chartering airplanes to obtain data (**Table 2**, Willcox et al., 2018). Consequently, governmental organizations with relatively large amounts of funding typically charter airplanes to capture new geo-spatial data which may in some situations be made available to researchers and members of the public at little or no cost on a *post hoc* basis (e.g., in the UK the Environment Agency makes all airplane-acquired remote sensing data available to citizens through data.gov.uk).

Important platforms for use in field studies are consumer-grade fixed-wing and rotor-based drones (take-off weight totals less than 30 kg; Anderson and Gaston, 2013; Krishnasamy et al., 2019; Rominger et al., 2021). They are typically also equipped with GPS so that the captured images can be geo-referenced. Drones may be useful for capturing floral data relevant to pollination research as they can be operated over spatial areas that roughly match the distances covered by many foraging pollinators (Osborne et al., 2008; Danner et al., 2016), with maximum operating distances of up to 10 km, depending on the aircraft model, and limited by legislative restrictions (Duffy et al., 2018). Their proximity to the Earth's

TABLE 4 | Examples of vegetation indices that are expressed as ratios between different portions of the electromagnetic spectrum reflected by vegetation on earth's surface.

Vegetation index (VI)	Vegetation feature	Measurement	Formula	Example
Normalized difference vegetation index (NDVI)	The amount of green vegetation cover, used to measure biomass, vegetation density	Reflectance in the near-infrared (NIR) (800–2,500 nm) and red (620–750 nm) portions of the light spectrum	$\frac{NIR-Red}{(NIR+Red)}$	• Dixon et al., 2021 • Hofmann et al., 2011 • Many examples in the literature
Leaf-Area Index (LAI)	The amount of green leaf area per unit ground area, used to characterize forest canopies	Average leaf area (based on vegetation type), the ground area sampled	$\frac{Leaf\ area\ \left(m^2\right)}{Ground\ cover\ \left(m^2\right)}$	• Zarate-Valdez et al., 2012 • Sun et al., 2018 • Many examples in the literature
Enhanced Bloom Index (EBI)	Flowering intensity, originally used to characterize almond flowers	Reflectance in the red (620–750 nm), green (500–565 nm), blue (450–485 nm)	$\frac{Red+Green+Blue}{(\frac{Green}{Blue})*(Red-Blue+1)}$	• Developed by Chen et al., 2019 • Dixon et al., 2021
Photochemical reflectance index (PRI)	An indicator of photosynthetic radiation use by plants, related to light use efficiency and plant health and function	Reflectance at 531 nm, reflectance at 570 nm	$(R_{531}-R_{570})/(R_{531}-R_{570})$	• Developed by Gamon et al., 1997 • Chen et al., 2020 • Many examples in the literature
Hyperspectral image-based vegetation index (HSVI)	The amount of green vegetation in urban areas, reduces the effects of shadows from buildings, the saturation due to the clustered nature of urban vegetation, and the effects of novel materials present in cities.	Reflectance at 760, 689, 861, 889, and 520 nm.	$\frac{\left[\left(2^{p760}-1\right)-p_{689}+a*(p_{861}-p_{889})\right]}{(p_{520}+p_{689})}$ Where p_{760}, p_{689}, p_{861}, p_{889}, *and* p_{520} represent spectral bands (i.e., reflectance) with 760, 689, 861, 889, and 520 nm, respectively.	• Developed by Sun et al., 2021

surface means that the grain of such observations has capacity to capture flower-scale information (e.g., Wan et al., 2018; Xavier et al., 2018; Chen et al., 2019; López-Granados et al., 2019). Furthermore, drones with high-quality inbuilt cameras are becoming more affordable (£500–£2000) for the everyday field ecologist, and there is the possibility to further modify these with customized sensors. Drones are particularly useful for monitoring pollinator resources as they can be deployed at shorter intervals compared to satellites, their measurements are less affected by cloud cover, their viewing angle can be adjusted in real-time and they can be used to capture high spatial resolution imagery in remote or inaccessible terrains (Landmann et al., 2015; López-Granados et al., 2019; Smigaj and Gaulton, 2021). Drones have also emerged as useful platforms for acquiring data describing volumetric vegetation structure – as an alternative to LiDAR. To do so requires a Structure from Motion (SfM) photogrammetry workflow to be applied to overlapping aerial photographs (examples can be found in Cunliffe et al., 2016; López-Granados et al., 2019; Smigaj and Gaulton, 2021). The workflow requires a drone with standard camera, plus optional ground control equipment. If validation is required this includes ground control targets and high accuracy geographic positioning system (GPS). A computer vision algorithm uses a bundle adjustment approach to determine 2.5D vegetation structures and stores this information in point clouds. Critical to the successful application of this method is the capture of images from distinct viewing angles and high convergence of images—i.e. high overlap in front and lateral extents, so that features with structure exhibit image parallax which can be modeled to generate a virtual rendering of their relative height and volume. This technique was facilitated by the use of drones and pioneered in geosciences for the measurement of stable structures (Jackson et al., 2020). It has also recently been applied successfully to vegetation for measuring crop height in agricultural fields (Holman et al., 2016), generating biomass estimates in drylands (Cunliffe et al., 2016), or for measuring forest structures with equivalent accuracies to LiDAR (Filippelli et al., 2019). Acquisition of such volumetric information can be achieved with very low hardware costs. This is relevant for pollination ecologists because such data can be combined with spectral information on floral resources to deliver information about the structural properties of foraging patches (e.g., ground elevation, tree/hedge height, building height) which could affect habitat use, microclimate (Duffy et al., 2021), and thus timing of flowering and navigational strategies employed by pollinators.

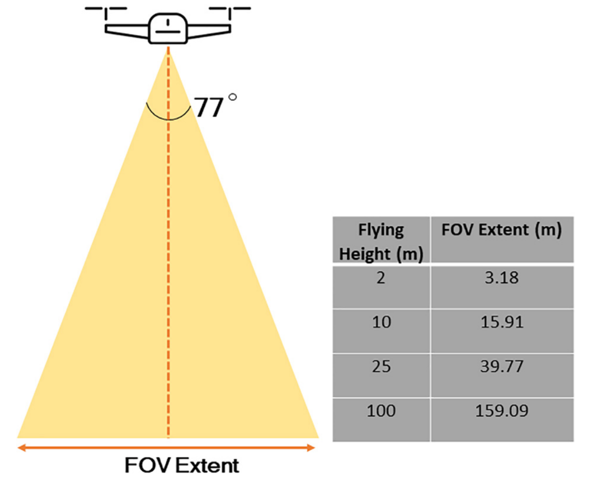
In addition to satellites, piloted aircrafts, and drones, ground-based sensors are frequently used to monitor the health and growth of individual plants. However, they capture information at very fine spatial scales (e.g., 3.5° field of view), which is relatively inefficient for assessing an entire floral patch or for capturing flowering patches across a landscape at all spatial scales relevant to pollinators' foraging behaviors. Although a discussion of ground-based sensors is omitted from this review for the aforementioned reasons, these approaches are widely used for monitoring individual crops in agricultural research (Hong et al., 2007; Raper et al., 2013; Quemada et al., 2014), and we acknowledge that data from close-range spectroscopy could also provide useful insights for discriminating different flowers based on spectral signatures, that could benefit (*via* calibration/validation) some of the other techniques discussed herein.

Remote sensing techniques exploiting drone, airplane and satellite-borne sensors are therefore mature with respect to generalized landscape ecology applications, but there is limited research that diverts these approaches toward characterization of floristic resources. The next sections will discuss the capabilities of state-of-the-art remote sensing techniques that can be used to examine relationships between pollinators and changing foraging landscapes. Furthermore, discussion is warranted on the level to which remote sensing approaches can deliver new insights into how future climate change and intensification of landscape management may affect pollination services in particular landscape types.

HOW ISOLATED ARE FLORAL RESOURCES?

Landscape fragmentation can isolate flowers in time and space. For example, as natural grasslands containing diverse floral resources are modified to create fields for agriculture or for human recreation, flowers offering preferred and valuable nutritional resources to pollinators can become separated from each other and from nesting or egg-laying areas within the landscape. As a result of this fragmentation and landscape homogenization, pollinators may be required to travel further on foraging trips to obtain a sufficient quantity and diversity of floral resources to sustain their survival and reproduction. Small-sized pollinators with smaller flight ranges, such as solitary bees, (Greenleaf et al., 2007; Garibaldi et al., 2011; Frasnelli et al., 2021), or insects with specialized diets (Winfree et al., 2007; Bommarco et al., 2010; Woodard and Jha, 2017), differ in their foraging strategies and choices and may particularly struggle to obtain food resources in highly fragmented habitats compared to large-sized, generalist pollinators that can travel farther to obtain food, such as bumblebees and honeybees (Gibb and Hochuli, 2002; Memmott et al., 2007; Martinson and Fagan, 2014; Wilson et al., 2021). However, even pollinators capable of covering large distances on foraging trips may face fitness consequences in highly fragmented and resource-poor environments, including reduced population, forager and colony sizes and changes to their foraging behaviors in the field. The effects of landscape composition on pollinators are particularly relevant due to the rapid expansion of agricultural activities and urbanization over the past 200 years, which have driven and accelerated fragmentation in many landscapes, such as forests and grasslands (Ghazoul, 2005; Libran-Embid et al., 2021).

However, how and along which spatial scales landscape composition affects the fitness and foraging behaviors of insect pollinators remains a largely open question. Remote sensing can provide new tools to map floral resources using multispectral imaging platforms that deliver data at distinct spatial grain. Research satellites are the most common platforms from which data on floral resources can be acquired. However, as discussed previously - most free-to-use data from public research satellites (e.g., Landsat, MODIS) deliver only relatively coarse spatial resolution data ranging from tens to hundreds of meters per pixel. Despite this, such data can have sufficient spatial resolution to identify patches of dense flowering vegetation found in meadows, urban parks, and mass flowering agricultural crops (Beduschi et al., 2018; Lane et al., 2020; Kowe et al., 2021), although it is not possible to distinguish between individual floral species within a single patch using such coarse-grain data.



Flying Height (m)	FOV Extent (m)
2	3.18
10	15.91
25	39.77
100	159.09

FIGURE 3 | The relationship between flying height and extent of image. As an example, the DJI Mavic 2 Pro, a relatively commonly used drone for capturing spatial information in scientific research, and the camera has a field of view of 77° and an image size in pixels of 5472 × 3468.

In contrast, there are commercial satellite products available which can deliver moderate- and fine- grain data (e.g., Geo-Eye1 multispectral resolution of 1.65 m, Worldview-3 multispectral resolution of 0.30 m), but the spatial resolution of these data would still be insufficient to resolve individual flower heads or inflorescences. The relatively high financial costs of purchasing such data have led to relatively few pollination studies employing commercial satellite data to study foraging landscapes (for examples see Beduschi et al., 2018; Chen et al., 2019). Additionally, satellite imagery can be used to identify distinct vegetation patches, such as densely forested areas, or cropped fields, that may serve as nesting and egg-laying habitats to pollinators or barriers to their dispersal. One of many examples in which research satellite data was used to characterize fragmentation of vegetation in pollinators' foraging habitats has examined how distinct types of landscape patches affected pollinators' dispersal from the nest site (Tscheulin et al., 2011). In this study, the authors obtained freely available satellite data (Landsat Thematic Mapper 5) and used algorithms to classify landscape patches surrounding their sampling sites. This information was paired with data on the proportions of differently sized pollinators along a transect. Therefore, it was possible to relate landscape composition surrounding the study sites to habitat use and the dispersal abilities of differently sized bee species, leading to observed differences in their spatial distributions across the landscape. Combining objective measures of spatial information at the landscape scale from remote sensing with data on pollinator abundance, size, diets, and navigation can lead to new insights into how landscape composition affects pollinators and pollination services.

One way to acquire scale-appropriate data describing detailed spatial variations in floral imagery is to obtain it from close range using low-flying drones (**Figure 3**), or in some cases, airplane-mounted sensors. The type of spatial information captured from airplane platforms may be particularly useful

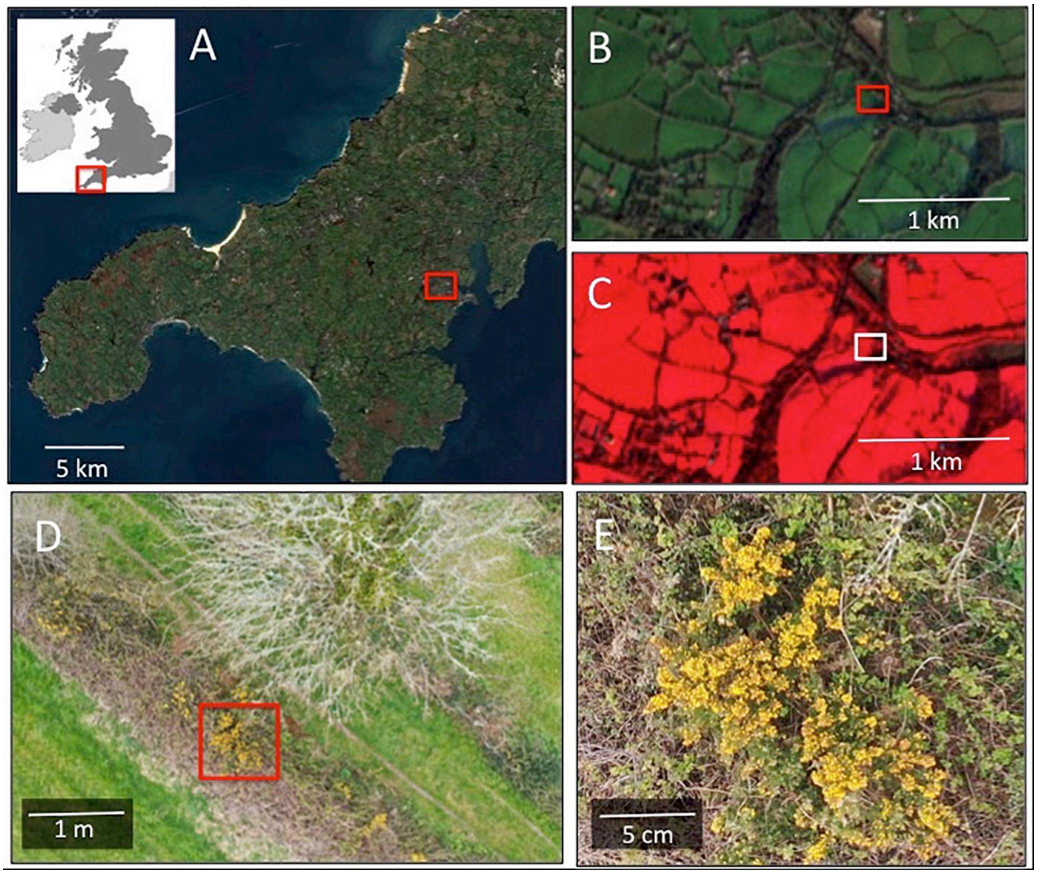


FIGURE 4 | Scale-varying views of flower-scapes from satellites to drones. **(A)** Shows a regional perspective imaged in optical (RGB) wavelengths from the Sentinel-2 satellite at 10 m spatial resolution. **(B)** Is a local perspective of the highlighted region in **(A)**, also at 10 m spatial resolution showing a neighborhood view comprising a mixed agricultural and residential area with various floristic resources including private gardens, woodlands and hedgerows. **(C)** Shows the same area as **(B)** but viewed using a false color composite (Bands 8, 4, 3). **(D)** Is a drone captured image from the region highlighted in **(B,C)** in optical wavelengths (RGB) acquired from a survey height of 25 m during spring budburst (April 2020). Gorse (*Ulex europaeus*; yellow) and blackthorn (*Prunus spinosa*; white) flowers can be seen in the hedgerow. The spatial resolution of this image is finer than 1 cm. **(E)** Is a highlight of the image shown in **(D)**, over the area highlighted showing in detail a bunch of gorse flowers.

to surveying floral species that are highly dispersed, enabling the capture of small patches of floral resources scattered across hundreds of km (Carson et al., 1995). Carson et al. (1995) for instance, investigated the abundance of hawkweed flowers in experimental plots using multispectral imagery (1 m spatial resolution blue-green, green, 2 bands in NIR) captured from an airplane. As this floral species occurs at low densities in nature, small patches of hawkweed comprised of only a few individual flowers (of 2–3 cm diameter approximately) would likely be impossible to distinguish from the surrounding vegetation using coarse spatial resolution data provided by satellite platforms such as Landsat.

From a drone perspective, the grain of data is even finer compared to those captured by airplanes – with a capability to measure individual flowers at sub-centimetric spatial resolution, depending on flying altitude, though there is a trade-off between spatial resolution and image extent (**Figure 3**). We therefore assert that it is particularly the fine-grained nature of drone data that lends itself strongly to the analysis of abundance and distribution of floral resources within a foraging patch, e.g., within forest, grassland, gardens, crop fields, and hedgerows (**Figure 4**). This information could be paired with data on the movements, foraging preferences, and individual and colony sizes of pollinators to provide insights on how changes to floral abundance along fine spatial scales affect pollinators (Woodard and Jha, 2017; Kremen et al., 2018; Lázaro and Tur, 2018). Fine-scale spatial data acquired by drones could be especially critical to facilitating investigations into habitat use by ecologically specialized pollinators and the effects of habitat fragmentation on their populations (Winfree et al., 2011). Although this approach remains novel within the field of pollination research, drones are increasingly used to study flowering plants within the agricultural sector, and this approach has been used in some conservation studies to map suitable habitats for animals threatened by habitat loss (Mangewa et al., 2019). Habel et al. (2018), for example, used a small commercial-grade multirotor platform (DJI Phantom 3, inbuilt RGB camera and CMOS Red + NIR sensor) to identify suitable habitats for three butterfly species that display varying degrees of diet specialization within a 45-ha managed grassland site. They found that high quality vegetation for the two specialized butterfly species were spatially restricted to a greater degree than for the generalist species, supporting the idea that habitat loss may have greater consequences for specialized species that rely on spatially restricted patches of vegetation for survival. Furthermore, the drone-obtained data showed that high quality vegetation for all three species were more frequently located in the verges of paths and roads than in open grassland, which may serve as important food resources for pollinators in urban and in agricultural landscapes.

In agricultural biology, data obtained from drones has been critical to assessing the growth of individual crops and predicting their yields (Wan et al., 2018; Chen et al., 2019; López-Granados et al., 2019). Fine-grain data acquired by drones could also be important to studying the efficacy of alternative agricultural practices, such as those seeking to enhance crop yields by providing a mix of diverse floral resources to attract pollinators. These studies could be carried out using relatively simple-to-use and cheap consumer-grade technologies as evidenced by Xavier et al. (2018), who used a drone (Solo-3DR) and GoPro Hero 4 RGB camera (12-megapixel photos) to capture spatial data on floral resources. The study assessed the suitability of wildflower strips in attracting pollinators in two distinct types of environments: one in which the wildflower strips were adjacent to woodland, and another in which the wildflower strips were adjacent to agricultural crops. The researchers quantified individual wildflower species arranged in mixed 34 m × 10 m plots and sampled pollinator abundance within these plots. They found that the abundance and cover of floral resources was positively correlated with the number of pollinator visits at both sites, with an increase of just 0.12 m^2 in floral cover resulting in 6–8 additional pollinator visits.

WHAT RESOURCES ARE AVAILABLE WITHIN FLORAL PATCHES?

An important factor in the ability of landscapes to sustain pollinator populations is the quality of foraging patches within the landscape. Not all foraging patches are equal by virtue of their variable floristic composition that can provide different resources to pollinators. Floral abundance and floral richness are two established metrics by which the quality of foraging patches is typically assessed in landscape ecology. These metrics correlate broadly with higher pollinator visitation rates (Hegland and Boeke, 2006; Lázaro et al., 2020). However, this comes often at a cost of resolving the differences between different pollinators, their movements and behaviors and their role in intricate plant-pollinator networks (Lima et al., 2020; Ropars et al., 2020; Chakraborty et al., 2021). To facilitate more integrated empirical and modeling approaches, such as combining remote sensing data with network data and behavioral observations, it is useful to differentiate between floral species across patches. Remote-sensing techniques are best placed to capture spatial data at fine-grain over sufficiently large areas for quantifying different floral resources at ecologically relevant spatial scales. By segregating flowers in the images from the surrounding vegetation on the basis of their spectral reflectance profiles, novel information on the richness and abundance of floral resources within flower patches can be obtained.

So far, most studies that have used remote sensing to quantify flower number within foraging patches have come from precision agriculture, where flowering phenology and abundance have been used to predict crop yields (Wan et al., 2018; Chen et al., 2019; López-Granados et al., 2019). However, the quantification of floral species is also important for comparing the quantity and diversity of resources provided to pollinators by different types of floral patches, from fields of monocrops to patches of natural grassland and urban green spaces (Vrdoljak et al., 2016; Kremen et al., 2018; Ziaje et al., 2018 Prado et al., 2021; Tew et al., 2021). To detect individual flowers at fine-grain using remote sensing, it is possible to utilize imagery from drones flown at low altitudes. Spectral data obtained through these remote sensing platforms are often processed using specialized VIs that have been developed to track small differences in the spectral reflectance of flowers across distinct color channels.

Using these VIs, the availability and development of distinct floral species can be precisely quantified and subsequently related to the quantity and quality of floral resources available within a foraging patch. Chen et al. (2019), for example, developed an enhanced bloom index (EBI) using multispectral data for quantifying the bloom intensity of individual almond flowers grown in orchards ranging in size between 21.5 and 222.5 hectares. EBI compared floral brightness in red, blue, and green channels against the low reflectivity of soil background reflectance in the red and blue channels and high reflectivity of leaves in the green channel (equation [1]):

$$\mathbf{EBI} = \frac{\mathbf{brighness}}{\mathbf{greenness} \times \mathbf{soil\ signature}} = \frac{\mathbf{R + G + B}}{\frac{\mathbf{G}}{\mathbf{B}} \times (\mathbf{R - B + \varepsilon})} \tag{1}$$

Where R, B, and G represent reflectance in the red, blue and green channels and ε represents an adjusting constant.

Furthermore, optical images (R,G,B) acquired by standard cameras on drones can be processed using SfM photogrammetry workflows to generate 3D models of vegetation patches. These models can help researchers quantify flowers in structurally complex floral patches which would be beyond the visual reach of a ground-based observer, such as in flowering tree canopies and along hedgerows, which can serve as important foraging resources for pollinators (Carl et al., 2017; Smigaj and Gaulton, 2021; **Figure 2**). This technique allows reconstruction of the structure and distribution of floral patches for investigating relationships with pollinator fitness, abundance, and behaviors. Carl et al. (2017), for instance, used a MAPIR Survey2 RGB camera (6.17472 mm × 4.63104 mm FOV, focal distance of 3.97 mm) mounted on a hexacopter drone to obtain RGB imagery of black locust tree (*Robinia pseudoacacia*) flowers, which serve as an important food resource to honeybees. This allowed the crown structure to be resolved, while flowers were distinguished from the surrounding vegetation using reflectance intensity in the blue channel. The precise quantification of flowers across the trees' 3D structures allowed the researchers to make predictions about the population of honeybees that the landscape could support. Similarly, Smigaj and Gaulton (2021) captured overlapping multi-spectral and RGB images using two different drones (DJI Matrice 600 with Micasense RedEdge-M multispectral camera, RGB, near red, NIR and DJI Phantom 4 Pro inbuilt RGB/NIR camera) to generate SfM-based models of hedgerow structures in an agricultural setting. The researchers were able to quantify and compare floral abundance within different hedgerow types using reflectance data in the red, green, blue and NIR channels, providing information on the quantity and quality of resources available to pollinators.

HOW DO FLORAL PATCHES CHANGE OVER TIME?

The synchronization of flowering and pollinator emergence times are critical to sustaining plant-pollinator relationships. The availability of flowers within a foraging patch is dynamic across time and is affected by change in micro- and macroclimatic conditions and the spatial heterogeneity of the landscape, with differences in light exposure which leads to changes in the growth and flowering phenology of plants (Alsanius et al., 2017; Ogilvie and Forrest, 2017; Swierszcz et al., 2019). These effects are easily illustrated in urban environments, where plants grow in small, heterogenous patches that are often located near anthropogenic structures such as roads and buildings, which create a variety of microclimatic conditions, resulting in differences in the growth and abundance of floral species (Habel et al., 2018; Jung et al., 2020). As such, some pollinators in highly fragmented urban environments may face favorable conditions for foraging on diverse flowers (Rollings and Goulson, 2019; Ropars et al., 2019; Staab et al., 2020; Tew et al., 2021). However, across broad spatial scales, many natural landscapes which previously would have contained diverse floral resources have been homogenized into agricultural monocrops and urban lawns. Therefore, the limited floral diversity available to pollinators may also limit floral availability across the foraging season, potentially leading to dips in floral resource availability for pollinators at various times across the season.

Across broader spatial scales, changes in local environmental conditions driven by climate change have altered many species' temporal distributions and the onset, peak and durations of flowering periods, changing the distribution of floral resources at given points in time (Chen et al., 2019). These environmental changes often have uneven effects on plants and pollinator populations, leading to "bottom-up" effects, limiting the food supply of pollinators, or "top–down" effects, limiting the reproductive potential of plants (Hegland et al., 2009). The flexibility of these relationships also remains unclear as pollinators may avoid the negative consequences of shifts in flowering emergence times depending on their ability to exploit a range of floral resources in the environment.

Answering questions about timing of phenological phenomena from remote sensing data requires, beyond all else, regular repeat survey coverage (**Figure 2**). The frequency with which flowers can be surveyed depends on the remote sensing platform utilized. The temporal resolution of satellite datasets are pre-determined by the satellite's orbital period and are typically used to examine vegetation along coarse and moderate temporal scales. Additionally, there are trade-offs between enhanced spatial and temporal resolution, as satellites in low orbit that provide fine spatial resolution data have longer re-visit periods. By comparison, airplane and drone pilots have much greater control over the temporal resolution of the data collected. Airplanes and drones can be deployed according to the user's demands (albeit at substantially higher costs with airplanes), allowing for very fine temporal resolution data to be collected. The ability to choose when an aircraft is deployed additionally allows researchers to select when floral surveys are carried out and select the optimal lighting conditions to maximize the visibility of flowers against the background. The latter is important to distinguishing flowers from green vegetation in photographs taken by RGB cameras mounted on drones flown at several meters in altitude (**Figure 5**). This is particularly relevant to drone-acquired imagery as many recreational drones carry inbuilt RGB cameras that could facilitate the capture of floral resources at low financial cost and with little need for further processing, increasing the accessibility of these technologies to scientists and conservationists with limited research funding.

Data obtained from satellites have been used to characterize temporal changes to flower patches over broad areas of landscape (e.g., hundreds to thousands of kilometers), and particularly in agricultural research to study changes to patches of mass flowering crops across various time scales (e.g., weeks, years, decades) (Zhang et al., 2003; Leong and Roderick, 2015; Dixon et al., 2021). Changes to mass flowering crops may be important to studying pollinator populations in agricultural environments, as crop fields dense in flowering plants may become deserts to pollinators within short periods of time. If such agricultural fields occupy extensive areas, the landscape may be unsuitable for sustaining pollinators in the long-term (Kovács-Hostyánszki et al., 2017). A growing number of studies are using satellite data to track changes in flowering phenology across landscapes in relation to the abundance or emergence times of pollinators. Leong and Roderick (2015), for example, analyzed changes in vegetation within natural and human-altered grassland landscapes in California. The researchers used spectral data obtained from MODIS to examine changes in normalized vegetation index (NDVI) and enhanced vegetation index (EVI) across agricultural, urban and natural landscapes comprising a 50 × 50 km region. They observed different trends in phenology across the three landscape types and corresponding differences in bee abundance over the 14-year (2000–2014) study period. Therefore, when paired with data on the richness, abundance and behaviors of distinct pollinator types, temporal data obtained from satellites can help uncover relationships between the availability of flower patches over time and the types of pollinators or pollination services that the landscape can support.

FIGURE 5 | The visibility of flowering bindweed (*Convolvulus*) when imaged under varying natural light intensities at approximately the same location **(A)** 9:30 (light intensity: 1792 lum/ft^2), **(B)** 12:00 (light intensity: 4096 lum/ft^2), and **(C)** 17:30 (light intensity: 2688 lum/ft^2) using an RBG camera (DJI Mavic Mini inbuilt camera) on October 9th, 2021. Light intensity was measured using a HOBO Pendant temperature/light data logger (Onset, Bourne, MA, United States). The ability to discriminate the flowers from the background can be impaired under some viewing and lighting conditions.

Drone data could be useful for examining how floral patches change in terms of their reward availability to pollinators (for a review see Bloch et al., 2017). Drones could be particularly useful to studying changes to flower patches over fine temporal scales (Neumann et al., 2019) especially when the floral species or its pollinators are only available for short periods of time (Xu and Servedio, 2021), and to track the development of individual flowers in patches containing mixed floral species. Shifts in flowering time within mixed floral patches may not be apparent when the whole patch is studied as a single unit. Investigations into the flowering dynamics of distinct floral species may be critical to understanding the use of the foraging patch as a food resource to different pollinator species over time (Simba et al., 2018; Kehrbergerand Holzschuh, 2019).

Changes to patches of mixed floral species can be measured using a variety of sensors, from consumer-grade RGB cameras to hyperspectral scanners, depending on a variety of environmental and study-specific factors, such as the reflectance spectra of the flowers studied and the lighting conditions under which the data are acquired. Few studies have attempted to investigate flowering in such heterogeneous environments at such fine spatial grain as it is difficult to obtain accurate floral counts due to the spectral complexity of the reflectance from the ground. Landmann et al. (2015), for example, used a hyperspectral remote sensor, the AISA/Eagle pushbroom scanner that produces images in 64 spectral bands (max spectral resolution of 2.9 nm), mounted on a drone to track the spatial availability of floral resources at a 100 km^2 study site in the African savannah. This hyperspectral dataset allowed the researchers to detect different flower types against the background. In addition to tracking shifts in the spatial distribution of floral resources over this time period, the researchers found an overall increase in floral resources from 2013 to 2014. This example shows how drones can help researchers detect small-scale shifts in floral resource availability over long periods for a better understanding of the consequences for the pollinator populations present within it. Furthermore, there is an increasing demand for information on how changes to floral resources affect pollinator populations, which are vital to the production of many human food crops. For example, actions by government agencies, such as the European Commission's EU Pollinators Initiative calls for research investigating the effects of climate and landscape change on pollinators and their food resources. Remote sensing approaches could contribute to increasing the scale, efficiency, and quantity of information required for such research.

DISCUSSION

Remote sensing offers unparalleled means of measuring flowering vegetation at various grains, extents and timescales. Alterations to floral resources can have long-lasting effects on pollinator fitness, abundance and behaviors over a range of spatial and temporal scales (Hegland et al., 2009; Ogilvie and Forrest, 2017) and can include changes to populations, forager sizes, and colony sizes, as well as shifts to their foraging strategies. The ability to use remote sensing data to rapidly and accurately acquire spatial information pertaining to pollinators' foraging habitats and their floral richness and abundance could vastly improve understanding of how landscapes affect insect pollinators and their populations. Remote sensing approaches can facilitate the capture of large portions or the entirety of pollinators' foraging or migration ranges, which may allow for more spatially representative sampling of floral resources and can reduce the time and labor costs associated with manual sampling. Pollinating insects vary widely in how far they travel to forage or during migrations, with small-sized pollinators typically traveling shorter distances up to 1.4 km (Nicholls et al., 2022). Larger-sized, generalist pollinators travel further (e.g., social bees typically travel 1–2 km while the bumblebee-tailed bumblebee and Western honeybee can travel 12–15 km), while some species of butterfly can travel hundreds to thousands of kilometers during migrations (e.g., Chowdhury et al., 2020). Additionally, the required sampling timescales vary depending on individual or colony lifespans. Therefore, both flight range and lifespan of an individual or colony will determine which remote sensing platform is most appropriate for capturing information pertaining to their food, nesting and mating resources. While a variety of manual methods exist for tracking insect movements in the field (see Kissling et al., 2014; Montgomery et al., 2021) using existing technologies for tracking individual insect movements is particularly challenging given their small body sizes paired with quick movements, leading to rapidly changing backgrounds against which they must be detected. Therefore, the objective is to pair remote sensing approaches with novel or traditional means of quantifying insect movements or behaviors in the field. Also, remote sensing approaches can provide an efficient and potentially accurate means for understanding the landscape context in which behaviors occur. Additionally, both natural and human-made structures have the potential to affect pollinator dispersal and habitat use, facilitating or reducing access to flowers across landscapes or changing microclimatic conditions (Morandin and Kremen, 2013; Habel et al., 2018). This is particularly relevant due to the rapid development of many rural habitats into agricultural fields or urban environments both in the UK and globally (Haddad et al., 2015; Wenzel et al., 2020). Not only can remote sensors capture changes to the spectral signatures of flowering vegetation over time, but changes to the volumetric characteristics of a landscape can be resolved using LiDAR sensors carried by drones or airplanes, or SfM photogrammetry, which generates models of 3D structures using overlapping images. However, the remote sensing platforms, sensors and data types utilized to measure floral resources must facilitate studying pollination at the spatial and temporal scales of interest.

Using remote sensing to measure floral resources *in situ* remains relatively novel within the field of pollination research. Most existing studies have used coarse-grain satellite imagery to examine one or a few species of mass flowering crops over large spatial areas and relate broad measures and categorization of floral availability (e.g., field, hedge, forest, urban green space coverage) to the abundance, physiology and behaviors of insect pollinators. The relative popularity of using coarse-grain satellite imagery in pollination research can be explained by the low (often free) cost of satellite imagery, the amount of publicly available information on how to process satellite data, prior knowledge of the coloration of the flowers under study and the narrow spectral variation within flower patches containing a single species, all of which makes it easy to broadly outline areas of floral growth. However, this information typically does not tell us about differences in abundance and flowering characteristics between individual plants, the availability of wild flowers and other sparsely distributed flowers across the landscape, or the floral diversity and volumetric character of foraging patches.

Although relatively novel within the field of pollination research, drones can capture imagery along very fine spatial scales, such that individual flowers can be detected and quantified. The use of drone imagery to quantify the spatial distributions and timing of distinct floral species could facilitate studies examining how pollinators interact with different floral types and vegetation structures along fine spatial scales in the field and their reliance on particular food resources for survival. Additionally, combining drone imagery with behavioral observations of insect pollinators could show how gradual shifts in flower number, species richness and flowering onset influence their populations from year-to-year or across decades and could be critical to modeling habitat requirements for different pollinators and identifying areas of suitable habitat across a landscape. Furthermore, drones can be deployed according to the researcher's demands, allowing the researcher to select the optimal environmental conditions under which to capture floral imagery. The ability to control when and how often flowers are sampled also facilitates repeated sampling along very fine temporal scales, whereas existing data acquired by airplanes and satellites have fixed temporal resolutions. However, there are trade-offs between spatial resolution and the extent of landscape that can be covered. Drones, for instance, can provide spatial data at very fine grain and cover much larger areas than traditional methods, such as transect measurements. However, they are inefficient for capturing vast areas of landscape in comparison to satellites and airplanes.

Sensors carried on-board remote sensing platforms are critical to defining what flower types and floral features can be analyzed. In agricultural research, the interest is focused on specific crops, and flower colors are therefore known for selecting appropriate sensors to maximize the visibility of the flowers against the background. However, in natural habitats and in urban environments, where differently colored flowers frequently exist in mixed distributions, selecting the appropriate sensors to capture flowering dynamics within the patch would be more difficult. One solution is to use hyperspectral sensors capable of simultaneously sampling multiple wavelengths of light and develop vegetation indices that maximize the visibility of distinct floral species against the background (Landmann et al., 2015). Although using hyperspectral remote sensing to study vegetation is relatively novel compared to the use of multispectral remote sensing, advancements in this field may be critical to capturing the health and flowering dynamics of different species at fine spatial and temporal grain. This may facilitate detecting changes in flowering over short periods of time and corresponding shifts to floral rewards, which can influence pollinators' foraging decisions (Ito et al., 2021). Furthermore, some studies have used hyperspectral datasets to establish relationships between species biodiversity and spectral diversity. The Spectral Variability Hypothesis, for example, argues that the reflectance spectra of vegetation are related to plant biodiversity (Palmer et al., 2000, 2002). Therefore, advances in hyperspectral remote sensing may help uncover relationships between the spectral diversity of a given area and its ability to sustain pollinator populations, and could be a ripe area for future exploration by pollination ecologists.

Another means of capturing floral richness at fine-grain is to use multispectral sensors or RGB cameras mounted on drones flown at low altitudes to capture floral imagery. While this option can be relatively cost-efficient and simple to deploy, the image quality can be greatly affected by environmental conditions in the field, which could potentially make it difficult to resolve flowers against the background.

One important barrier to using drones in ecological fieldwork is the increased adoption of legislation limiting the use of drones over public and private land and airspace. As the debates over who has the right to use airspace over privately- and government-owned lands intensify, the potential benefits to using drones for assessing landscape change are often overlooked. The conflicts over the use of airspace come at a time when recent developments in drones, sensors, and processing software have revolutionized researchers' abilities to analyze the spectral and structural properties of vegetation along fine spatial scales. Simultaneously, the increase in cost-effective consumer-grade drones and cameras could allow for their widespread use in pollination research. The costs of drones can be further reduced by using DIY drone kits or by altering existing pieces of hardware to capture floral resources[1], making them accessible to researchers on low budgets (Moudrý et al., 2019). Along with reductions in cost, the rise in the number of hobbyists operating drones has allowed online communities to flourish. Such communities may stimulate innovation and make operating drones increasingly accessible to beginner pilots.

CONCLUSION

Across the globe, anthropogenic activities are accelerating changes to landscapes in which pollinating insects forage. These changes can affect the size, shape and number of foraging patches in the landscape and the quantity and quality of floral resources they contain. Spatial and temporal changes to floral resources may have diverse consequences for pollinator behaviors, population sizes, reproductive strategies, and pollination services. However, the extent to which changes to floral resources affect different types of pollinating insects and along which spatial and temporal scales remains relatively unknown. Traditional methods for floral sampling, such as manually counting flowers using transects and quadrats can be difficult to use along diverse spatial and temporal scales, as they can be laborious, time-consuming, limited by floral features that are detectable by the human eye and may not accurately reflect floral distributions present across a pollinator's entire foraging or migration range. However, drones and satellites are well-suited to capturing changes in the richness and abundance of floral resources through RGB images, spectral, and structural data. Additionally, many of the methods for capturing and processing drone and satellite data established in geographical and computer sciences have now become streamlined, simplified and reduced in cost. This has made them much more accessible to ecologists and conservationists who often operate on small research budgets and may not have specialist knowledge of remote sensing technologies. This trend is exemplified by the recent widespread availability of consumer-grade drones, free or cost-effective apps for piloting drones, recreational drone use and online communities aimed at improving drone hardware and software. Satellites, airplanes and drones can be used to generate datasets on floral resources and can revolutionize our understanding of the spatial and temporal scales along which pollinator species are affected by environmental change.

AUTHOR CONTRIBUTIONS

DG drafted the manuscript. All authors conceived the project, contributed to writing, and approved the final version.

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[1] http://diydrones.com/

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A Spark in the Dark: Uncovering Natural Activity Patterns of Mormyrid Weakly Electric Fish

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To understand animal ecology, observation of wildlife in the natural habitat is essential, but particularly challenging in the underwater realm. Weakly electric fishes provide an excellent opportunity to overcome some of these challenges because they generate electric organ discharges (EODs) to sense their environment and to communicate, which can be detected non-invasively. We tracked the EOD and swimming activity of two species of mormyrid weakly electric fishes (*Marcusenius victoriae* and *Petrocephalus degeni*) over diel cycles in the laboratory, and we recorded EODs and environmental dissolved oxygen (DO) concentration and temperature over several months in a naturally hypoxic habitat in Uganda. Under laboratory conditions, both species showed increases of activity and exploration behavior that were closely synchronized to the onset of the dark phase. In the wild, fish preferred structurally complex habitats during the day, but dispersed toward open areas at night, presumably to forage and interact. Nocturnal increase of movement range coincided with diel declines in DO concentration to extremely low levels. The fact that fish showed pronounced nocturnal activity patterns in the laboratory and in the open areas of their habitat, but not under floating vegetation, indicates that light intensity exerts a direct effect on their activity. We hypothesize that being dark-active and tolerant to hypoxia increases the resistance of these fish against predators. This study establishes a new technology to record EODs in the field and provides a window into the largely unknown behavior of mormyrids in their natural habitat.

Keywords: behavioral activity, habitat use, electric organ discharge, hypoxia, swim behavior

INTRODUCTION

From the morning songs of birds to the seasonal migrations of salmon, rhythmic patterns of behavior are ubiquitous in nature across all taxa of animals (Wulund and Reddy, 2015; Häfker and Tessmar-Raible, 2020). Rhythmic behavior is driven by cyclical (abiotic) shifts in environmental conditions, such as the day/night cycle, the lunar cycle, and seasonal changes, e.g., in temperature and rainfall. The predictable nature of these cycles has made it possible for organisms to adapt to, and anticipate, environmental change in order to optimize the use of resources and environmental conditions (Häfker and Tessmar-Raible, 2020).

Many aquatic habitats are characterized by rhythmic changes in environmental conditions, although these may differ from terrestrial cycles. Light and temperature, reliable terrestrial diel time cues (Hut et al., 2013), can be less predictable underwater. In deep and/or turbid water bodies, ambient light levels may reach values where day and night are indistinguishable. The large heat capacity of water and other factors, such as rainfall or vertical mixing, can affect daily temperature cycles (Hut et al., 2013; Häfker and Tessmar-Raible, 2020). Fluctuations of dissolved oxygen (DO) concentration on the other hand, are mostly negligible on land but can be subject to pronounced rhythms in water bodies. Reduced input of photosynthetically produced oxygen during the night affects diel shifts in DO concentration. Seasonal effects, such as ice cover and rainfall can shift DO concentration over a longer timescale (e.g., Greenbank, 1945; Talling, 1957; Kannan and Job, 1980; Townsend, 1999). Alongside DO levels, pH and CO_2 concentration can fluctuate substantially on a diel and seasonal timescale (Semesi et al., 2009; Baumann et al., 2015). In tropical ecosystems, increased rainfall during the wet season can change inundation levels, flush nutrients and pollutants into water bodies and thereby change their hydrochemistry drastically (Adebisi, 1981; Hamilton, 2010).

Adaptive behavioral rhythms are just as commonly found in marine and freshwater habitats as on land (Naylor, 2010; Häfker and Tessmar-Raible, 2020). Among these, rhythms that occur on a diel timescale have been studied most intensively. Many aspects of fish behavior follow such diel rhythms, for example, shelter use (Vanderpham et al., 2012), swimming activity (Tabata et al., 1989; Hurd et al., 1998), food intake (Boujard and Leatherland, 1992; Sánchez-Vázquez et al., 1995), foraging behavior and migration (Hobson, 1973; Neilson and Perry, 1990; Ibbotson et al., 2006), and the formation of fish assemblages (Rooker and Dennis, 1991; Arrington and Winemiller, 2003). On a longer timescale, migration and reproductive behavior have been found in many species to be linked to lunar, seasonal, and annual rhythms (reviewed in Volpato and Trajano, 2005).

The study of activity rhythms in the field is challenging, and this is particularly true for the study of aquatic animals, mostly due to the logistical challenges and limitations of underwater sampling methods that are associated with them. For example, most studies on fish activity rhythms that are carried out in tropical regions are based on direct observation (Volpato and Trajano, 2005; Frehse et al., 2020, 2021). These studies are subject to a sampling bias that favors species that are diurnal and live in clear water. Hence, natural behavioral patterns of more elusive fish species, such as nocturnal fishes, remain largely unknown. In this respect, weakly electric fishes offer unique opportunities for behavioral observations in their natural habitat. They generate an electric field around their body by emitting species-specific electric organ discharges (EODs, Lissmann, 1951), which they use to navigate and forage (Lissmann, 1958; Lissmann and Machin, 1958), and to communicate with conspecifics (Moller, 1970; Moller and Bauer, 1973; Carlson, 2002). By recording their EODs with electrodes, the presence, activity state, number, and (if the EOD waveform is known) species of weakly electric fish can be detected in a water body without the need for visual or acoustic sampling (e.g., Lissmann and Schwassmann, 1965; Henninger et al., 2018, 2020; Madhav et al., 2018; Migliaro et al., 2018).

Weakly electric fishes are widespread in tropical freshwater habitats in Central and South America and throughout Africa where they constitute important food web components (Lundberg et al., 1987; Moller, 1995; Crampton, 1996). They are generally considered nocturnal animals, and this has been documented in some species that show nocturnal increases of locomotor and EOD activity in the laboratory (Harder et al., 1964; Bässler et al., 1979; Cobert, 1984; Franchina and Stoddard, 1998; Markham et al., 2009; Migliaro and Silva, 2016). Field studies generally support the notion of nocturnality in weakly electric fishes (Lissmann, 1961; Lissmann and Schwassmann, 1965; Kruger, 1973; Moller et al., 1979; Henninger et al., 2018, 2020; Madhav et al., 2018; Migliaro et al., 2018). However, a close examination of this body of research shows that there are examples of weakly electric fish that remain active during the day (e.g., in dark habitats, Kruger, 1973; Hopkins, 1980), and that nocturnal activity patterns have often been observed in shallow and/or clear water habitats, where light conditions change drastically from day to night (Lissmann, 1961; Lissmann and Schwassmann, 1965; Henninger et al., 2018, 2020). Thus, it seems that the activity of weakly electric fishes in the wild corresponds more closely to ambient light levels than to the phase of the diel cycle. It is therefore likely that activity patterns of many of the >450 species of weakly electric fishes (Crampton, 2019) depend on light conditions among other exogenous and endogenous factors, and that these animals might not always classify as nocturnal. Previous studies have shown that fishes can display extraordinary plasticity in their diel and circadian activity rhythms (reviewed in Reebs, 2002). Other factors than illumination, such as social stimuli (Franchina et al., 2001; Silva et al., 2007) and temperature (Dunlap et al., 2000; Ardanaz et al., 2001), can affect activity, and the interplay of these and other, hitherto unknown environmental influences, might shape natural behavioral patterns that differ from those observed in the laboratory. An environmental factor whose influence on diel behavior has been studied little in the field is ambient DO (Pollock et al., 2007). Many weakly electric fishes experience seasonal, diel, or chronic hypoxia (Chapman et al., 1996b; Crampton, 1998). Usually, DO reaches the lowest levels during the night due to respiration and lack of photosynthesis. The degree to which weakly electric fish can tolerate hypoxia might be limited by the energetic costs for generation and sensing of EODs (Salazar and Stoddard, 2008; Salazar et al., 2013; Lewis et al., 2014; Sukhum et al., 2016). With the large ionic currents underlying EOD generation (Markham et al., 2009), electric organs are considered to be energetically similarly expensive tissue as brains, but make up a larger proportion of body mass than brain tissue (Markham et al., 2016). Little is known about how environmental rhythms, such as diel patterns of hypoxia, affect the activity of weakly electric fish, and how representative laboratory studies are of natural activity patterns of fish.

The aim of this study was to investigate and compare activity and habitat use of mormyrid weakly electric fish in the laboratory and in the wild. *Marcusenius victoriae* and *Petrocephalus degeni* occur in a severely hypoxic swamp habitat in Uganda and show a high degree of hypoxia tolerance (Chapman and Chapman, 1998; Clarke et al., 2020; Moulton et al., 2020). They belong to different subfamilies of the Mormyridae, Mormyrinae and Petrocephalinae, respectively, and differ in their electrosensory anatomy, overall morphology, and social behavior (Carlson, 2016). *Marcusenius victoriae* are medium sized fish (usually 7–15 cm standard length) that are found predominantly alone in nature and show a high degree of intraspecific competition for shelters (Carlson, 2016). *Petrocephalus degeni* are shorter fish (usually 5–9 cm standard length) that are often found in groups and show pronounced social affiliation behavior (Carlson, 2016). Both species produce very short (ca. 300–500 μs), bi- or triphasic EOD pulses with a species-specific waveform (**Supplementary Figure 1A**). The diel variation of DO in their swamp habitat has not been quantified yet, and no detailed description of their diel habitat use and activity rhythms exists.

We conducted laboratory and field work to assess behavioral patterns under controlled and natural conditions. We captured fish from their swamp habitat and acclimated them to standardized laboratory housing conditions for one year before measuring diel patterns of EOD and locomotor activity in a 12:12 h light:dark environment under constant temperature and DO. To assess the behavioral patterns of *M. victoriae* and *P. degeni* in their habitat, we developed and built autonomous EOD recording devices. We measured the diel cycling of environmental temperature and DO, and recorded EODs in three habitats within a swamp lagoon system between July and September 2019 to document diel patterns of activity and habitat use, and abiotic environmental cycles.

MATERIALS AND METHODS

Laboratory Activity Measurements

Activity of laboratory-housed *Marcusenius victoriae* and *Petrocephalus degeni* was measured at Humboldt-Universität zu Berlin, Germany. The fish were wild caught in the Lwamunda Swamp that surrounds Lake Nabugabo in Uganda (**Figure 1**) and transferred to the animal housing facility of the Institut für Biologie of Humboldt-Universität zu Berlin, where they were held under controlled conditions for at least one year prior to experiments. *Marcusenius victoriae* were housed individually in 70 l tanks, and *P. degeni* were kept in groups of up to three fish in 70 l tanks. The light regime was set to a 12:12 h light:dark cycle with 30 min dusk/dawn periods during which light levels gradually transitioned between light and dark. All fish were in non-breeding condition.

Activity was quantified in a shuttle-box system (**Figure 2**; Loligo Systems Inc., Viborg, Denmark). The shuttle-box system consisted of two circular compartments (diameter of 50 cm each), that were connected by a central passage (8.5 cm wide and 14 cm long). Two opaque PVC tubes (5.5 cm inner diameter, 16 cm length) were placed symmetrically, one per compartment, to serve as shelter for the fish during the experiments. A piece of plexiglas was glued to the bottom of the shelter tubes to secure their position against disturbances. Water temperature was maintained by a thermostat (ITC-308, Shenzhen Inkbird Technology Co., Ltd., Shenzhen, China) that controlled the power supply to two silicone rubber heating mats on either side of the shuttle-box system and one heat radiator that was placed near the setup. The temperature sensor of the thermostat was submerged in the passage between the two compartments of the shuttle-box system. Two pumps (Universal Pumpe 1048, EHEIM GmbH & Co., KG, Deizisau, Germany) created a weak circular current in both compartments to homogenize temperature gradients in the system. The shuttle-box system rested on a self-constructed LED panel, which illuminated the system from below with white light during the day and infrared light during the night. The LED panel was controlled by a programmable LED timer (TC420, Shenzhen Leynew Technology Co., Ltd., Shenzhen, China). The light:dark cycle was set to synchronize with the light regime in the animal housing facility. Light intensity in the shuttle-box was ca. 48 lux during the light phase and 0 lux during the dark phase (**Supplementary Figure 2**). During the dark phase, the setup was illuminated with infrared light (940 nm wavelength) to enable video tracking of the animals. A camera (Grasshopper3 GS3-U3-41S4C-C 1, Teledyne FLIR LLC., Wilsonville, OR, United States) was mounted above the shuttle-box to record swimming activity of the fish. Videos were recorded at sample rates of 25–40 frames per second using Matlab R2018. To minimize disturbance, the setup was visually isolated from the surrounding area with lightproof fabric that was mounted on an aluminum frame around the shuttle-box system, LED panel, and camera. A Faraday cage made of steel mesh was mounted on the same aluminum frame and connected to an electric ground to reduce electric noise. EODs were recorded with carbon rod electrodes that were submerged in both compartments of the shuttle-box. During experiments with *M. victoriae*, one pair of electrodes was used in each compartment. Due to the smaller peak-to-peak amplitude of EODs of *P. degeni*, two pairs of electrodes in each compartment were necessary to reliably record their EODs during activity measurements. EODs were band-pass filtered (100 Hz – 20 kHz), amplified (10 x gain, DPA-2FS, NPI, Germany), digitized at a sample rate of 50 kHz (NI-PCI 6259, National Instruments, Austin, TX, United States), and stored on a computer using Matlab R2018. Videos and EOD recordings were synchronized using a LED light on the side of the LED panel, which signaled the beginning of each new EOD recording.

For each trial, one fish was introduced in a randomly chosen compartment of the shuttle-box in the late afternoon, before the onset of the dark phase, and left undisturbed for 42 h. Over the duration of the trial, movements and EOD activity were recorded continuously. After conclusion of the trial, the fish was removed from the shuttle-box system, weighed and measured, and returned to its home tank. Between two trials, the shuttle-box was cleaned with salt water and rinsed to remove residues from the previous trial. All experiments were approved by the German Landesamt für Gesundheit und Soziales (project number G0278/17).

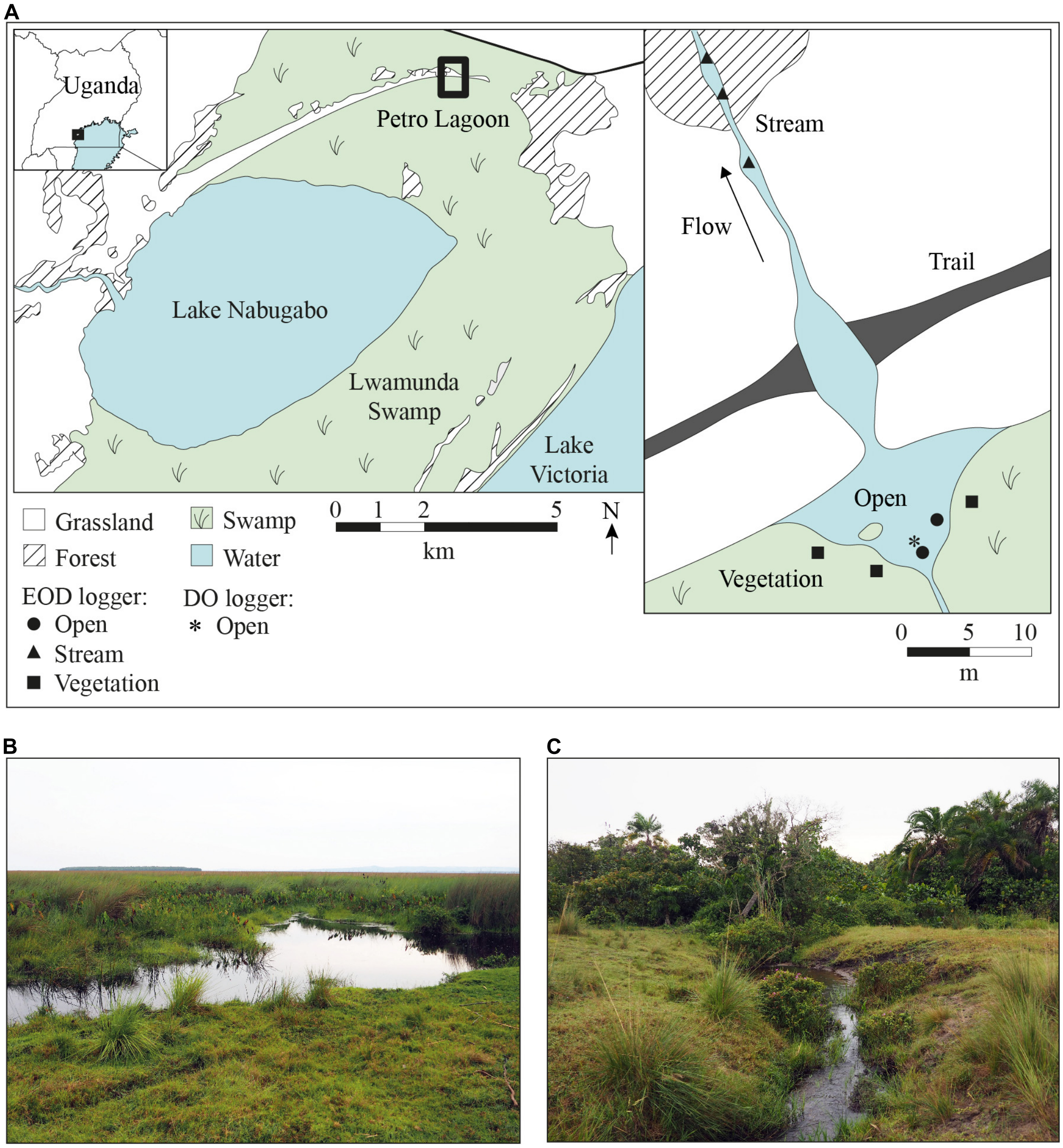

FIGURE 1 | Map of Lake Nabugabo and Petro Lagoon, Uganda. **(A)** Lake Nabugabo is separated from Lake Victoria by the extensive Lwamunda Swamp, which surrounds Lake Nabugabo to the north, east and south. To the west, Lake Nabugabo is bordered by forest and grassland. The map on the right shows a close up of Petro lagoon, where water from the swamp collects in the pond area and flows outwards from the swamp to seasonally inundated grassland. The outflow is intersected by a trail that crosses through a shallow section of the stream. EODs were recorded in three habitats in the lagoon (open, stream and vegetation). Positions of EOD loggers are indicated by filled symbols. The asterisk marks the position of the DO and temperature logger. **(B)** The pond area of Petro Lagoon where the open and vegetation habitat are located. **(C)** The small stream-like area of the lagoon where water flows seasonally from the wetland to the inland. Map data were obtained from satellite images (Google Earth, https://earth.google.com/web/, accessed 26.07.2021) and our own measurements at Petro Lagoon.

Shuttle-Box Data Analysis

Videos recorded from the shuttle-box were overlaid with a mask to obscure the parts of the image that did not show the shuttle-box system and increase tracking speed using FFMPEG ver. 4.4[1]. Fish position was then tracked using the Biotracker 3 software (Mönck et al., 2018). The resulting dataset was further processed using R. High-velocity jumps of the track position (>400 cm/s) that occurred, e.g., when fish traversed their opaque shelter tubes or when the tracking position was transiently lost, were corrected by connecting the two positions before and after the jump with a series of equally interspaced steps. Small jitters of the track position were then smoothed using a second-order Savitzky–Golay filter (Savitzky and Golay, 1964). Tracking data were annotated with categorical information about the position of the fish based on the x and y coordinates of the track position. For this, three zones in the shuttle-box tank were

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[1] https://ffmpeg.org/ (accessed December 2021).

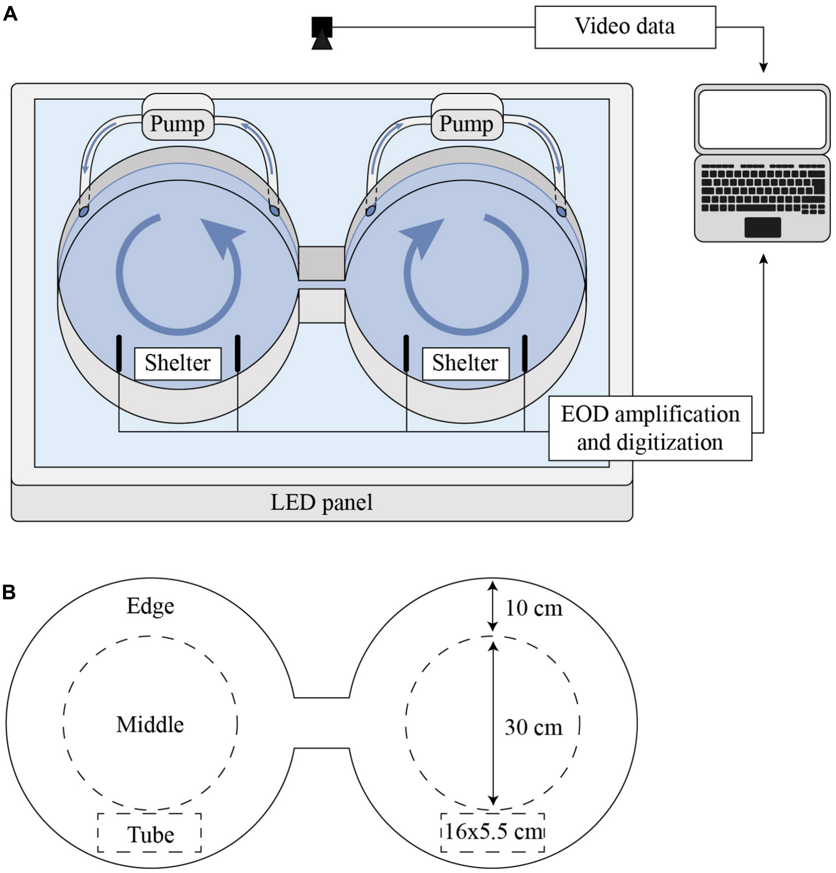

FIGURE 2 | Schematic of shuttle-box setup for behavioral activity measurements. **(A)** Experimental setup. The setup was illuminated from below by a LED panel and videos were recorded from above. EODs were recorded with carbon rod electrodes in both compartments that were arranged to both sides of each PVC shelter tube and, in experiments with *P. degeni*, with an additional pair of electrodes per compartment that was arranged on a perpendicular axis. Pumps created a slow circular current in both compartments. The heating system is not shown here for better clarity. **(B)** Definition of zones in shuttle-box tank. Edge: within 10 cm of shuttle-box wall; middle: more than 10 cm away from shuttle-box wall; tube: in PVC tube.

defined: shelter tube (tube), within 10 cm of the edge (edge), more than 10 cm away from the edge (middle, **Figure 2B**). To quantify swimming behavior, the mean swimming speed and the percentage of time spent in the tube, middle, and/or edge of the shuttle-box system were summarized for every 10 min of the trial.

EODs were extracted offline from shuttle-box recordings using Python ver. 3.8.8[2] with a custom-written Python script and peak-finding functions from the Thunderfish library[3]. Signals were identified using a peak-finding algorithm, which detected the positive and negative peak of each EOD pulse above a preset amplitude threshold of 0.1 V. A short data fragment (1 ms) centered around the zero crossing of each EOD was extracted, and sample rate was interpolated to 200 kHz for waveform analysis. Several EOD features were extracted from each EOD: peak-to-peak duration, peak-to-peak amplitude, amplitude ratio of positive/negative peak, and the frequency of the highest power of the FFT-transformed pulse. To quantify changes in electric signaling during the trial, EOD rate, inter-pulse-interval coefficient of variation (IPI CV), EOD amplitude CV, and EOD waveform features were summarized for every second and every 10 min of the trial.

For analysis of location preference, resting EOD rate, and the correlation of EOD rate and velocity, only data from the last 24 h of each trial were used to reduce the effect of acclimation to the shuttle-box system. Location preference was computed as the percentage of total time that fish spent at different locations of the shuttle-box. Resting EOD rate was calculated from sequences of the recording when fish spent at least 5 s in their shelter tube and EOD amplitude remained constant (coefficient of variation < 0.12), suggesting low locomotor activity (Silva et al., 2007).

In situ Recordings

Study Site

We recorded EOD activity in the natural habitat of *M. victoriae* and *P. degeni* between July and September 2019 in the Lwamunda Swamp, a wetland surrounding Lake Nabugabo in Uganda

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[2] https://www.python.org/ (accessed December 2021).

[3] https://github.com/bendalab/thunderfish (accessed December 2021).

(**Figure 1**). Separated from Lake Victoria about 5000 years ago (Stager et al., 2005), Lake Nabugabo is a small satellite lake (surface area = 33 km^2, mean depth = 3.13 m, Nyboer and Chapman, 2013) with a unique composition of fish species, such as a small number of haplochromine cichlid species that are endemic to Lake Nabugabo and nearby satellite lakes (Trewavas, 1933; Greenwood, 1965; Kaufman and Ochumba, 1993) and four species of mormyrid weakly electric fishes (Ogutu-Ohwayo, 1993; Chapman et al., 2002). In the years following the introduction of the predatory Nile Perch in the 1960s (Ogutu-Ohwayo, 1993; Pringle, 2005), Lake Nabugabo suffered a dramatic decline or disappearance of several native species from the waters of the main lake (Ogutu-Ohwayo, 1993; Chapman et al., 2003). The lake is surrounded by an extensive wetland area (Lwamunda Swamp), which is dominated by the emergent grass *Miscanthidium violaceum* that transitions to water lilies (*Nymphaea lotus* and *N. carulea*) or hippo grass (*Vossia cuspidata*) at the lake edge (Chapman et al., 1996a*;* Chrétien and Chapman, 2016). Various fish species occur deep within the wetland (see species list in Chapman et al., 1996b), including *P. degeni and M. victoriae* (Chapman et al., 1996b, 2002; Chapman and Hulen, 2001; Moulton et al., 2020). These fishes are protected from predation by Nile perch, as this species does not penetrate the dense swamp and is sensitive to hypoxia (Chapman et al., 2002). Inundation levels, water temperature and DO in the wetland show seasonal and diel variation, though DO remains generally low (previously reported diurnal ranges: 1.06–2.63 mg O_2 L-1, Reardon and Chapman, 2008; 0.69–0.82 mg O_2 L-1, Moulton et al., 2020), and water conductivity is very low (10–20 μS cm^1, measured manually during sampling). All research conducted in Uganda was approved by the Uganda National Council for Science and Technology (research clearance nr. 10601).

Electric Fish Loggers

We developed an autonomous data-logging device to record electric signals in the natural habitat of weakly electric fishes. The electric fish logger consists of a Teensy 3.5 microcontroller board (PJRC.COM, LLC., Sherwood, OR, United States) that is connected to a DS3231 real-time clock module (AZ-Delivery Vertriebs GmbH, Deggendorf, Germany) for timekeeping functionality, and powered by four AA batteries. The electronic components of the logger are housed in a PVC tube with an inner diameter of 45 mm, that is sealed on one side with a glued-on lid and on the other side with a screw lid, which allows replacement of batteries and the microSD card. Two small carbon disks (1 cm diameter, 4 mm long) serve as electrodes to measure EODs. The disks are glued into the lids of the logger housing using aquarium silicone and connected to the analog-digital converters of the Teensy board via copper wire.

The logger continuously records differential voltage through its electrodes with a dynamic input range between –3.3 V and +3.3 V. Voltage data were digitized at a sample rate of 100 kHz with a 12-bit resolution, and stored on a microSD card. The program for the logger was written using the Arduino IDE[4] and the Teensyduino plugin[5]. The code for the logger program has been archived and is maintained online on GitHub, alongside a more detailed documentation[6].

The detection range of the electric fish logger depends on the amplitude of the signal that is generated by the fish. Due to the dipole character of the electric field, the recorded EOD amplitude depends on the distance and the position of the fish relative to the electrodes (see Benda, 2020 for a comprehensive review of electric field geometry). As the two poles of the electric field are located on the head-to-tail axis of the fish, EOD amplitude is largest when the measurement electrodes align with this axis, and practically reaches zero when the fish is perpendicular to the line connecting the two measurement electrodes. Thus, there is no fixed detection range for the loggers, and we rather estimated detection probability. To do this, we recorded freely swimming specimens of each species ($n = 4$ per species, see **Supplementary Material**) in a rectangular tank (200 cm × 100 cm). Detection probability decreased with distance, following an exponential decay function, and was overall lower for *P. degeni*, approaching zero at 50 cm radial distance from the logger (**Supplementary Figure 3**). Detection probability for EODs of *M. victoriae* reached about 10% at the maximum distance of 180 cm. All fish used for detection probability estimation in the lab were in non-breeding condition.

In situ Electric Organ Discharges Sampling

Eight electric fish loggers were deployed in a small lagoon (Petro Lagoon, 0°19′07′′S, 31°56′48′′E) in the Lwamunda Swamp in three different habitats: under floating vegetation with ca. 1 m distance to the edge of vegetation cover (vegetation, $n = 3$), under the open water surface of the lagoon with at least 1 m distance to the nearest vegetation (open, $n = 2$), and in the middle of the small stream-like area of the lagoon where water flows seasonally from the wetland to the inland (stream, $n = 3$). These habitats were chosen to capture a variety of environmental conditions that is representative of the conditions experienced by fish in the lagoon (**Table 1**). To secure the logger position against perturbations, each logger was attached to a stick that was driven firmly into the ground. Each logger was placed approximately in the middle of the water column and with at least 3 m distance to the nearest logger.

In situ recordings were made on six sampling sessions in 2019 (July: 23–24 and 29–30, August: 8–9 and 21–22, September: 2–3 and 19–20). Electric fish loggers were deployed in the morning and left to record continuously for at least 24 h. After retrieval of the loggers, EODs were extracted from recordings as described above, using Python ver. 3.8.8 (see footnote 2, accessed December 2021) with a custom-written Python script and functions from the Thunderfish library (see footnote 3, accessed December 2021). Only EODs with peak-to-peak amplitudes above 0.066 V (1% of the dynamic range of the logger) were extracted. EODs were then grouped together into encounters based on their zero-crossing timestamp using R ver. 4.1.0[7]. An

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[4] https://www.arduino.cc/ (accessed December 2021).

[5] https://www.pjrc.com/ (accessed December 2021).

[6] https://github.com/muchaste/EOD-Logger (accessed December 2021).

[7] https://www.r-project.org/ (accessed December 2021).

TABLE 1 | Characteristics of different habitats in Petro Lagoon situated in the Lwamunda Swamp of the Lake Nabugabo System.

Habitat	Loggers (n)	DO	Water flow	Water depth (cm) (cm)	Vegetation cover	Shelter options
Vegetation	3	Low	Very low	33 (22 - 45)	Full cover by swimming mats of vegetation	Structurally complex root masses
Open	2	Low	Very low	66 (56 – 77)	No cover	No shelter
Stream	3	Low	Continuous	33 (12 – 55)	Shaded by overhanging trees and shrubs	Vegetation and roots along the stream edges

DO, dissolved oxygen; Water depth is represented as mean (range).

encounter was defined as a coherent EOD pulse train with at least 10 pulses and a maximum inter-pulse interval (IPI) of 5 s. These selection criteria served to eliminate very brief and/or erroneous fish detections that occurred due to electrical noise, e.g., through lightning discharges. For each encounter, EODs were assigned to either *M. victoriae* or *P. degeni* based on their EOD waveform, using mixture discriminant analysis (MDA) and EOD features. To create MDA models, EOD features from fish that had been recorded under laboratory conditions were used (see **Supplementary Material**). Three features, peak-to-peak duration, peak 1/peak 2 amplitude ratio, and the frequency of the highest power of the FFT-transformed pulse, were sufficient for robust species discrimination with a fidelity of 98.3% (**Supplementary Table 1**). The MDA model was then applied to predict the species for each EOD pulse in the field recordings. After species prediction via MDA, the EOD pulse train of each encounter was inspected visually to determine the number and verify the species of the fish. Encounters with single fish that could not be assigned with confidence (i.e., less than 70% of pulses could be assigned to either species) were removed from analysis.

As individual fish of a given species could not be identified unambiguously based on their EOD features, it is likely that some animals were encountered multiple times. This is even more likely for *M. victoriae*, which have been described as territorial (Carlson, 2016). Although, in theory, absolute animal densities may be estimated despite double counting of individuals (Campos-Candela et al., 2018; Follana-Berná et al., 2020), the data required to satisfy the assumptions underlying such methods (e.g., fish must display home ranging behavior and home range centers must be homogeneously distributed, Campos-Candela et al., 2018) are not available for this study system. Thus, data on fish distribution are presented as frequency of encounters, which depends on fish activity as well as fish density. Frequency of encounters was calculated as the total number of fish that were encountered per logger over intervals of 30 min. Duration of encounters (from first to last EOD) and EOD rate during encounters were extracted to compare encounter characteristics between habitats and periods of the diel cycle. For the comparison of encounter characteristics, only events with a single fish were used, as they occurred most consistently across time and space.

In situ Temperature and Dissolved Oxygen Sampling

One oxygen and temperature logger with optical oxygen sensor (miniDOT, PME, Vista, CA, United States) was placed in the open habitat of the lagoon to record water conditions on the same sampling days as *in situ* EOD recordings were made. Water temperature and DO concentration were sampled at least every 30 min for the duration of EOD recordings. After each sampling day, data were collected from the logger, and the first and last 30 min of measurements were deleted to remove disturbances in the measurements that were caused by placing and retrieving the logger. Previous manual measurements that were taken during the daytime at this site between 2005 and 2018 showed that temperature varies on average by 0.2 ± 0.5°C and air saturation varies by 1.8 ± 7.9% between the water surface and at a water depth of 50 cm (n = 690 measurements). As we took the measurements for this study in the middle of the water column, we expect our values to be representative of large parts of the water column where the fish are located. In addition to continuous measurements, diurnal temperature and DO were recorded in all habitats twice per sample day using a handheld meter (Handy Polaris, OxyGuard, Farum, Denmark).

RESULTS

All values are represented as mean ± standard deviation (SD) if not otherwise stated.

Activity Patterns in the Laboratory

The activity patterns of *M. victoriae* and *P. degeni* in the lab corresponded closely to ambient light levels, with long phases of inactivity and strong shelter preference during the light phase and increased activity and exploration behavior during the dark phase of the trial (**Figure 3**).

During the light phase of the trial, *Marcusenius victoriae* spent 93.1 ± 18.5% and *P. degeni* spent 98.7 ± 2.4% of the time in a shelter tube, which decreased during the dark phase to 36.4 ± 27.5% and 12.5 ± 10.0% percent, respectively. During the dark phase, *M. victoriae* showed no preference for either shelter tube or unsheltered areas of the shuttle-box (edge and middle). However, when fish were located outside of the shelter tube, they spent significantly more time close to the edge of the shuttle-box than in the middle. Throughout the light cycle, *M. victoriae* emitted EODs at lower rates in the shelter tube than in the unsheltered areas of the shuttle-box. *Petrocephalus degeni* spent significantly more time in the unsheltered areas of the shuttle-box during the dark phase without a preference for the edge or

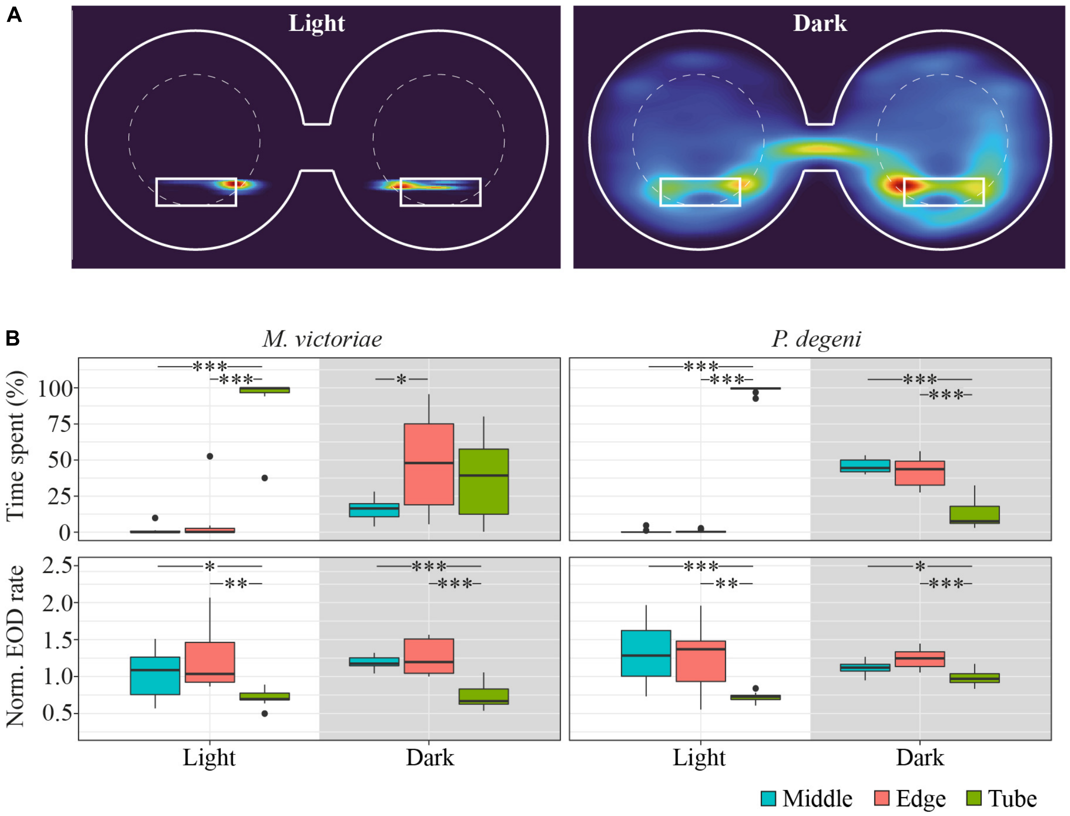

FIGURE 3 | Example heatmap, location preference and EOD rates in the shuttle-box. **(A)** Heatmap of swim activity of one *P. degeni* during the light and the dark phase. Warmer colors indicate that the fish spent more time at this location. The margins of the shuttle-box tank and the two shelter tubes are indicated with solid white lines, the area boundaries between edge and middle are indicated with dashed white lines. Two-dimensional kernel density estimation was used to convert X-Y coordinates from tracked video data (downscaled to 1 frame per second) into a probability density function for the location of the fish. **(B)** Top row: proportion of time fish spent in different locations in the shuttle-box as percentage of total time. Bottom row: normalized EOD rates, averaged per fish, period of the light cycle, and area of the shuttle-box (*M. victoriae*: $n = 11$; *P. degeni*: $n = 10$; light phase EOD rates: Conover test; rest: Wilcoxon test: $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.001^{***}$). Overall *M. victoriae* emitted EODs with a rate of 8.0 ± 0.9 Hz (day) and 11.1 ± 1.1 Hz (night); *P. degeni* with 8.0 ± 0.4 Hz (day) and 12.3 ± 0.3 Hz (night).

the middle of the shuttle-box tank. Similar to *M. victoriae, P. degeni* emitted EODs at lower rates in the shelter tube during the light phase. However, and in contrast to *M. victoriae*, there was no difference in EOD rates between tank areas during the dark phase (**Figure 3B**).

Swim speed and EOD rate of both species increased sharply during the 30-min dusk period, peaked at the onset of complete darkness, and remained at elevated levels for the entire duration of the dark phase (**Figure 4A**). The dark phase proportion of the total distance that fish swam within 24 h was 96.3 ± 7.8% for *M. victoriae* and 97.7 ± 4.6% for *P. degeni*. There was no evidence of anticipation of the dark phase (i.e., no increase of EOD rate or swim activity before the dusk period). During the light phase, EOD rate decreased steadily in contrast to swim speed, which dropped quickly close to 0 body lengths per second (BL s^{-1}) at the onset of the light phase and remained there until the dusk period. Despite this difference, EOD rate was overall positively correlated with swim speed throughout the light and dark phases for both species (linear regressions: $p < 0.001$; **Figure 4B**).

There were some differences between species in swim and EOD activity. Swim activity of *P. degeni* was significantly higher than that of *M. victoriae* during the dark phase (1.30 ± 0.40 vs. 0.44 ± 0.28 BLs^{-1}, Wilcoxon test: $p < 0.001$), and *P. degeni* increased swim speed and EOD rate at the end of the dark phase, an effect that was not as prominent in *M. victoriae*. IPI coefficient of variation (IPI CV) of *P. degeni* showed a clear diel pattern indicating that EOD rate was more variable during their active phase. By contrast, IPI CV of *M. victoriae* did not differ between light and dark phase, and some individuals showed greatly irregular IPIs during the inactive phase, mostly due to longer cessations of EOD generation. The resting EOD rate of *P. degeni* was significantly higher at night than during the day (t-test: $t = -3.57$, df = 9, $p < 0.01$; **Figure 4C**). This effect was not observed in *M. victoriae*.

Environmental and Behavioral Patterns at Petro Lagoon

Temperature and Dissolved Oxygen

Temperature and DO in the open habitat of the Petro Lagoon fluctuated over the diel cycle (**Figure 5A**). DO reached minimum values at night (5.1 ± 5.4% air saturation at 11.5 h after sunset)

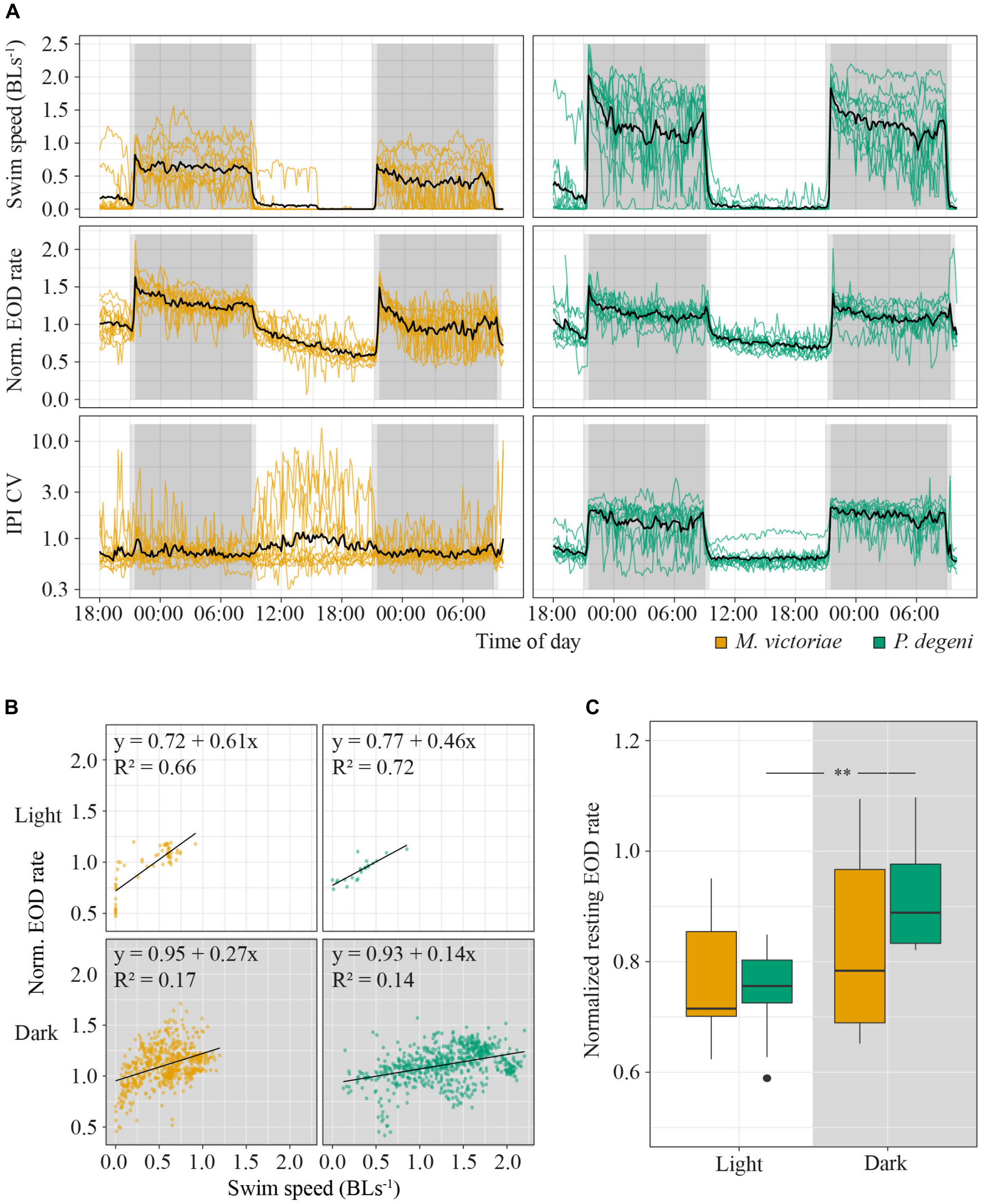

FIGURE 4 | Behavioral activity of *M. victoriae* (*n* = 11) and *P. degeni* (*n* = 10) under laboratory conditions. **(A)** Swim speed in body lengths per second (BL s^{-1}, top row), normalized EOD rate (middle row), and coefficient of variation of inter-pulse intervals (IPI CV, bottom row) of *M. victoriae* (left) and *P. degeni* (right) in the shuttle-box. All variables were averaged per fish over 10-min intervals. Colored lines represent individual fish, black lines represent the average over all fish, gray shaded areas represent periods of dusk/dawn (light gray) and complete darkness (dark gray). Notice that IPI CV is shown on a logarithmic *y*-axis. **(B)** Linear regressions of swim speed and normalized EOD rate. Each point represents a measurement from an actively swimming fish that was averaged over a 10-min interval. Points from different fish are not distinguished. **(C)** Box plot of normalized EOD rate of fish that rested in their shelter tubes during the day and night. Resting EOD rate was averaged per fish (*t*-test: $**p < 0.01$).

and increased in the morning. Rainfall and/or disturbance of the water surface is visible as peaks in DO values and contributed to the increase of mean DO in the morning. Water temperature followed a more pronounced oscillation than DO, reaching its maximum in the late afternoon (24.9 ± 1.1°C at 3.5 h before sunset) and its minimum in the early morning (21.6 ± 0.7°C at 0.5 h after sunset). Day length remained constant throughout the sampling period (12:06 – 12:07 h, Sunrise: 06:47 – 06:47 am, Sunset: 06:50 – 07:03 pm[8]).

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[8] https://www.worldweatheronline.com/masaka-weather-history/masaka/ug.aspx (accessed January 2022).

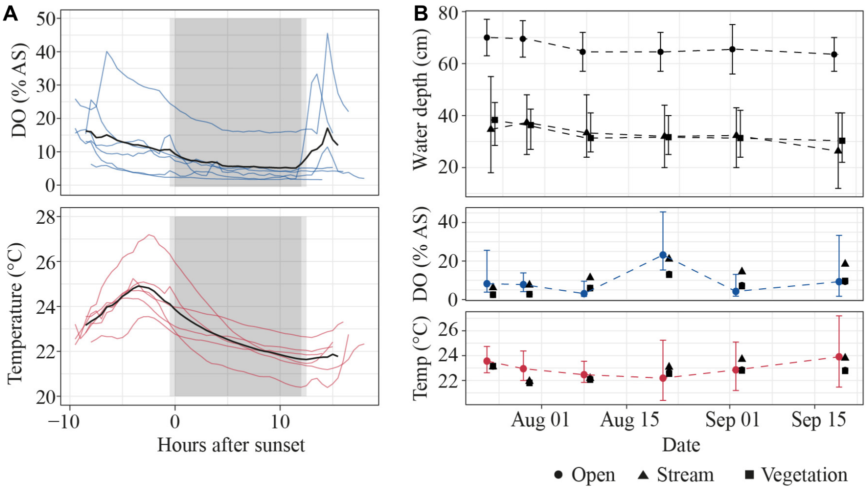

FIGURE 5 | Environmental conditions at Petro Lagoon. **(A)** Daily changes in DO (in% air saturation, top) and water temperature (bottom). Thin lines indicate individual sampling days, the thick black line shows the mean over all sampling days (*n* = 6). Light gray boxes indicate 30-min dusk/dawn periods, dark gray boxes indicate night periods. **(B)** Variation of water level (top), DO (% air saturation, middle), and temperature (Temp., bottom) across sampling days. Symbols represent mean values, error bars represent the range of values. Manual measurements that were taken during the daytime are shown as black symbols in the DO and temperature panel. The shape of the symbols represents the different habitats.

TABLE 2 | Average number of encounters, EOD rates, and durations, grouped by species, habitat and phase of the diel cycle.

Phase	Species	Open	Stream	Vegetation
Encounters per 30 min				
Day	*M. victoriae*	–	–	0.4 ± 1.1
	P. degeni	–	0.1 ± 0.8	0.9 ± 2.7
Night	*M. victoriae*	2.2 ± 4.2	–	1.8 ± 3.4
	P. degeni	0.3 ± 1.6	0.4 ± 1.5	1.8 ± 3.9
EOD rate (Hz)				
Day	*M. victoriae*	–	–	10.3 ± 4.3
	P. degeni	–	16.3 ± 6.3	12.9 ± 6.9
Night	*M. victoriae*	12.1 ± 6.6	–	10.4 ± 5.4
	P. degeni	16.6 ± 6.1	15.6 ± 4.8	12.1 ± 4.7
Duration (s)				
Day	*M. victoriae*	–	–	25.6 ± 17.6
	P. degeni	–	8.7 ± 7.6	16.5 ± 15.5
Night	*M. victoriae*	13.5 ± 12.1	–	25.7 ± 16.9
	P. degeni	10.7 ± 9.4	10.5 ± 8.9	16.7 ± 10.9

EOD rates and durations were extracted from encounters with single fish. Values are represented as mean ± SD.

Across sampling days, DO was highest on August 21st, due to continuous rainfall on that day, but remained low on average (9.2 ± 7.3% air saturation) and was extremely low on August 8th (2.7 ± 1.4%, **Figure 5B**). Daily water temperature remained at a relatively constant average value (23.0 ± 0.6°C) despite diel fluctuations. Water depth also remained relatively constant in all habitats and was highest in the open habitat. Manual measurements that were taken during the day showed little difference in temperature and DO between the open and vegetation habitat. However, DO and temperature tended to be slightly but systematically higher in the stream habitat (by 6.4 ± 2.4% air saturation and 0.4 ± 0.7°C, **Figure 5B**) than other areas of the lagoon.

Presence of Electric Fish in the Different Habitats

We collected more than 1,122 h of electric recordings in the open, stream and vegetation habitat at Petro Lagoon between July 23rd and September 20th 2019. We registered 2849 encounters with at least one *M. victoriae* and/or *P. degeni*, most of which happened during the night (2218). Both species were encountered equally often (*M. victoriae*: 1433, *P. degeni*: 1416). By far the highest number of encounters was recorded in the vegetation habitat (1137 and 852, respectively), followed by the open habitat (581 and 78, respectively), and the stream (0 and 201, respectively).

The frequency with which encounters occurred in the different habitats varied between species and periods of the

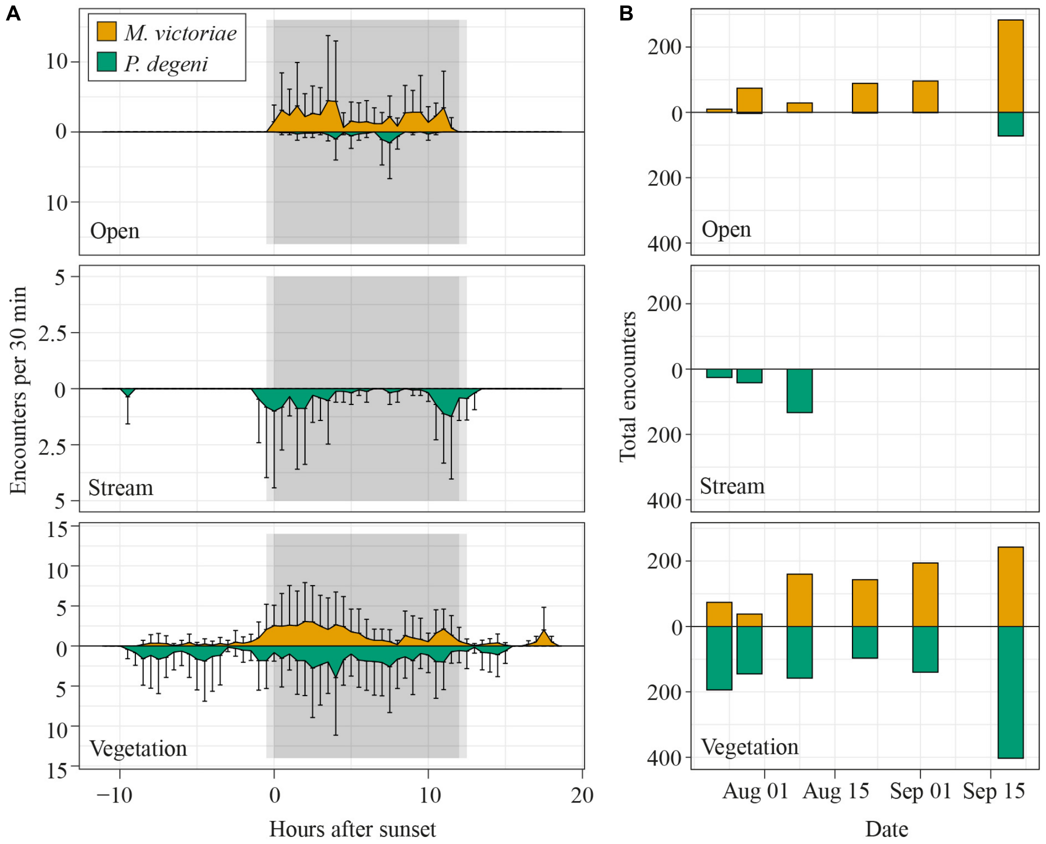

FIGURE 6 | Diel patterns of EOD activity in Petro Lagoon. **(A)** Average number of fish encounters per 30 min in the open (top), stream (middle) and vegetation (bottom) habitat. To calculate the average number of fish encounters per 30 min, the number of fish was extracted from each encounter, summed up for each logger over intervals of 30 min and averaged over the number of loggers per habitat (open: 2, stream: 3, vegetation: 3) and all sampling days ($n = 6$). Error bars representing SD extend only into one direction for better readability. Notice that the y-axis scaling differs between the different panels. **(B)** Total number of fish that were encountered per sample day in the open (top), stream (middle), and vegetation (bottom) habitat. Data for *M. victoriae* are shown above the x-axis, those for *P. degeni* below.

diel cycle (**Table 2** and **Figure 6A**). In the open habitat, both *M. victoriae* and *P. degeni* were encountered only at night, and *M. victoriae* accounted for the majority of the nocturnal encounters. Under the floating vegetation, both species were encountered throughout the diel cycle. However, the frequency of encounters with *M. victoriae* increased more than fivefold from daytime to nighttime, whereas *P. degeni* were encountered more continuously during the day and the night under the floating vegetation (**Table 2**). In the stream habitat, only *P. degeni* were encountered, and primarily at night.

The number of daily fish encounters varied considerably across sampling days (**Figure 6B**). Encounters with *M. victoriae* increased in the open habitat and vegetation habitat from 84 to 526 encounters. Numbers of encounters with *P. degeni* remained relatively constant under the floating vegetation except for a peak of 403 encounters on the last sampling day. In the stream habitat, *P. degeni* was encountered only during the first three sampling days, and in the open habitat only on the last sampling day. Overall, 35% of all fish encounters were made on the last sampling day.

Co-occurrence of Fish

In 18.7% of encounters, more than one fish was detected (co-occurrence; **Figure 7A**). On average, the proportion of encounters during which multiple *M. victoriae* were detected simultaneously was higher at night (17.4 ± 10.9% of encounters) than during the day (2.0 ± 2.8%; U-test: $U = 2$, $p < 0.05$). *Petrocephalus degeni,* likewise, co-occurred more often at night (10.8 ± 8.4%) than during the day (2.4 ± 4.1%; U-test: $U = 3$, $p < 0.05$). There was no difference between the two species in the percentage of encounters that included more than one conspecific. In 50.9% of co-occurrences, fish from both species were present.

Encounter Duration and Electric Organ Discharges Rate

Encounter duration and EOD rate differed among habitats and periods of the diel cycle, suggesting that fish displayed higher activity in the open and stream habitat and around sunset and sunrise (**Table 2** and **Figures 7B,C**).

In the vegetation habitat, where most encounters were detected throughout the diel cycle, EOD rates of *M. victoriae*

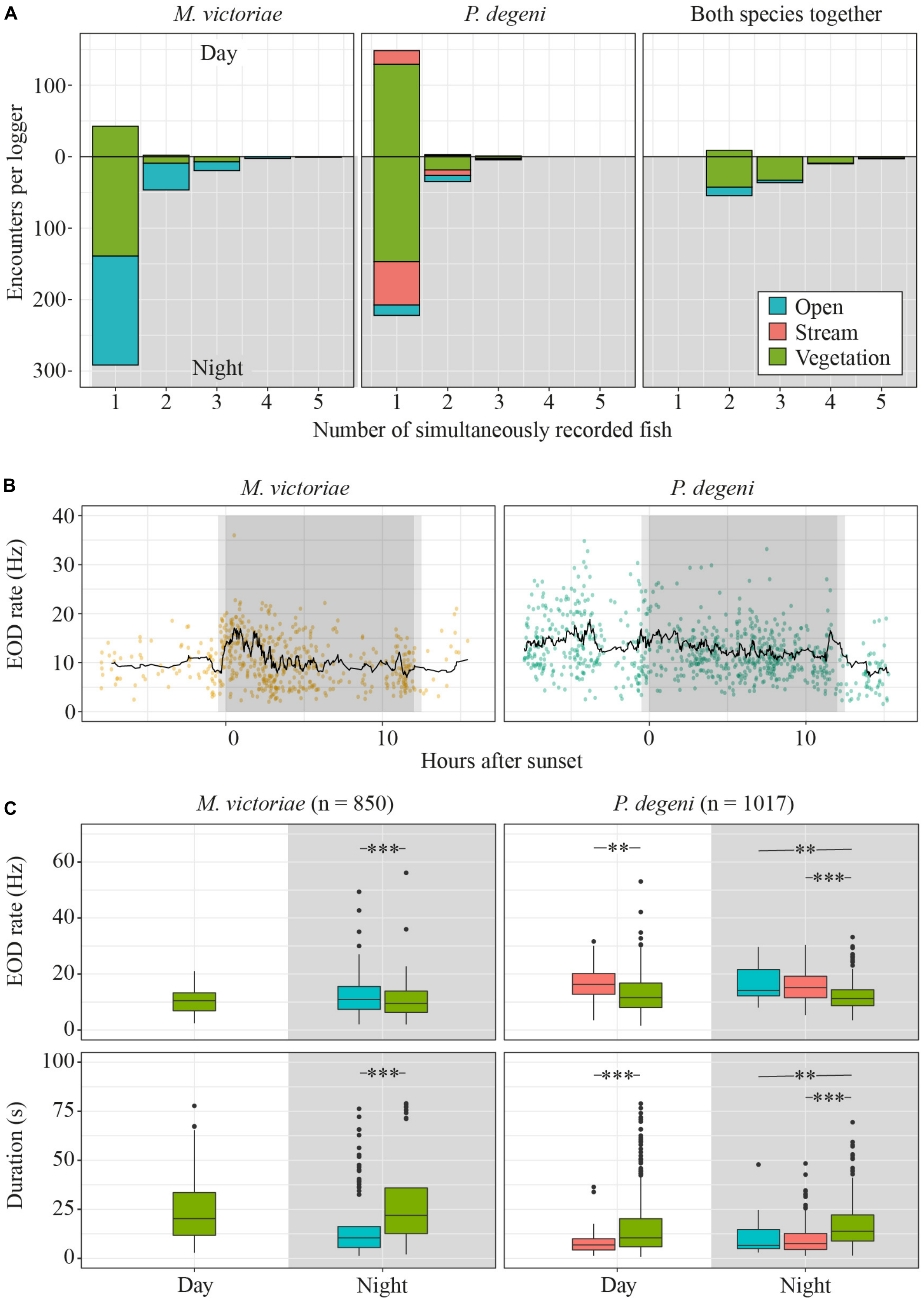

FIGURE 7 | Diel differences in encounter characteristics. **(A)** Total number of encounters by species, habitat, daytime and number of fish that were present during encounter. **(B)** EOD rates of encounters with single *M. victoriae* (left) and *P. degeni* (right) in the vegetation habitat. Each encounter is represented by a dot, the black line represents the rolling mean over 20 encounters. **(C)** Boxplots of EOD rate (top row) and duration (bottom row) of encounters by phase of the diel cycle, species and habitat. Extreme outliers (more than 3 × interquartile range above the 3rd quantile or below the 1st quantile) were removed from the duration plot to improve readability. Outlier removal did not affect the outcome of statistical testing (*t*-test: $^{**}p < 0.01$; $^{***}p < 0.001$).

peaked within one hour of sunset and again shortly before sunrise. EOD rates of *P. degeni* were highest during the day but showed a small peak before sunrise (**Figure 7B**). These changes were transient and did not lead to elevated nocturnal EOD rates, as were observed in the laboratory. Both species emitted EODs at a lower rate under the vegetation than in the other habitats, both during the day and at night, and encounters lasted longer under the vegetation than in the other habitats (**Figure 7C**). Overall, the EOD rates of *M. victoriae* in the field were comparable to those from the laboratory (day: 10.3 ± 4.3 Hz vs. 8.0 ± 2.3 Hz; night: 11.1 ± 6.0 Hz vs. 11.1 ± 3.3 Hz). EOD rates of *P. degeni* were significantly higher in the field than in the laboratory during the day (13.0 ± 6.8 Hz vs. 7.7 ± 2.8 Hz, U-test: $U = 3069$, $p < 0.01$), but not during the night (13.6 ± 6.8 Hz vs. 11.3 ± 4.8 Hz).

DISCUSSION

In this study, we tracked the activity of two species of mormyrid weakly electric fishes in the laboratory and recorded their diel activity patterns in a chronically hypoxic habitat using newly developed recording devices. In both settings, *M. victoriae* and *P. degeni* showed distinct, daylight-dependent behavioral patterns that were characterized by increased activity and exploration behavior under dark conditions. Diel patterns of co-occurrence suggest that the study species interact socially primarily during the night in their habitat. Although the behavior observed in the laboratory and in the wild was largely congruent, *P. degeni* showed higher daytime activity in naturally dark habitats than in the laboratory, and the increase of activity during the dark phase was much more pronounced and consistent in the laboratory than in the wild for both species. These findings suggest that the activity of the study species depends more on the environmental light conditions than on the phase of the diel cycle. It is likely that sinking nocturnal DO and temperature further affect activity patterns that are more varied in the wild than what is observed in the laboratory.

Spatial and Temporal Patterns of Behavior

Habitat Use and Swim Behavior

In the laboratory, fish spent nearly 100% of the time in their shelters during the light phase and increased their behavioral activity with the onset of the dark phase, which resulted in higher swim speed and a change in their movement range from sheltering to active exploration of their environment. In the wild, fish were almost exclusively detected under floating vegetation during the day and dispersed into the open areas of the lagoon at night. Interestingly, *P. degeni* showed a stronger preference for open areas in the shuttle-box during the dark phase than *M. victoriae*, whereas in the wild, *M. victoriae* were encountered more often in the open area at night, and *P. degeni* seemed to prefer the vegetation habitat both during day and night. As the sheltered areas in the shuttle-box were limited to two tubes, it is possible that this was an effect of the overall higher swim activity of *P. degeni* compared to *M. victoriae*. The different activity levels of the two species could also help to explain the fish dispersion patterns at Petro Lagoon. Given that DO was overall low but highest in the stream habitat, it could have been expected that fish move into the stream at night to reduce hypoxic stress. Thus, it came as a surprise to find that only *P. degeni* were encountered here, and only on the first three sampling days. It is possible that their higher activity level makes *P. degeni* more vulnerable to hypoxia and/or better adapted to an environment with flowing water. The more sluggish *M. victoriae*, on the other hand, might be better adapted to stagnant environments. Another possible explanation for the lack of fish in the stream is that fish had to cross a shallow section of the lagoon in order to reach it. Due to its low water depth, this section may have represented a movement barrier for the larger *M. victoriae* in the beginning of the sampling period. Over the sampling duration, water depth decreased slightly throughout the lagoon, which could have kept *P. degeni* from venturing into the stream on the later sampling days.

The temporal patterns of fish encounters suggest that fish actively moved into the open and stream areas of the lagoon at night and returned to the floating vegetation around sunrise. The transient increase of EOD rates under the vegetation during sunset and sunrise could be a result of this movement activity. This seems to align well with the activity peaks that were observed in the laboratory at the onset and toward the end of the dark phase. The question is, for what purpose do fish emerge at night and venture into unprotected areas? We suggest that fish use the open habitats to forage, interact, and carry out ASR at night at a lower risk of predation (e.g., Hobson, 1973; Nagelkerken et al., 2000; Hammerschlag et al., 2010; Sikkel et al., 2017). Although the low DO in the swamp protects fish from large predators, such as the Nile perch (*Lates niloticus*, Chapman and Chapman, 1998; Chapman et al., 2002), there is some risk of predation during the daytime due to the presence of visually oriented piscivorous birds, such as the hamerkop (*Scopus umbretta*) and the pied kingfisher (*Ceryle rudis*, **Figure 8**). These predators hunt predominantly in the open and shallow regions of the lagoon (pers. observation), turning them into dangerous territories for fish during the day. Further, the complete absence of mormyrids in the open and stream habitat during the day indicates that there is little diurnal foraging activity in these areas. At night, fish can exploit the untapped resources at a lower risk of predation. The non-visual sensory modality of *M. victoriae* and *P. degeni* facilitates prey capture in the dark (Emde and Bleckmann, 1998; Emde, „ 1999), and gives them an edge over other visually oriented nocturnal foraging species. Encounter duration and EOD rates suggest that the activity level of fish was higher in the open and stream area than in the vegetation, which aligns well with foraging behavior as increased EOD rates increase the temporal acuity of electrosensory information, and can aide in the localization of prey and/or avoidance of predators. Similar nocturnal exploration and foraging activity has been described before in weakly electric fishes (Lissmann and Schwassmann, 1965; Kruger, 1973; Moller et al., 1979; Friedman and Hopkins, 1996; Henninger et al., 2018; Madhav et al., 2018; Henninger et al., 2020). For example, Henninger et al. (2020) observed in a creek in Panama that three gymnotiform species, *Apteronotus rostratus*, *Eigenmannia humboldtii* and *Sternopygus dariensis*, move actively

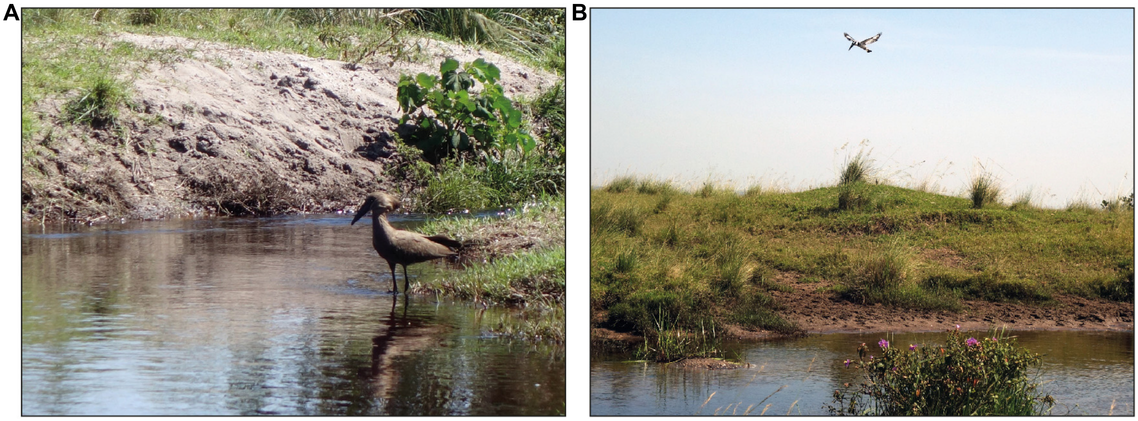

FIGURE 8 | Piscivorous birds that forage at Petro Lagoon during the day. **(A)** A hamerkop (*Scopus umbretta*) lurks for prey at a shallow area of the lagoon. **(B)** A pied kingfisher (*Ceryle rudis*) hovers over the open area of the lagoon. Photo credit: Stefan Mucha.

only during the night, traveling upstream after sunset and downstream in the second half of the night. In an early study in Brazil, Lissmann and Schwassmann (1965) demonstrated that sandfish (*Gymnorhamphichthys hypostomus*) rest, buried in the sand, during the day and emerge at night to forage and interact. In South Africa, Kruger (1973) found that *Marcusenius macrolepidotus* (formerly *Gnathonemus macrolepidotus*), a close relative to *M. victoriae*, moves at night from bottom waters into the littoral zone, presumably to feed on chironomids. Moller et al. (1979) recorded mormyrids in the Swashi River in Nigeria, which hid during the day in a river inlet and moved actively in and out of the river at night. The behavior of *M. victoriae* and *P. degeni* described here matches these observations well.

We saw an increase in total fish encounters toward the end of the sampling period, especially on the last sampling day. Our last measurements were made just at the onset of the short rainy season, and there was a slight upwards trend in DO and temperature on the three last measurement days. The increase of encounters might be a result of higher behavioral activity due to higher energy availability and temperature. Another intriguing possibility is that the increase of fish encounters was caused by seasonal breeding migrations. Some mormyrid species breed at the onset of the rainy season (Hopkins and Bass, 1981; Kirschbaum, 1995). Decreasing water conductivity, increasing water levels, and artificial rainfall on the surface all promote gonad growth in those species (e.g., Kirschbaum and Schugardt, 2002). Although not much is known about the natural breeding behavior of our study species, it is possible that the increase of encounters with *M. victoriae* and the high number of encounters with *P. degeni* on the last sampling day indicate breeding behavior. This would be an exciting finding, as it would mean that these fish reproduce under severely hypoxic conditions. A longer-term survey of the fish distribution in the Lake Nabugabo system during the rainy seasons, e.g., with electric fish loggers, should give better insights into this important aspect of the ecology of *P. degeni* and *M. victoriae*.

Electric Activity

In the laboratory, EOD rate was closely correlated to swim activity in both species and showed clear diel rhythms. The two species differed in their temporal patterns of EOD generation. *Petrocephalus degeni* emitted EODs at more regular intervals during their inactive phase and became more irregular during their active phase. This was not the case in *M. victoriae*. In contrast, some *M. victoriae* showed greatly irregular IPIs with pauses that exceeded 10 s and could reach up to > 120 s during their inactive phase. Similar differences in EOD generation have been described by Carlson (2016) who found that *M. victoriae* respond to EOD playback with pauses of electric signaling whereas *P. degeni* tend to generate EOD bursts. These differences in pulsing behavior likely reflect a variety of differences in electrosensory anatomy and social behavior between these two species (Carlson, 2016). For example, cessations of EOD generation have been associated with aggressive and submissive behavior (Carlson, 2002), traits that might be more typical of *M. victoriae* (Carlson, 2016). Pausing EOD generation during their inactive phase could reduce their detectability by electrosensory predators and/or aggressive conspecifics.

In the field, EOD rates of *M. victoriae* increased after sunset, however, this effect was much more transient than in the laboratory. The EOD rates of *P. degeni* were highest during the day and showed only a transient increase toward the end of the night. Overall, the clear pattern that was observed in the laboratory could not be reproduced in the field. We identified a variety of factors that may have influenced EOD activity patterns. First, the lower water temperatures likely reduced EOD rates during the night (e.g., Toerring and Serrier, 1978). Second, nocturnal hypoxia could have led to reduced EOD rates (Clarke et al., 2020; Moulton et al., 2020), if it was not compensated for, e.g., by aquatic surface respiration (ASR), during which fish ventilate the well-oxygenated surface water layer (Lewis, 1970; Chapman and Chapman, 1998). Third, *P. degeni* maintained a higher level of activity during the day under the floating vegetation than in the tubes of the shuttle-box. This effect could not be discerned in *M. victoriae*. It appears that the availability of an extensive sheltered habitat stimulated diurnal activity (e.g., exploration and foraging) of *P. degeni*. A possible factor for the species difference in diurnal activity could be their different social behavior, with the group-living *P. degeni* exhibiting higher diurnal activity than the solitary *M. victoriae*, although this remains speculative.

Social Interaction

Unlike many other fishes, which rely primarily on visual cues for social interaction (e.g., reviewed by Rowland, 1999), weakly electric fishes can use their active electrosense to communicate and interact with conspecifics, and thus are well equipped to do so at night (Moller et al., 1989; Scheffel and Kramer, 1997; Carlson and Hopkins, 2004). The sampling design we used did not allow detailed observation of social interactions, and an in-depth analysis of EOD recordings from co-occurrences was beyond the scope of this study, which focused on the temporal and spatial dynamics of fish behavior. However, some conclusions can be drawn from the statistics of recorded co-occurrences of fish. Here, it is important to consider the different detectability of the two species. While co-occurring *P. degeni* had to be within approximately 1 m of each other to be detected simultaneously in a logger recording, *M. victoriae* could be more than 3 m apart from each other and still be detected together. Thus, based on co-occurrences, the degree of social interaction is likely somewhat overestimated in *M. victoriae* (co-occurrence without interaction) and underestimated in *P. degeni* (detection of one fish out of a group). Notwithstanding these limitations, the fact that co-occurrences increased by 850% (*M. victoriae*) and 450% (*P. degeni*) strongly suggests that fish encountered and interacted with conspecifics more frequently at night. Nocturnal bouts of social interaction have also been described in the phylogenetically distant gymnotiforms and could be a common characteristic of weakly electric fishes (Silva et al., 2007; Stoddard et al., 2007). *Marcusenius victoriae* has been described as a territorial species, which is solitary and displays high intraspecific competition for shelters (Carlson, 2016). Our recordings suggest that territoriality and competition for shelters is most prominent during the daytime, when *M. victoriae* were recorded exclusively in the vegetation, and were alone in ca. 98% of those encounters. During the night, *M. victoriae* seem to show a higher degree of social affiliation and/or acceptance of conspecifics in their proximity. Interestingly, most co-occurrences of *M. victoriae* were recorded in the open habitat, which may suggest that they favor interacting in a more "neutral zone" than in the habitat where fish occupy their shelters during the day. *Petrocephalus degeni*, on the other hand, has been described as a gregarious species (Carlson, 2016), and can be kept in group tanks more easily than *M. victoriae* (pers. observation). There was no difference between the two species in the proportion of conspecific co-occurrences, however, given the considerable difference in detectability, this finding supports the notion that *P. degeni* occurs naturally in social groups.

During most encounters with multiple fish, both species were present. In some cases, the low-amplitude EODs of a *P. degeni* were only revealed due to the presence of another fish whose EOD amplitude crossed the detection threshold, which was frequently *M. victoriae*. Nevertheless, this implies that both species occur in spatial proximity and likely are connected through interactions and/or competition for resources. More detailed behavioral observations, e.g., using a grid of electrodes (Henninger et al., 2018, 2020; Madhav et al., 2018), could give interesting insights into the nocturnal social behavior of these sympatric species.

Environmental and Endogenous Rhythms

Influence of Light

Behavioral rhythms can be governed by environmental cues and endogenous biological clocks, and the question, which one of these two components drives an observed behavioral rhythm cannot be answered without thorough study of the endogenous circadian rhythms of an organism (Aschoff, 1960). In the laboratory, where temperature and DO were kept constant, behavioral activity of *M. victoriae* and *P. degeni* corresponded closely to ambient light levels: locomotor activity and EOD rate peaked precisely at the onset of darkness. Although activity level was not directly quantified in the field, the patterns of fish encounters corroborate this finding. In the open area where day-night changes in illumination are most pronounced, the number of fish encounters followed a clear diel pattern. In the vegetation habitat where light conditions remain mostly dark throughout the diel cycle, this pattern was less obvious, and *P. degeni* showed the same level of activity during the day and the night. Both observations suggest that light intensity is an important factor for behavioral activity, however, they are insufficient to disentangle the effects of endogenous clocks that are entrained by light and the direct effect of light as environmental cue on the behavior of *M. victoriae* and *P. degeni*.

There was no evidence that fish anticipated the dark phase, which could have served to suggest the existence of a circadian clock (Reebs, 1994). Two findings, however, could indicate the effect of an endogenous rhythm on behavioral activity in *P. degeni*. First, they showed an increase in swim speed and EOD rate toward the end of the dark phase, both in the laboratory and in the field. A similar phenomenon has been documented in *G. petersii* (Bässler et al., 1979). Second, their EOD rate at rest was significantly higher during the night than during the day. A similar increase in nocturnal EOD baseline rate has been found in the pulse-type gymnotiform *Gymnotus omarorum*, which lives under naturally constant darkness (Migliaro et al., 2018). This phenomenon has been hypothesized to be under endogenous control, although this has yet to be confirmed under laboratory conditions. The presence of a circadian rhythm could be tested by subjecting fish to constant light and/or dark conditions for several days while assessing whether free-running behavioral rhythms can be detected (Aschoff, 1960). In a study with the pulse-type gymnotiform *Brachyhypopomus pinnicaudatus*, Stoddard et al. (2007) found circadian rhythms in electric signal parameters that are likely caused by melanocortin peptides, which act directly on electrocytes, and through serotonergic projections to neuronal pacemaker structures. However, in a recent field study, Henninger et al. (2020) also found no evidence for anticipation of the active phase in wild gymnotiforms.

Temperature and Dissolved Oxygen

Interestingly, the nocturnal increase of activity and movement range co-occurred with extreme nocturnal hypoxia at Petro Lagoon. In line with previous observations and surveys, which have consistently found both species in this site since the 1990s under hypoxic conditions (Chapman et al., 1996b, 2002; Chapman and Chapman, 1998; Carlson, 2016; Clarke et al., 2020; Moulton et al., 2020), the continuous *in situ* measurements of EODs, DO and temperature show for the first time that these fish remain present, and likely are most active, throughout the most extreme nocturnal hypoxia periods. This is an intriguing finding as, although both species investigated here have been found to be hypoxia-tolerant (Chapman and Chapman, 1998; Clarke et al., 2020; Moulton et al., 2020), the degree of nocturnal hypoxia found here far exceeded their respective physiological thresholds of oxyregulation (P_{crit}). P_{crit} is defined as the oxygen partial pressure at which an organism can no longer maintain a stable rate of oxygen consumption (oxyregulation) and begins to conform to environmental DO (oxyconformation; Beamish, 1964; Ultsch et al., 1978). In the study site, air saturation dropped as low as 2.7 ± 1.4% (on August 8, 2019), which is well below any P_{crit} measured in swamp-dwelling *P. degeni* (Chapman and Chapman, 1998: ca. 5.8%, Clarke et al., 2020: ca. 7.0%) and *M. victoriae* (Moulton et al., 2020: ca. 9.5%). In fact, mean PO_2 at this site remained below the lowest published P_{crit} for more than 50% of the diel cycle on three of the six sampling days. Ackerly et al. (2018) showed that a DO concentration of ca. 17.5% already negatively affected the critical swim speed and EOD rate of *M. victoriae*. Temperature decreased during the night at Petro Lagoon, which could reduce the rate of metabolic processes and somewhat buffer hypoxic stress. However, it seems unlikely that this effect would suffice to compensate for extremely low nocturnal DO, which suggests that environmental hypoxia significantly limited the performance of both species, particularly during their most active phase. This raises the question as to how these fish manage to maintain and even ramp up their levels of electrical and locomotor activity during extreme hypoxia. One possible explanation is that fish compensate for the nighttime hypoxia by using ASR (Chapman and Chapman, 1998). Since ASR requires that fish have free access to the water surface, fish have to emerge from the swimming vegetation to perform ASR. This might drive the nocturnal migration of both species into the open habitats. At night, ASR could be particularly well suited to increase oxygen supply as the absence of diurnal predators reduces the otherwise high risk of predation (Kramer et al., 1983). A similar behavior has been identified in Amazonian fishes that emerge from their macrophyte cover to perform ASR during periods of severe nocturnal hypoxia (Saint-Paul and Soares, 1987).

The diel fluctuation of DO and temperature could entrain endogenous circadian rhythms, and therefore affect behavioral activity on another level. The circadian clock has been shown to be closely related to the hypoxia-signaling pathway in mammals (e.g., Adamovich et al., 2017) and zebrafish (*Danio rerio*, Egg et al., 2013; Pelster and Egg, 2018; Sandbichler et al., 2018). Hypoxia-inducible factor 1α (HIF-1α) is a key regulator of the physiological hypoxia response. It dimerizes with HIF-1β under hypoxic conditions and regulates the transcription of genes by binding to hypoxia-responsive elements (Wenger et al., 2005; Schödel et al., 2011). The expression of HIF-1α is tightly controlled by the circadian clock and follows a circadian rhythm (Egg et al., 2013). On the other hand, hypoxic conditions lead to decreased expression levels of clock genes such as *period1* (Egg et al., 2013; Sandbichler et al., 2018). Adamovich et al. (2017) suggest that, mediated through HIF-1α, hypoxia is a resetting cue for circadian clocks. In fish that have adapted to chronic environmental hypoxia, such as *M. victoriae* and *P. degeni*, an interesting question for further studies would be, whether adaptation to hypoxia has affected this interaction, or whether the expression of clock genes remains constantly dampened by hypoxia.

Many fish species seem to possess peripheral clocks in their tissues that are light sensitive without the need for specialized light sensing organs such as eyes or the pineal gland (Whitmore et al., 2000; Frøland Steindal and Whitmore, 2019). The finding of such peripheral clocks illustrates that the concept of a centralized "master-clock" that governs rhythmic behavior does not extend to fish, which rather possess a full-body light sensitivity (Frøland Steindal and Whitmore, 2019). As oxygen levels are not homogeneously distributed throughout body tissues, it is likely that these peripheral clocks are differentially affected by hypoxia. Although this has not been shown in electric fish yet, their well-understood neural circuits and non-invasively detectable EODs make electric fishes promising subjects for future studies of biological rhythms and the influence of light, DO and temperature.

Recording Natural Activity Patterns With Electric Fish Loggers

Mormyrids are often considered nocturnal animals, however, this view is at least partially based on laboratory studies (Harder et al., 1964; Moller, 1970; Bässler et al., 1979; Cobert, 1984). As we show here, natural activity patterns can deviate from laboratory activity patterns. A closer look into the literature demonstrates that mormyrids exhibit a variety of natural activity patterns that would not all classify as nocturnal. Using capture by gill net in Loskop Dam, South Africa, Kruger (1973) found that *M. macrolepidotus* migrate at night from bottom waters to the littoral zone. However, based on stomach content analysis, he also concluded that they feed predominantly during the day at the bottom of the reservoir. In the Ivindo River district in Gabon, Hopkins (1980) recorded EODs of 23 mormyrid species in the field and noted that mormyrids in shallow water remained sheltered during the day and made occasional foraging excursions at night whereas fish that lived in deeper waters appeared to move around during the day, as well. Our study shows that *P. degeni* remain active during the day under floating vegetation. In all of these studies, mormyrid activity seems most clearly determined by ambient light levels, and might not follow a diel pattern in continuously dark habitats. This differentiation may seem trivial, however, it has far-reaching biological implications. Nocturnality suggests a fixed and recurring behavioral rhythm that is likely to be under the control of endogenous oscillations whereas direct light-sensitivity facilitates more opportunistic behavioral patterns and might be independent of endogenous clocks. Understanding the mechanisms that underlie natural activity patterns of fishes is a crucial step to understanding the dynamics of aquatic ecosystems. Weakly electric fishes offer great opportunities in this regard. Their EODs give insight into the activity, habitat use and social interaction of elusive, tropical fishes, that are impossible to gain through visual observation methods. Previous studies have used electrode arrays (Henninger et al., 2018, 2020; Madhav et al., 2018), or single/handheld electrodes (Lissmann and Schwassmann, 1965; Moller et al., 1979; Hopkins, 1980; Friedman and Hopkins, 1996; Migliaro et al., 2018) to record EOD activity of weakly electric fishes in the wild. The methods that these studies employed necessitated the supervision of a researcher, which naturally limited the timespan over which observations were made. Here, we introduce a new and unsupervised method to continuously record the EODs of weakly electric fish. Our approach is based on the deployment of autonomously operating logging devices. In comparison with the previously used electrode arrays, observations from individual loggers lack the spatial resolution that is necessary to track animals and document detailed behavioral interactions. Nevertheless, there are certain benefits to this approach. Distribution of individual loggers allowed us to sample from different habitats and cover a larger area than would have been possible using an electrode array, and it enabled us to make observations over longer timespans than previously possible. The low price per unit (ca. 70 €) makes this technology accessible and reduces the financial risk of loss of equipment in the field. Open source microcontroller technology, such as the Teensy, and the Arduino programming language are used by a large community of scientists and non-scientists. These features make the EOD logger a promising and open tool for studies that focus on longer-term temporal patterns, or that are conducted in challenging environments that are difficult to access, such as swamps and lakes. For future applications, it is conceivable that the recording program can be adapted to allow the connection of multiple EOD loggers to record with higher spatial resolution from areas of interest.

CONCLUSION

This study shows nocturnal behavior of two mormyrid fishes that persist in a habitat with extreme nocturnal hypoxia. Activity increases corresponded directly to ambient light levels indicating that light is an important environmental cue and/or zeitgeber for endogenous clocks. Compared to laboratory observations, nocturnal activity increases were less pronounced in the field, which is possibly due to lower nocturnal water temperatures, hypoxia, and a higher environmental complexity that fosters a higher diurnal activity level. Habitat use shifted between a strong preference for complex habitats during the day to increased activity in open and unprotected areas during the night, when DO concentrations are lowest. Co-occurrences suggest that social interactions take place in both species mostly during the active phase at night, and in the case of *M. victoriae* mostly in the open areas of the habitat, although further investigation is needed for a detailed description of the nocturnal social behavior of the study species. Given their preference for structurally complex habitats, freshwater ecosystems with dense vegetation are likely to play an important role in the ecology of the study species.

DATA AVAILABILITY STATEMENT

The original data presented in the study are publicly available at: doi: 10.6084/m9.figshare.19526155.v3.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Gesundheit und Soziales, Berlin, Germany.

AUTHOR CONTRIBUTIONS

All authors participated in the design of this study. SM developed the EOD logger, performed the field work and laboratory experiments with *P. degeni*, and data analyses. FO conducted the laboratory experiments with *M. victoriae*. SM drafted the manuscript and all authors took part in its revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.870043/full#supplementary-material

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The vertical light-gradient and its potential impact on animal distribution and behavior

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The visual environment provides vital cues allowing animals to assess habitat quality, weather conditions or measure time of day. Together with other sensory cues and physiological conditions, the visual environment sets behavioral states that make the animal more prone to engage in some behaviors, and less in others. This master-control of behavior serves a fundamental and essential role in determining the distribution and behavior of all animals. Although it is obvious that visual information contains vital input for setting behavioral states, the precise nature of these visual cues remains unknown. Here we use a recently described method to quantify the distribution of light reaching animals' eyes in different environments. The method records the vertical gradient (as a function of elevation angle) of intensity, spatial structure and spectral balance. Comparison of measurements from different types of environments, weather conditions, times of day, and seasons reveal that these aspects can be readily discriminated from one another. The vertical gradients of radiance, spatial structure (contrast) and color are thus reliable indicators that are likely to have a strong impact on animal behavior and spatial distribution.

KEYWORDS

vertical light-gradient, spatiotemporal distribution, animal behavior, behavioral choice, vision, behavioral state

Introduction

As humans, it is easy to relate to the impact that visual environments have on our mood and its profound effects on what we desire to do. We want to remain in beautiful environments but cannot wait to get away from places we find visually displeasing. Our preferred activities clearly differ between sunny and overcast days, as well as between the intense mid-day sun, the warm light of the setting sun or the blueish light at late dusk. These things feel almost too obvious and natural to realize that it is in fact a superior behavioral control at play.

All animals need to select habitats and continuously pick the right activities from their behavioral repertoire. These choices differ between diurnal, crepuscular, and nocturnal species and depend on their size, food source, main threats, and numerous other factors. The species-specific choice of where to be and what to do under different times of day and different environmental conditions is one of the fundamental pillars shaping ecological systems (Davies et al., 2012). Sensory information from the environment together with internal physiological conditions (hunger, fatigue, etc.) provide the information that sets behavioral states, which in turn determines the activities that each species is prone to engage in at any one time (Gurarie et al., 2016; Mahoney and Young, 2017; McCormick et al., 2020). If an environment changes, it will alter the input to behavioral states, and potentially change the activities or whereabouts in a species-specific manner.

The external information used to set behavioral states is obviously multimodal. Temperature and olfactory cues are undeniably important (Abram et al., 2016; Breugel et al., 2018), but vision arguably provides the largest amount of information for a vast number of species. Animals use vision to find suitable habitats, position themselves optimally in the habitat and select the behaviors that make best sense in the current place, time, and environmental condition. Especially for assessing the type of environment and determining the time of day and the current weather conditions, vision provides rich and reliable information.

However, it is not immediately obvious which aspects of visual information provide input to behavioral states. The position and movement of visible structures are used for orientation behaviors and active interactions with the environment, and they are often considered to be the main or only relevant type of visual information (Carandini et al., 2005; Sanes and Zipursky, 2010; Clark and Demb, 2016). But these aspects of visual information do not say much about the type of environment, time of day, season, or weather conditions. However, the visual structures used for active vision are seen against a background, which is often considered as redundant visual information. To reduce the amount of information and economize on both time and cost of processing, early visual processing in the nervous system is believed to remove much of the visual background (Wandell, 1995; Olshausen and Field, 2004). The absolute intensity and spectral balance, or large gradients of these across the visual field, are typically considered as mere disturbances to the visual information. But the visual background is far from superfluous. It specifically contains information that can be used to read the type of environment and the current conditions.

Until recently, there has been a lack of methods for quantitatively describing the visual background. With the environmental light field (ELF) method (Nilsson and Smolka, 2021), there is now a tool by which visual environments, in particular the general background, can be comprehensively quantified (**Figure 1**). As a function of elevation angle, the ELF method measures the absolute intensity (photon radiance), the intensity variations (contrasts), and the spectral balance. In principle, the ELF method describes the vertical gradients of intensity, visual structure and spectral balance in the environment. These are the features that remain when scene-specific information is removed. The vertical light gradients depend on the type of environment, its quality, and the current conditions (time of day, weather, season, etc.) (Nilsson and Smolka, 2021). This is exactly the information animals need for assessing their environment and selecting relevant behaviors. If we can quantify the visual background in different habitats and how it varies with time of day and current environmental conditions, we can tap into the information that animals most likely use for deciding where to be and what to do.

Here, we compare the vertical light gradients from different environments, seasons, times of day, and weather conditions. Based on these comparisons, we characterize the visual background information to identify aspects of the information that animals may use for assessing the environment and setting behavioral states. We specifically test whether different landscapes and their current condition can be reliably determined by information extracted from the vertical gradients of intensity, spatial structure (image contrasts), and spectral balance.

Materials and methods

Vertical light gradients were measured from raw images taken with digital cameras (Nikon D810 or D850) equipped with a 180° lens generating circular images (Sigma 8 mm F3.5, EX DG Fisheye) (see Nilsson and Smolka, 2021 for details). The camera was oriented horizontally, such that the image covered all elevation angles from straight down (−90°) to straight up (+90°). To extend the dynamic range, daylight and twilight scenes were captured using bracketing with three different exposures (automatic exposure ±3 EV steps). Night-time scenes with only starlight were captured with the camera on a tripod and a single exposure of 8 min, followed by another 8 min exposure with the shutter closed (to quantify and subtract sensor-specific dark-noise). For comparisons of different environments and different weather conditions, we sampled some 10–30 different scenes within each environment to produce "average" environments with a minimum of scene-specific bias. For comparisons over time (24 h), we used a single scene with the camera mounted on a tripod. Under-water measurements were made using a custom-built under-water housing with a hemispherical dome port centered around the anterior nodal point of the fisheye lens (to obtain identical angular mapping in terrestrial and aquatic environments).

Images were analyzed using open-source software described in Nilsson and Smolka (2021). This software uses radiometric calibration files to compute the median radiance, together with the 50–95% range of data, as a function of elevation angle. Calculations were made separately for the red, green and blue

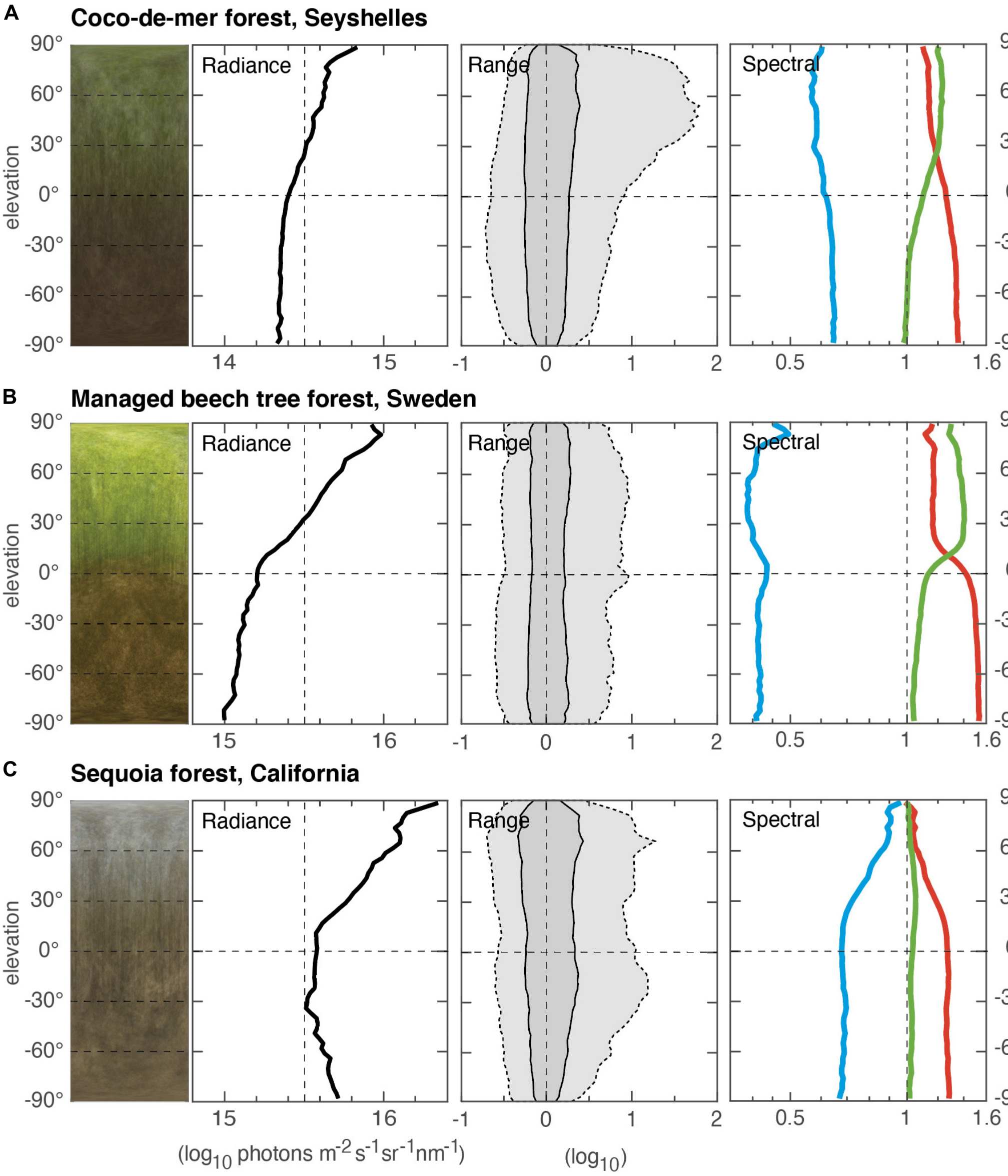

FIGURE 1

Vertical light gradients in three forest environments recorded with the ELF method (Nilsson and Smolka, 2021). For each environment, radiance data sampled from many different positions (scenes) in the environment is plotted as a function of elevation angle. An average image (compressed in azimuth) from the contributing scenes (180° by 180°) is shown to the left, followed by panels showing the intensity (radiance) on an absolute log-scale, the intensity range on a relative log-scale (dark gray, 50% of all intensities; light gray, 95% of all intensities), and to the right, the contribution of red, green, and blue light plotted on a relative log scale. **(A)** A wet tropical forest at the center of Praslin Island, the Seychelles (measurement based on 51 scenes, 29 December 2017, start 15:16, duration 51 min). The dense forest is dominated by coco-de-mer and other palm trees with leaves at multiple levels. **(B)** A managed beech tree forest, close to Maglehem, southern Sweden (measurement based on 13 scenes, 14 May 2015, start 13:55, duration 4 min). Smaller trees and bushes are largely cleared, leaving largely open space under the dense but translucent canopy. **(C)** Dense sequoia forest, Sierra Nevada, California, United States (measurement based on 31 scenes, 17 April 2015, start 12:02, duration 42 min). All environments, **(A–C)**, were measured on clear, sunny days when the sun was at least 45° above the horizon.

channels. Calculations were performed at the full resolution of the camera, but the output was binned for 3° bands of elevation (and 180° azimuth), effectively generating a resolution of 3° for elevation angles. Calibrations were made in photon flux per nm wavelength, providing comparable values for white-light and individual spectral channels. Radiances are given as $\log_{10}$ values to provide manageable numbers over the full range of natural intensities from night to day. Using *median* rather than *mean* values, returns "typical" radiances for each elevation angle, where any strong light sources are represented mainly as peaks on the upper bound of the 95% range. The computed data was plotted in three different panels showing the vertical gradients of (1) median radiance (intensity) for white-light, (2) range of intensities (50–95% data range, normalized to the median radiance) and (3) the relative radiances (relative color) of red, green and blue light (normalized to the median white-light radiance).

For spectral sensitivity of the red, green and blue channels of Nikon D810, see supplementary information to Nilsson and Smolka (2021). The spectral sensitivities of Nikon D850, used for some of our measurements, is very similar and displayed in **Supplementary Figure S1** for this manuscript. For both camera models, the median wavelengths of the spectral channels are: blue 465; green 530; and red 610 nm. As computationally demonstrated in the supplementary information of Nilsson and Smolka (2021), the spectral balance curves for environment measurements are not very sensitive even to larger changes in the spectral sensitivities of the camera, implying that other radiometrically calibrated models or makes of RGB cameras (with color image sensors having red, green, and blue pixels) would provide close to identical measurements. The reason for this robustness is that the spectral curves summed over 180° azimuth bands are always very broad in natural environments.

To give a visual impression of the vertical gradients, the software also generates an equirectangular image projection of the scene or average environment (horizontally compressed in our figures).

Results

For more than a decade, we have used the ELF method to measure vertical light gradients in a large range of natural environments from across the world. The measurements have been performed at different times of day, weather conditions and seasons. From this large collection of data, we here use selected typical measurements to identify the features of vertical light gradients that carry information about the type of environment, time and weather.

In **Figure 1** we compare three different forest environments (a dense tropical forest dominated by coco-de-mere and other palm tree species, a managed beech tree forest, and a sequoia forest). The vertical light gradients differ significantly and in many ways between the different forests. The radiance gradient (in the text referred to as intensity gradient) has a steeper slope (more light from above than from below) in the managed forest, with a dense canopy but little undergrowth, compared to the tropical forest with multiple layers of vegetation. In the sequoia forest, the intensity gradient has an obvious C-shape that we have seen also in other coniferous forests with cone-shaped trees (spruce). By a large margin, the tropical forest is the darkest (note that the intensity values are logarithmic, and a difference of one represents 10 times difference in intensity). Other differences are seen in the range plots, where the glossy but opaque leaves of the tropical forest generate a characteristic P-shape caused by high intensity spots of specular reflections centered around the direction of the sun. This phenomenon is not seen in the beech forest with its more translucent leaves. The color gradients are also markedly different between all three types of forest, especially the relative amount of blue light and the inflection of the red and green curves. These spectral differences depend heavily on the fractions of light reflected or transmitted through the foliage.

Semi-open environments (**Figure 2**) differ from dense forests by significant amounts of direct skylight/sunlight or diffuse light from clouds. This causes a characteristic shift in the spectral balance (color) at the skyline. On clear days (at which all three data sets in **Figure 2** were acquired), the sky is always dominated by blue light, with less green and even less red. Below the skyline, this order is reversed because vegetation and other organic material are often poor at reflecting blue light but efficient in reflecting red. Fresh green leaves favor green, whereas red dominates in light reflected from dirt, leaf litter and non-photosynthesizing parts of plants. The intensity and range gradients depend much on the type of vegetation, how tall it is, how far apart trees and bushes are and how dry or lush the vegetation is. The color of the ground is also a matter of great variation. An obvious example (**Figure 2**) is the Australian bushland environment, which has a bright ground dominated by dry mineral dirt, whereas the environments from Sweden and Cape Verde have a much darker ground dominated by organic matter.

In fully open environments, where little or no vegetation lifts the skyline above the horizon, the vertical light gradients are split sharply by the horizon (**Figure 3**). On clear days, the sky intensity gradient is revealed in full, with the highest intensities just above the horizon. If the ground reflects much light, as in the Californian lakebed, intensities below the horizon may significantly exceed those of the sky (note that direct sunlight does not contribute much to sky median intensity, but it does illuminate the ground). The elevation of the sun is visible mainly as an intensity peak on the 95% data range of the range graphs. A bare ground also leads to narrow range profiles, whereas vegetated ground, as in a meadow, generates a broader range and also much lower intensity compared to the sky. The color gradients in the sky depend on solar elevation and air humidity, but below the horizon they reveal the color of the ground.

Aquatic visual environments are very different from terrestrial ones (MacIver et al., 2017; Nilsson, 2017), and this is true also for the vertical light gradients (**Figure 4**). The horizon is typically much less pronounced, but because light is refracted as it enters water, the 180° terrestrial hemisphere is compressed to a cone of 97°, called Snell's window (see Cronin et al., 2014). In shallow pelagic environments, Snell's window makes a sharp intensity shift at an elevation angle of 48.5°, although movements of the water surface may blur this shift. In shallow epibenthic environments the lit seafloor makes Snell's window much less obvious. With increasing depth, Snell's window is replaced by a smooth vertical gradient, as exemplified

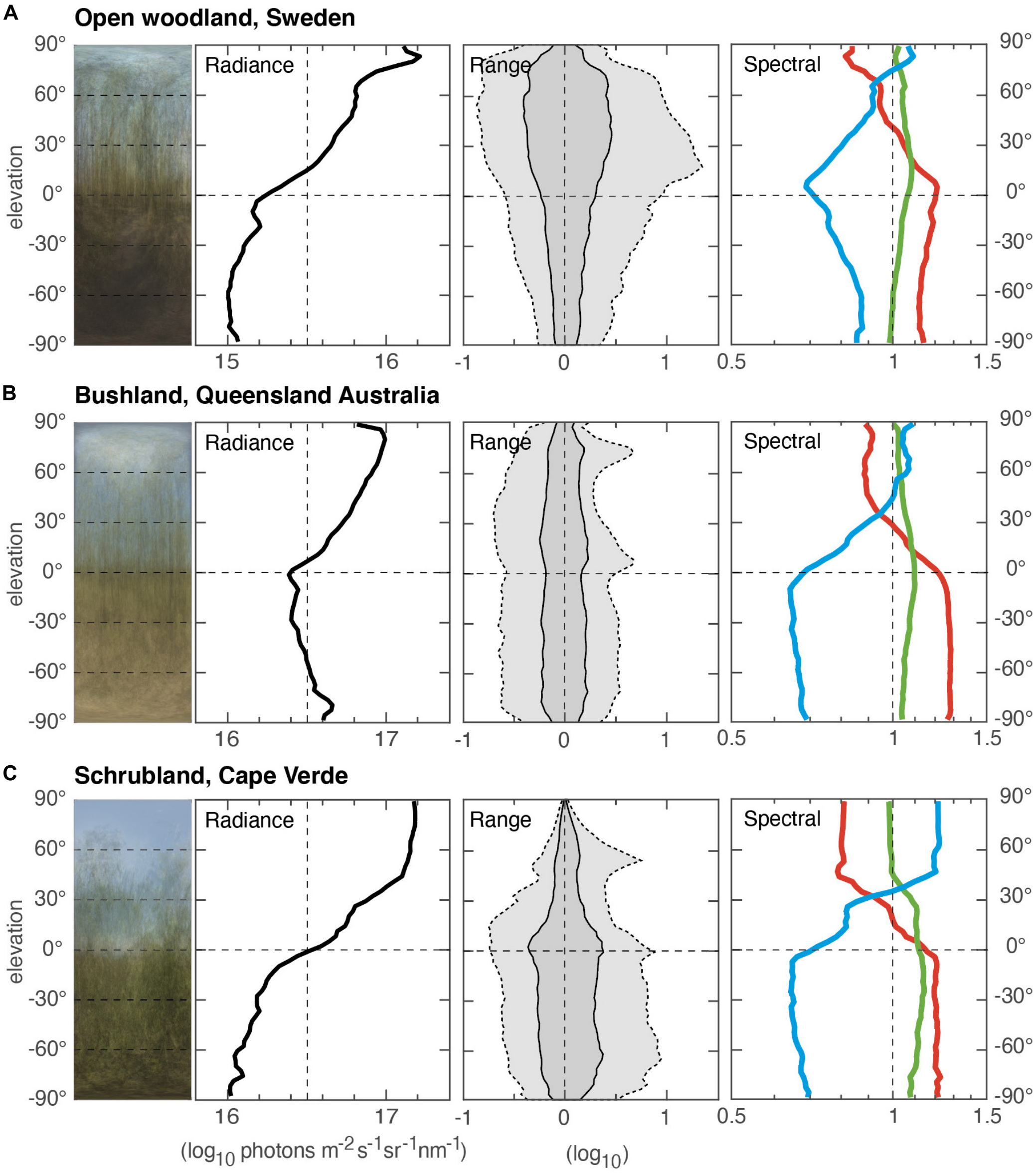

FIGURE 2

Three different semi-open environments. **(A)** Open woodland, close to Kivik, southern Sweden (measurement based on 20 scenes, 29 March 2020, start 15:12, duration 5 min). **(B)** Dry bushland at Mt. Garnet, Queensland, Australia (measurement based on 26 scenes, 3 December 2012, start 10:03, duration 14 min). Here, eucalypt trees are separated by dry grass. **(C)** Shrubland at Serra Malagueta National Park, Santiago, Cape Verde (measurement based on 14 scenes, 6 December 2016, start 15:08, duration 6 min). In this lush mountainous terrain, low trees are mixed with bushes. All environments, **(A–C)**, were measured on clear, sunny days when the sun was at least 45° above the horizon.

in the Cape Cod environment of Figure 4. In general, aquatic environments display much narrower range gradients because light scattering reduces contrasts in water (except for high-amplitude light-flicker at the edge of Snell's window). The spectral gradients may be very complex in shallow epibenthic habitats, but simpler in pelagic and deeper habitats. Different types of water preferably transmit specific wavelength regions, and this makes the vertical color gradients depend strongly on depth.

In all naturally lit environments, the time of day causes major changes to the vertical light gradients. Figure 5 illustrates a single scene from an Australian desert monitored round the clock under a constantly clear sky. Apart from the obvious intensity shifts covering a total of eight orders of magnitude (100 million times difference in intensity), the vertical light gradients in the sky change slope and spectral balance depending on solar elevation. The large intensity shifts occur at solar elevations between −20° (below the horizon) and 10° (above

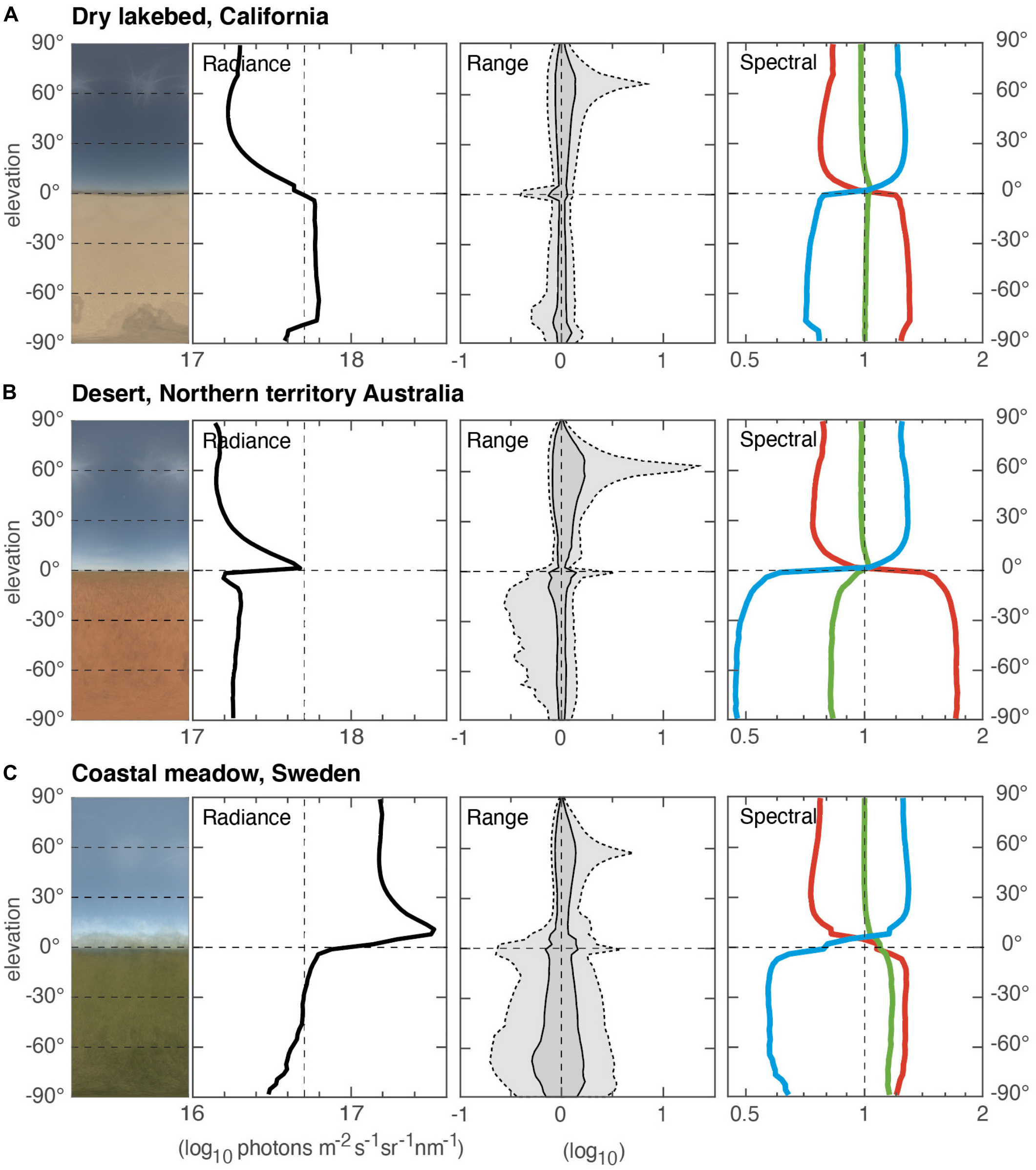

FIGURE 3
Open environments of different types. **(A)** Dry lakebed near Lancaster, California, United States (measurement based on 14 scenes, 13 April 2015, start 11:25, duration 7 min). **(B)** Desert with red dirt near Tilmouth Well, Northern Territory, Australia (measurement based on 23 scenes, 6 April 2019, start 12:37, duration 6 min). **(C)** Coastal meadow at Hovs Hallar, southern Sweden (measurement based on 22 scenes, 30 May 2020, start 16:17, duration 5 min). All environments, **(A–C)**, were measured on clear, sunny days when the sun was at least 45° above the horizon.

the horizon). For solar elevations above about 10°, the slope of the vertical intensity gradient provides a potential time signal in addition to the solar position. During dusk and dawn (solar elevations between 0° and −20°) there are also changes in the vertical intensity gradient in the sky, as well as a reliable change in the spectral balance between red, green, and blue light. When the sun's elevation is between −5° and −10° (below the horizon), there is a strong dominance of blue light (the blue hour). Under overcast conditions, changes in the vertical intensity gradient are largely lost, but the changes in spectral balance remain. When the sun is 18° or more below the horizon (astronomical night), and there is no moonlight, stars and airglow are the main light sources, causing a typical dominance of red light (Johnsen, 2012; Jechow et al., 2019; Warrant et al., 2020). During astronomical night, there is no time signal apart from the movement of the celestial hemisphere.

In **Figures 1–5** measurements have been made under clear skies. Under overcast conditions, all these measurements would

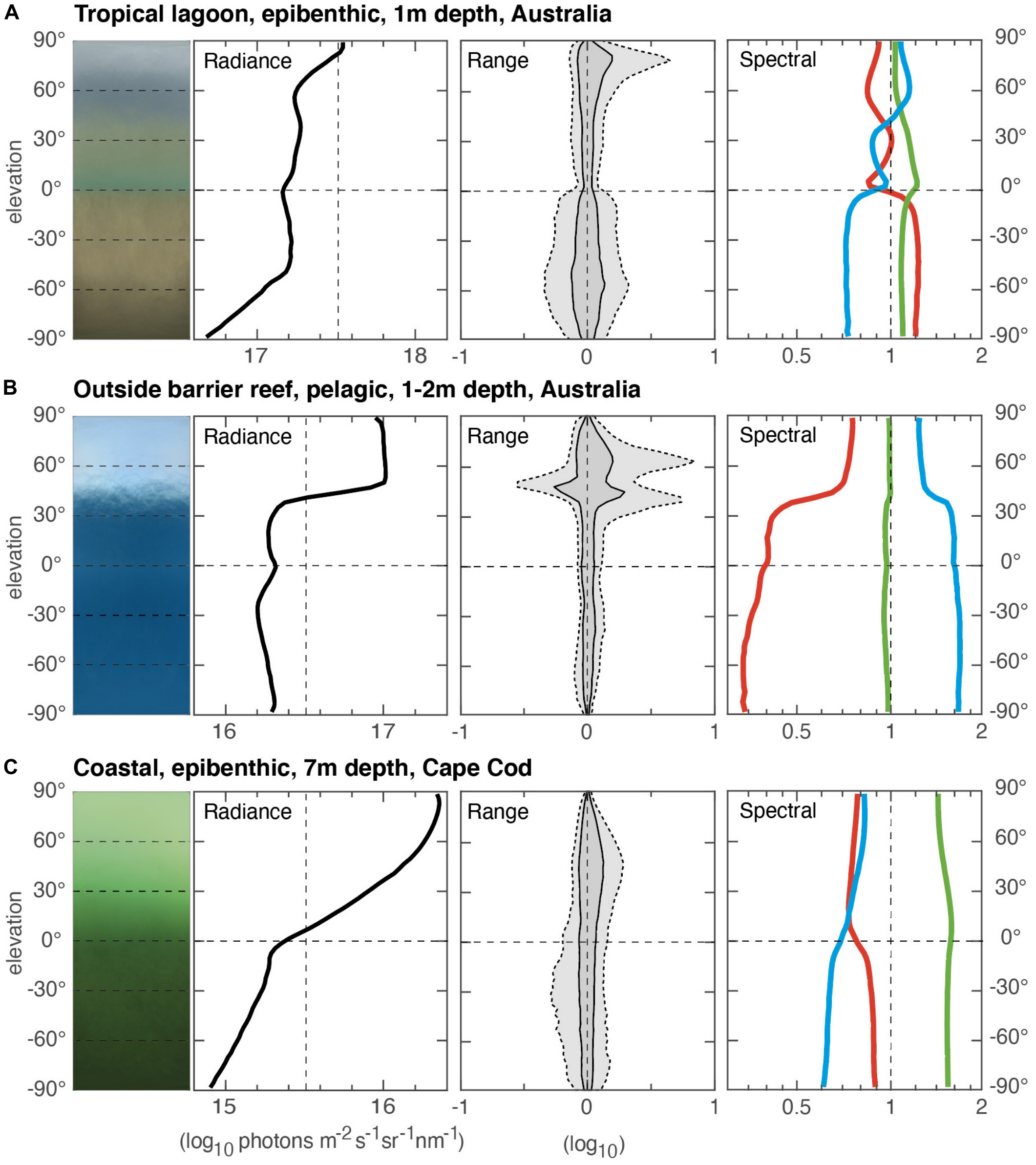

FIGURE 4

Shallow aquatic environments. **(A)** A tropical lagoon at Lizard Island, Queensland, Australia (measurement based on 40 scenes, 7 September 2015, start 12:17, duration 12 min). The environment was sampled at 1 m depth close to the bottom in the shallow lagoon. **(B)** Outside the barrier reef, at 1–2 m from the surface, over deep water with no visible seafloor, Queensland coast, Australia (measurement based on 27 scenes, 4 September 2015, start 14:31, duration 11 min). **(C)** Coastal epibenthic environment at 7 m depth outside Cape Cod, Massachusetts, United States (measurement based on 24 scenes, 19 October 2016, start 15:52, duration 8 min). All environments, **(A–C)**, were measured on clear, sunny days. In panels **(A,B)**, the sun was at least 45° above the horizon, and in panel **(C)**, 20°C above the horizon.

have been different. The changes in vertical light gradients from clear sky to overcast (100% cloud cover) are major and similar in terrestrial and shallow water environments, although dense forests are less affected. In open or semi-open environments during the day, overcast conditions cause a massive reduction of intensity below the skyline (Figure 6), with no corresponding drop of sky radiance. This is caused by the loss of direct sunlight, which under clear skies makes up about 80% of the irradiance (the blue sky making up the remaining 20%). A heavily overcast sky may be darker than a blue sky, but a lightly overcast sky may even be brighter. Typical for overcast conditions is also that the range of intensities is dramatically reduced. A third consequence of overcast skies is that the vertical color gradients change to almost neutral above the skyline. The relative contribution of blue light decreases above the skyline but increases below the skyline. Overcast skies thus cause major

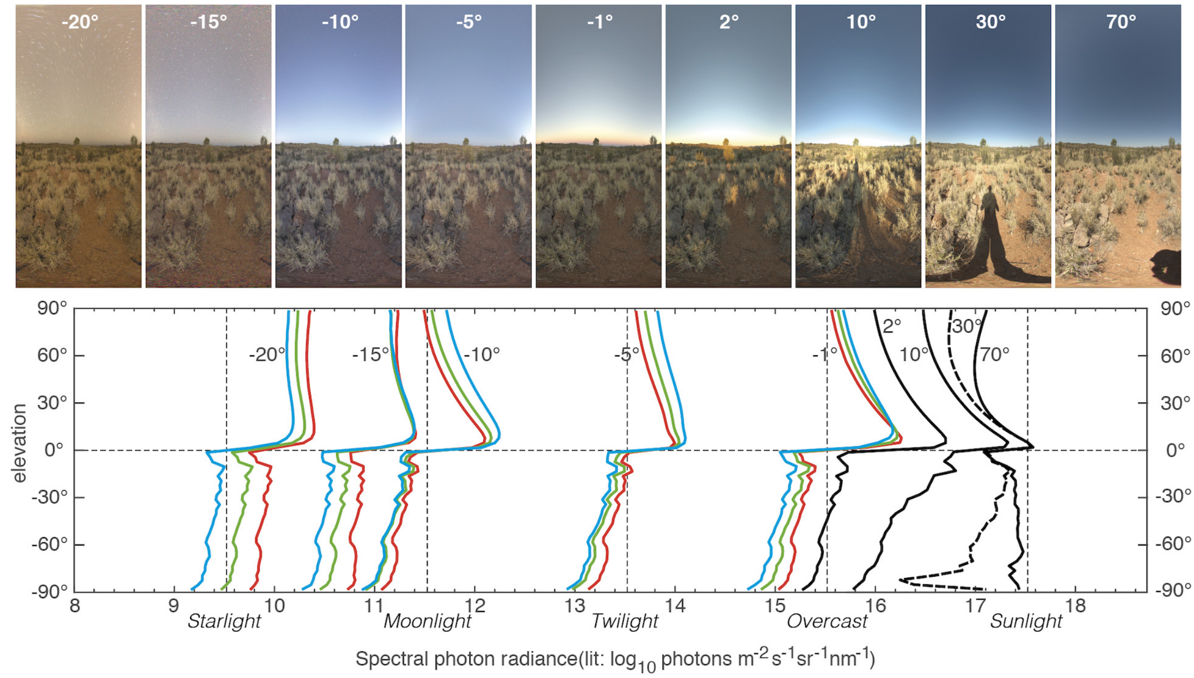

FIGURE 5

Vertical light gradients at a single scene in arid country south of Alice Springs, Northern Territory, Australia, sampled at different times, both day and night (17–18 October 2017). Images of the scene are shown above for each of the solar elevations plotted in the graph below. Absolute radiances are plotted with the sun at different elevation angles from −20° (below the horizon) at night to 70° above the horizon during the day. Night-time measurements are plotted with separate curves for red, green, and blue to show the shifts in spectral balance. To avoid clutter, daytime measurements are not divided into separate curves for different spectral bands (Full daytime graphs are provided in **Supplementary Figure S3**). The curve for 30° is shown as a dashed line for clarity. Measurements were taken at new moon to avoid influence of moonlight at night. The location was selected to be entirely free of detectable light pollution. All displayed measurements are of the antisolar hemisphere. The solar hemisphere was also recorded and found to be similar, but at daytime, angles ±20° of the sun's elevation are noticeably brighter. The labels, *Starlight*, *Moonlight*, *Twilight*, *Overcast*, and *Sunlight* are not related to the displayed data, but provided by the analysis software as guides for typical radiances. All measurements provided in the figure were recorded under completely clear skies.

changes in vertical light gradients. Partial cloud cover results in intermediate changes, which are closer to clear conditions if the sun or moon is not occluded.

Vertical light gradients change also with the seasons, especially in areas of deciduous vegetation or winter snow cover (**Figure 7**). Leaves in deciduous forest canopy change the spectral balance as the leaves mature and eventually acquire autumn colors. Springtime vegetation on the ground, before the canopy takes too much of the light, is obvious in the vertical gradients of April and May in **Figure 7**. Dry or wet leaf litter on the forest floor causes additional variations in the vertical light gradient, and snow cover obviously has a major effect.

Discussion

Visual assessment of the environment

It is obvious that animals equipped with vision can be expected to use this sense to assess their environment for choosing their whereabouts and activities. But which kinds of visual information do animals use for assessing their environment? There can be two principal answers to this question: identified objects and non-object-based visual information (Nilsson, 2022). Seeing identified objects, such as prey or other food items, conspecifics, predators, or objects that can be associated with any of these, are all potentially very useful for assessing the situation and choosing suitable activities (Hein, 2022). There can be no doubt that animals capable of object discrimination also use this visual modality to assess their environment.

However, detection, identification and classification of objects require several layers of devoted neural circuits for visual processing as well as cognitive abilities to translate the information into adaptive behaviors. Non-object based visual information is simpler, requires fewer levels of neural processing and was present before object vision evolved (Nilsson, 2020, 2021). The ancient visual roles that are not based on object vision must have involved assessment of the environment, and because they exploit very different types of visual information, such visual mechanisms are likely to remain important even after object vision evolved.

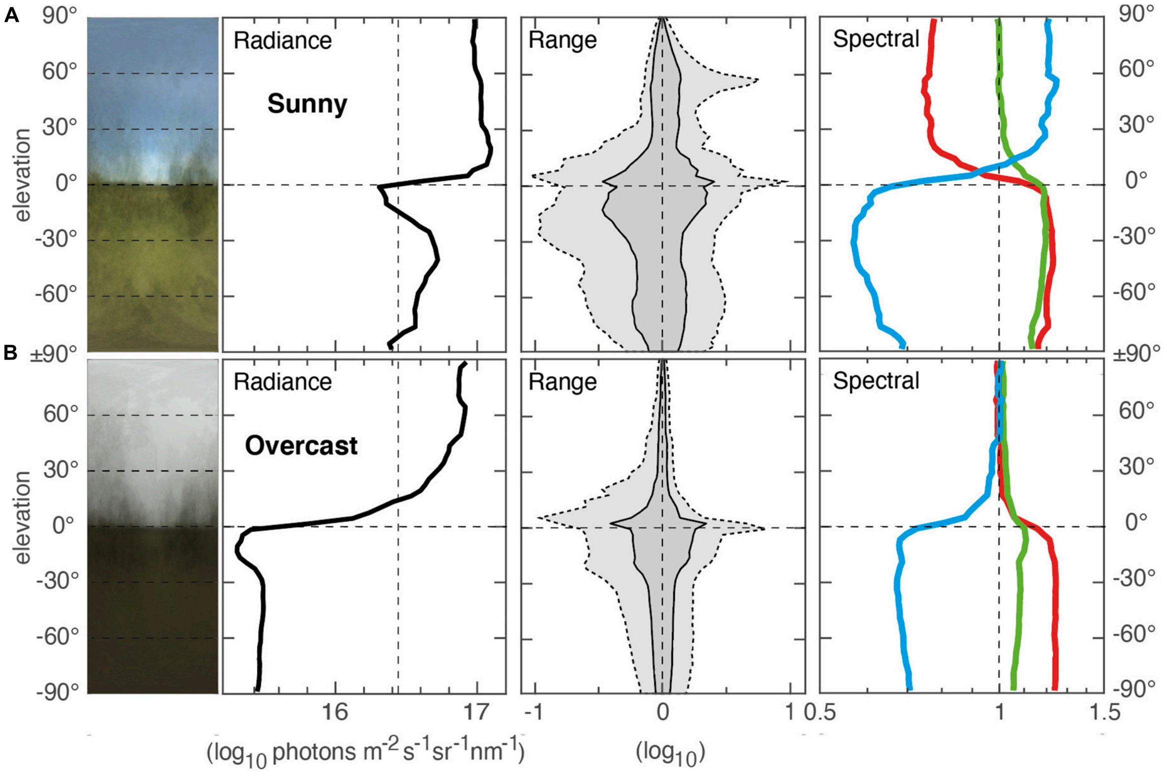

FIGURE 6

Vertical light gradients in a semi-open environment near Dalby, southern Sweden measured in clear sunny conditions **(A)** and overcast conditions **(B)**. Measurements were based on 21–25 scenes, sampled on 21 July 2016, start 13:30 m, duration 7 min **(A)** and 17 September 2016, start 12:44, duration 6 min **(B)**. A single scene at another environment, recorded at sunny, partially cloudy and fully overcast is provided in Supplementary Figure S4.

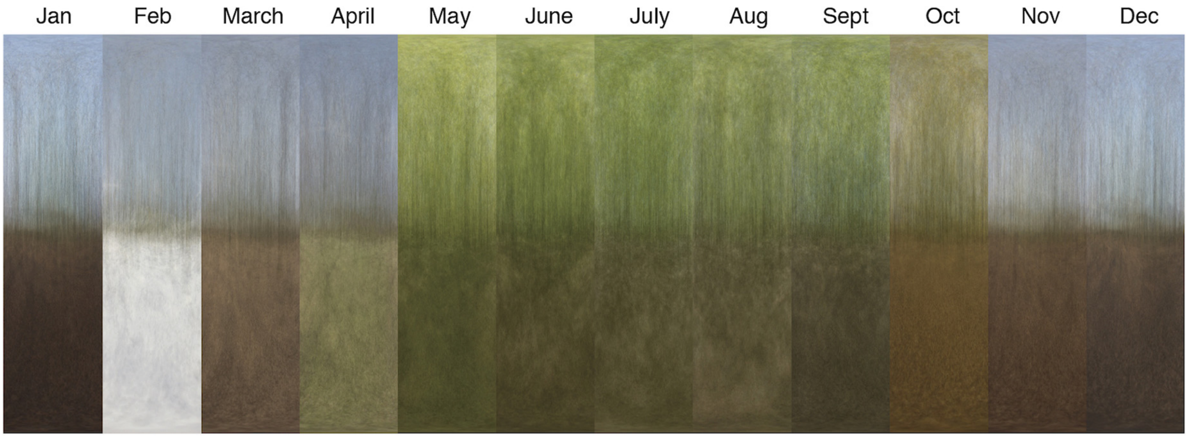

FIGURE 7

Average images (±90° elevation) of a beech-tree forest near Dalby, southern Sweden, sampled in clear sunny conditions each month of the year. The images were sampled randomly from a trail through the forest, resulting in 30–36 scenes contributing to each image. For these measurements, full results with light gradient graphs are found in Supplementary Figure S5.

Visible structures that are not identified objects are fundamental for orientation, control of movement and many other visually guided behaviors (Rodiek, 1998; Tovée, 2008). For assessing the visual environment, non-object-based vision can help determine the type and quality of the environment to aid in habitat selection and choice of activity. It may also be used to determine weather conditions and time of day, all of which are important factors for choosing the most appropriate location and behavior. Without object identification, the visual world is a spatial distribution of intensities. These may vary from up to down and from left to right, i.e., with elevation angle and with azimuth. It is the variations with elevation angle, i.e., the vertical light gradients, that can be expected to differ systematically between different types of environment, times of day, or weather conditions. The azimuth direction provides additional information on the range of intensities at each elevation angle, which is a proxy for the distribution and abundance of visible structures.

Vertical light gradients thus represent much of the non-object based visual information that is available for assessing the environment. Being both simple and informative, vertical light gradients are potentially essential for a wide range of animals in their ability to assess the type of environment, the time of day and the weather conditions. It follows that vertical light gradients may have a fundamental impact on the distribution and activity of animals.

In this manuscript we have presented measurements of vertical light gradients from different environments, terrestrial as well as aquatic, different weather conditions, and times of day and seasons. The gradients were divided into three components: intensity, intensity range (a proxy for spatial structure), and spectral composition. The intensity component can be further divided into the absolute position on the intensity scale, and the relative intensities at different elevation angles. In total, we thus identify four different components of information contained in the vertical light gradients (**Table 1**). With access only to one of these four components, it would be difficult or impossible to discriminate between different environments and tell them apart from differences that are due to weather or time of day. But combining all four components, such discrimination is generally straightforward. Color vision is needed for determining the spectral balance, and it makes the assessment of vertical light gradients more robust. Many vertebrates and arthropods, from both aquatic and terrestrial environments, can quickly relocate over longer distances, and in these two animal groups, color vision is common.

Many insects, reptiles and birds have excellent color vision which includes a UV channel (Kelber and Osorio, 2010; Cronin et al., 2014; Osorio, 2019). Our measurements were made with a consumer camera and are thus restricted to red, green, and blue channels, but it would clearly be interesting to include also the UV band (300–400 nm) in the measurements. Non-primate mammals are dichromats and would not be able to discriminate between red and green, and many nocturnal species have reduced or lost their color vision (Osorio and Vorobyev, 2008; Jacobs, 2009).

Although color vision is arguably an asset for evaluation of the environment, vertical light gradients are useful also for color-blind species. Most cephalopods (octopus, squid, and cuttlefish) are color blind, but instead have polarization vision (Shashar, 2014). This is a common modality also in arthropod vision (Labhart, 2016). For these animals, the polarization properties of light, as a function of elevation angle, is a potential additional component of the vertical light gradient.

Our measurements were computed for 3° resolution of elevation angles. To make reliable assessments of place, time, and weather, it would suffice to sample with much lower resolution. For nearly all vertebrates and many arthropods, the visual acuity would allow at least the same resolution as in our measurements. Many invertebrates, such as polychaetes, gastropod snails, onychophorans, and millipedes have small low-resolution eyes with an angular resolution in the range of 10°–40° (Nilsson and Bok, 2017), and even for these animals, vertical light gradients would contain useful information for habitat selection and behavioral choice. Interestingly, such low-resolution vision excludes the possibility of object vision, making vertical light gradients the only option for visual assessment of the environment (Nilsson, 2022). In many insects, the dorsal ocelli, and not just the compound eyes, provide sufficient spatial resolution to be used for reading vertical light gradients (Berry et al., 2007a,b; Hung and Ibbotson, 2014; Taylor et al., 2016).

Vertical light gradients from forests, semi-open and open environments (**Figures 1–3**) reveal large and obvious differences between types of environment and significant differences also between different environments of the same general type. This implies that robust visual cues are available to animals for habitat selection and choice of activities. Within a forest, the vertical light gradients may also differ significantly between different locations, such as the forest edge, local open patches and areas with different plant compositions. Consequently, vertical light gradients are potentially important cues determining the distribution of animals over a large range of spatial scales.

In addition to terrestrial environments, we also presented a small selection of aquatic environments (**Figure 4**). These are clearly very different to the terrestrial world, largely because of the noticeable loss of contrast with distance. This means that aquatic visual scenes have much fewer objects and other structural details, and are far more dominated by the vertical light gradients of intensity and spectral balance. In aquatic habitats, the vertical light gradients have additional dependencies on depth and water quality, which adds new types of important information. This also means that type of environment, time and weather may be more entangled and more difficult to separate in aquatic light gradients. Here we only present a small sample of aquatic measurements to show that variations are large also in the under-water world. A more rigorous analysis of vertical light gradients in aquatic habitats will be published separately elsewhere.

To make measurements comparable in **Figures 1–3**, they were all made close to noon and under clear skies. The

TABLE 1 Four components of the vertical light gradient identified in this investigation.

Component	Type of information
1. Absolute intensity	Position of the intensity gradient on the absolute intensity scale. This directly follows the irradiance (amount of light illuminating the environment), which varies over eight orders of magnitude between day and night, and over a few orders of magnitude between different weather conditions and environments (depth in aquatic environments can cause enormous changes)
2. Relative intensity	The shape of the intensity profile, i.e., the relative amount of light from different elevation angles
3. Range	The variation of intensities (amount of contrast) at each elevation angle. This is a proxy for the amount of spatial structure at different elevation angles. The two ranges (50%, dark gray and 95%, light gray) describe different aspects of this component
4. Spectral balance	The relative amount of major spectral bands (colors) at different elevation angles

The first two components can both be read from the intensity graph of **Figures 1–6**, as the general position on the intensity scale and the shape of the curve.

measurements would be partly different at other times of day or other weather conditions, but the different environments can still be discriminated from each other because time of day and weather affect the vertical light gradients in very specific ways (**Figures 5**, **6**). This means that information about the time and weather can be extracted from the vertical light gradients irrespective of the type of environment. However, there are exceptions where the type of environment, time or weather cannot be entirely separated, or some aspects cannot be read from the vertical light gradients. In dense forests the vertical light gradients change rather little with weather, and also with time, if dusk and dawn are excluded. Cloud cover can mask changes that depend on time, and at starlight intensities most animals are color blind (Cronin et al., 2014; Warrant et al., 2020). Under these conditions, animals may resolve the ambiguities by other sensory modalities or by their biological clock.

The difference between clear and overcast sky (**Figure 6**) is obvious, but there are of course other weather conditions affecting the visual world. Partial cloud cover, with or without the exposed sun, thin high-altitude clouds, different amounts of air humidity, precipitation, fog and suspended particles will all cause specific changes to the vertical light gradients. At night, the lunar phase will have an impact on the vertical light gradients, offering cues for regulating nocturnal behavior and setting lunar rhythms. Vertical light gradients also contain information on seasons (**Figure 7**), which are strongly linked to the conditions of the vegetation. Likewise, extended droughts or wet periods will cause changes in the vegetation that are detectable in the vertical light gradient. Finally, anthropogenic alterations of the environment or climate change will influence the vertical light gradients and this may mediate effects on animal distribution and behavior. We conclude that vertical light gradients provide simple but reliable visual cues that can be used by animals to assess a broad range of essential qualities and conditions in their environment.

With an exposed sun or moon, vertical light gradients depend on the azimuth angle. When the sun or moon is far from zenith, solar and antisolar (or lunar and antilunar), directions display different sky gradients as well as different gradients below the horizon. This can be used by animals for directional cues, but for information on the type of environments, time of day or weather conditions, averaging over azimuth orientations (compass directions) offers a way to eliminate such bias. Recording many scenes at random azimuths then corresponds to animals integrating vertical light gradients with time constants much longer than those used for active vision.

How can the information content of vertical light gradients be further investigated?

Our database of vertical light gradients currently includes about 1,200 terrestrial and 100 aquatic environments distributed across the globe, at different times of day and different weather conditions. The material presented in this manuscript and in Nilsson and Smolka (2021) are samples from this large databank of measurements. The technique is simple to use, and many other labs are now contributing to these quantifications of the visual world. How can this wealth of new information be exploited?

There are of course limits to the conclusions that can be drawn from samples selected manually from a large database. A more systematic and unbiased approach would improve the use of vertical light gradients as a tool in various disciplines of ecology, physiology and neurobiology. However, it is not immediately clear how to analyze an entire database of vertical light gradients. The approach of choice will depend on the questions that are asked. The ability to independently determine the type of environment, weather, depth, etc., from the vertical light gradients is of great biological significance. A relevant question is thus whether vertical light gradients can be used to independently classify the type of environment, time of day and weather, and for aquatic environments also depth and possibly even water quality. It would further be important to know if there are conditions where it is difficult or impossible to fully read any of the factors that contribute to the vertical light gradients.

One approach is to apply software that quantifies similarities between different vertical light gradients to generate a phylogenetic tree of measurements. The degree by which measurements from different environments, times of day, weather, etc., assemble in different clades would thus quantify the reliability of vertical light gradients for informing about the environment and its conditions. Failures to place measurements in clades that correspond to different environments and conditions would indicate the limits of useful information from vertical light gradients. We have made preliminary test with this approach and found that existing Matlab routines for phylogenetic analysis can be adapted to successfully classify different types of terrestrial environments (see **Supplementary Figure S2**). An advantage of this approach is that environment classification is part of the results and is thus unbiased. It is of course also possible to use the same approach to test the support for arbitrary classifications made beforehand.

A principally different approach would be to tag measurements with environment type, time of day, degree of cloud cover, etc., and train a neural network to classify measurements from a subset of the database, and then validate how well it can classify the remaining database. This approach can be adapted to arbitrary measurement classifications and allows investigations of how well different classes can be discriminated. There will of course always be a potential bias introduced by subjective classifications of weather or type of environment (whereas time of day or water depth can be objectively classified). This implies that strict classification criteria would have to be established.

Both the above approaches allow for quantification of the minimum amount of information on the vertical light gradients that is needed for successful discrimination between types of environments, time of day, weather, depth in water, etc. Our measurement routines generate a 3° resolution of elevation angles, but it is quite possible that much lower resolution suffices. It is also possible that some components of the vertical light gradients or angular spans are more important than others for reliable classifications. It would be of particular interest to identify the parts (elevation angles and intensity/range/spectral components) of vertical light gradients that are most essential for biologically relevant discrimination of environments and conditions. This would facilitate behavioral experiments on the effects of vertical light gradients.

The biological significance of vertical light gradients

We have concluded that vertical light gradients carry rich and essential information about the environment. This information is available to all animals with spatial vision, and it would be remarkable if it does not have a major influence on animal behavior and choice of habitat. The spatial distribution of light in natural environments is known to be reflected in the retinal design of both vertebrate and insect eyes (Zimmermann et al., 2018; Lancer et al., 2020; Qiu et al., 2021), but how animals use the overall distribution of light to assess their environment is practically unknown. The amount of light illuminating the environment (the illuminance or irradiance) is well-known to regulate behavior and to entrain biological rhythms across the animal kingdom (Hertz et al., 1994; Cobcroft et al., 2001; Kristensen et al., 2006; Chiesa et al., 2010; Tuomainen and Candolin, 2011; Pauers et al., 2012; Alves-Simoes et al., 2016; Farnworth et al., 2016; Kapogiannatou et al., 2016; Blume et al., 2019; Storms et al., 2022). Accordingly, artificial light at night (light pollution) is emerging as a major factor influencing the spatiotemporal distribution of animals (Polak et al., 2011; Owens et al., 2020; Hölker et al., 2021; Miller et al., 2022). However, with rare exceptions such as Jechow et al. (2019), it is the ambient light intensity (illuminance), not the spatial distribution of light, that is documented as the environmental cue. In contrast, many established effects of light may very well be sensed as vertical light gradients by the animals. It is clear that illuminance readings cannot be used to discriminate between the effects of time, weather, environments, or water depth, but the combined components of vertical light gradients can resolve these ambiguities. There are thus reasons to believe that, for many animal species, vertical light gradients represent the actual cue of which the general illuminance is just one component related to the absolute position of the intensity profile on the radiance axis. It is therefore possible that the majority of known responses to light intensity are in fact responses to vertical light gradients.

Given the potentially major and important effects on animal behavior, responses to vertical light gradients deserve attention, but how can such responses be investigated? For the vast majority of animal species, it can be expected that the spatiotemporal distribution and behavioral choices depend on the input from multiple senses, previous experiences and internal physiological states. To isolate the contributions of vertical light gradients, the first option would be to monitor behavioral choice in laboratory environments where the vertical light gradients can be manipulated when all else is kept constant. However, reproducing natural light gradients in a laboratory setting is not entirely trivial. Projection of light on a planetarium hemisphere is problematic because reflected light will illuminate all other elevation angles. Full control of the vertical light gradients requires bright light emitting diode (LED) monitors in all directions, at least above the horizontal plane, in addition to a direct light source simulating the sun or moon, and carefully controlled reflective properties of the ground and surrounding objects. The emission of red, green, and blue light must be individually controllable, and the produced light environments must be calibrated by measurements. The controlled environment must further be large enough to allow for a natural choice of behaviors of the tested species.

There are good reasons to attempt the type of laboratory test outlined above on a broad selection of species. Such experiments will reveal the impact of vertical light gradients and how much it differs between species. By manipulating the vertical light gradients, it will also be possible to uncover the components of the light environment that are most important for behavioral control. With a good knowledge of the effects of vertical light gradients, these can be applied to predict the consequence of changes in both natural and artificial environments, to identify desirable and undesirable changes, and to mitigate negative effects on the spatiotemporal distribution of animals. More specifically, vertical light gradients can be used to identify conditions that elicit swarming, aggregation, or other biologically important behaviors. It is also possible that vertical light gradients are used for endocrine control, to make the animals physiologically prepared for the preferred activities. Knowledge of the influence of vertical light gradients may also be used to create more appropriate conditions for physiological and behavioral animal experiments (Mäthger et al., 2022), and to improve artificial indoor lighting for humans and in animal husbandry.

There is also a neurobiological side of visual assessment. Vertical light gradients are likely to provide access to previously unexplored mechanisms that set behavioral states in animals. All animals constantly have to decide where to be and what to do. The choices could be to forage at the current location or search for a better place, to avoid or engage in aggressive mate interactions, to continue resting or initiate activity. At any moment, depending on the type of environment, time of day, weather condition or other visible cues, some activities are suitable, but others are not. The changing tendency to engage in some activities but not in others is often referred to as "behavioral states." This concept is gaining ground in both ecology and neurobiology (Berman et al., 2016; Gurarie et al., 2016; Mahoney and Young, 2017; Naässel et al., 2019; Russell et al., 2019; McCormick et al., 2020). The contribution of vertical light gradients in the setting of behavioral states must of course involve the visual system, but the neural processes that result in a choice of activity remains poorly know.

In most animals, the master-control of behavior is likely to involve several rather different processes. A general tendency to prefer some activities and avoid others can be set by internal physiological states together with sensory information about external conditions such as vertical light gradients. Behavioral tendencies are likely to involve a combination of systemic hormones and local release of neuromodulators that together alter the properties of decision-making circuits in the brain, i.e., set an appropriate behavioral state. For animals with at least some cognitive abilities, the actual decision of initiating or terminating a behavior will involve an assessment of the situation. In terms of vision, such an assessment would be based on the distribution of recognized objects (animals or other relevant items), combined with memories and experiences, and the outcome would be used to time and actuate behavioral decisions (Hein, 2022). Most species will also have protective reflexes that can override any behavioral decision. Once a behavior is initiated, it can be guided with continuously updated sensory information in a closed loop (Milner and Goodale, 2008).

The neural circuits that act as a master–control of behavior are poorly known in both vertebrates and arthropods. The neural pathways that carry information on vertical light gradients must be different from "ordinary vision" used for orientation and object vision. The reason for this statement is that "ordinary vision" relies on image contrasts and their movement (Rodiek, 1998), whereas vertical light gradients instead represent the background against which objects and contrasts are seen. To relay information on the movement of objects and other image contrast, the neurons need to be fast and provide high spatial resolution. To record the visual background, the opposite is true: it is contained largely in low spatial frequencies, and the information is extracted by integrating over time. Low spatial resolution means few neurons, and long integration time (slow speed) implies thin neurites. So, even if reading vertical light gradients may be a fundamentally important visual function, its neural correlates may have escaped attention because they are expected to make up only a small and inconspicuous part of animal visual systems.

Conclusion

The mechanisms that animals use to visually assess the environment and its conditions have been largely overlooked. Here we show that vertical light gradients contain vital information on the type of environment, time, weather, and other factors that animals are likely to rely on for finding suitable habitats and optimally deploying their behavioral repertoire.

Responses to vertical light gradients can be expected to be species-specific and contribute to define the niche of each species. Unraveling these responses will open a new dimension in behavioral ecology and provide an understanding of the mechanisms behind choices of habitat and activity. Measurements of vertical light gradients are easily acquired and offer a powerful tool for making predictions on the spatiotemporal distribution of animals, timing of specific behaviors and responses to a changing world.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

D-EN conceived the project with input from JS and MB. D-EN wrote the first draft. JS and MB refined the text. D-EN and JS prepared the **Supplementary material**. All authors contributed data and approved the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.951328/full#supplementary-material

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Mechanisms of flower coloring and eco-evolutionary implications of massive blooming events in the Atacama Desert

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The Atacama Desert, one of the driest places on earth, holds a rich biodiversity that becomes most appreciable in years when unusual rainfall accumulation triggers a phenomenon of explosive development of ephemeral herbaceous and woody desert species known as "desierto florido" or "blooming desert." Despite the scientific importance of this unique phenomenon only few studies have addressed the mechanisms of flower phenotypic divergence under the fluctuating environment provided by this recurrent event. We investigated the mechanisms of floral color diversity in *Cistanthe longiscapa* (Montiaceae), a dominant species across the ephemeral blooming landscape of Atacama Desert. Our analyses show that the variation in colors of *C. longiscapa* flowers result from petals containing betalain pigments with different absorption spectra. The different pigment composition of petals causes flower color differences in the visible and ultraviolet (UV) range of the spectrum. Through color vision models we show that *C. longiscapa* flowers are highly polymorphic in their color appearance for insect pollinators. Our results highlight the variable nature in flower color of *C. longiscapa* varieties blooming simultaneously in a geographical restricted area. Given the importance of color in attracting floral visitors, the observed color variability could contribute to increased cross pollination in extreme desert conditions, while accounting for complex and fluctuating histories of plant-pollinator interactions.

KEYWORDS

Atacama Desert, polymorphism, flower color, floral pigments, pollinators, color vision

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Introduction

The Atacama Desert, one of the driest places on earth, stretches along the western border of South America from coastal regions between 18 and 30°S up to 5,000 m (Arroyo et al., 1988; Schulz et al., 2012). Arid (precipitation of $\leq$50 mm/y) and hyperarid climates ($\leq$ 5 mm/y) can be recognized within this region, which in spite of its persistent aridity, undergoes pronounced rainfall fluctuations at interannual and interdecadal time scales. While interannual rainfall variability seems to be modulated to a large extent by El Niño-Southern Oscillation (ENSO), the pronounced low frequency changes during the past century, characterized by the occurrence of 'humid' (1920–1945; 1976–2002) and extremely dry (around 1910 and 1945–1975) periods, appears to be linked to large-scale climatic fluctuations in the Pacific region associated with the Interdecadal Pacific Oscillation (IPO) (Schulz et al., 2012). Rainfall fluctuations are directly associated with changes in the abundance of plants and animals, with intensive rainfall events inducing drastic changes in the desert landscape.

Despite representing one of the harshest environments of the planet, there is a rich biodiversity of plants and animals adapted to the unique extreme conditions of the Atacama Desert (Rundel et al., 1991). The colonization of the Atacama Desert by some plants and animals required a long lag time, occurring much later than the formations of its arid climates (Guerrero et al., 2013). In line with this, the evolution in some clades inhabiting this region seems to have occurred relatively recently (Guerrero et al., 2013). The highly diverse biota of the Atacama Desert becomes most appreciable in years when unusual accumulation of precipitation induces an explosive development of ephemeral herbaceous and woody desert species (Armesto et al., 1993; Vidiella et al., 1999; Jaksic, 2001). During these events, known as "desierto florido" or "blooming desert," different annual and geophyte species share dominance in a sequential appearance, triggering outbreaks of insects and vertebrates associated with these ecosystems (Vidiella et al., 1999; Chávez et al., 2019). Despite its scientific importance, little is known about the eco-evolutionary processes triggered by this unique phenomenon. For instance, plants native to this ecosystem have evolved traits associated not only with unpredictable dynamics of rainfall tolerance, but also traits to ensure pollination during short but massive blooming in the Desert (González and Pérez, 2010). Most of our current understanding of flower signals arises from non-arid biomes, while studies on the mechanisms underlying floral diversification and plant-pollinator interactions under such extreme environments are still scarce (Trunschke et al., 2021).

Variability in floral traits represents an essential condition to induce processes of floral isolation mediated by pollinators (Johnson, 2010). Pollinators rely on attributes such as color, shape and floral volatiles to detect and discriminate between flowers (Hirota et al., 2012; Reverté et al., 2016; Martínez-Harms et al., 2018). Variations in floral attributes can cause pollinators to behave selectively toward floral variants (Vickery, 1992; Salzmann and Schiestl, 2007; Dormont et al., 2014), inducing processes of floral isolation ("ethological isolation" *sensu* Grant, 1949) that can contribute to the formation and maintenance of reproductive barriers to hybridization. Variations in floral traits are common between species and can also occur naturally within species or as the result of hybridization events (Ippolito et al., 2004; Martínez-Harms et al., 2018, 2020). The eco-evolutionary mechanisms contributing to maintain intra-specific variations in floral attributes include processes such as selection by biotic and abiotic factors and non-adaptive processes such as equilibrium between gene flow and drift (reviewed in Narbona et al., 2018; Sapir et al., 2021). On the other hand, hybridization is considered a major source of genetic diversity, promoting phenotypic variability in plants (Goulet et al., 2017). Under such scenarios, pollinators can mediate processes of floral isolation that can lead to divergence in floral traits and the formation of new races or species. Understanding the mechanisms by which variations in floral characters arise and their consequences on floral visitors can provide valuable insights about the evolutionary processes derived from plant-pollinator interactions.

Previous studies have shown that differences in floral color can induce pollinators to behave selectively toward certain flowers (Vickery, 1992; Schemske and Bradshaw, 1999; Hirota et al., 2012). Flower color involves two optical phenomena: (*i*) the reflection and scattering of light by floral structures, and (*ii*) wavelength-selective absorption by floral pigments (Kay et al., 1981; Lee, 2007; Vignolini et al., 2012; Stavenga and van der Kooi, 2016; van der Kooi et al., 2017; Wilts et al., 2018). Although much remains to be studied about the optical properties of flowers, we know that due to the different refractive indices of the petals' cellular components, incident light is reflected and scattered (Vignolini et al., 2012; Stavenga and van der Kooi, 2016; van der Kooi et al., 2017; Wilts et al., 2018). Pigments, on the other hand, have been extensively characterized with respect to their spectral properties, biosynthesis and biological functions. Pigments accumulate and localize at different subcellular compartments of floral tissue, reducing light scattering and providing specific hue depending on their absorption spectrum (Exner and Exner, 1910; Kugler, 1963; Kay et al., 1981; Stavenga and van der Kooi, 2016). Therefore, variations in flower color can result from differences in the structure of flowers, differences in the floral content of pigments or a combined effect of the two phenomena.

Here we investigate the mechanisms of flower coloring of *Cistanthe longiscapa* (Barnéoud) Carolin ex M.A.Hershkovitz (Montiaceae), a dominant species across the ephemeral blooming landscape at the hyperarid Atacama Desert. A massive blooming of this species took place near the city of Caldera, Chile, during September-October 2021 (**Figures 1A,B**), that consisted of two distinctive large patches dominated by purple and yellow flower color forms of *C. longiscapa* (**Figure 1C**). The flowering event called the attention of the general public all over the world and was widely reported in social media (France24, 2021; Reuters, 2021).

It is important to note that the yellow flower variant was initially described as *Calandrinia litoralis* Phil (Philippi, 1860) and was later synonymized with *C. longiscapa* based on herbarium material (Hershkovitz, 1991). In the areas where patches of yellow and purple flowers were close to each other reddish, pinkish and whitish flowers could be found in more abundance (**Figures 2A–H**, **4** and **Supplementary Figure 1**), floral variants that could have resulted from the cross pollination of the dominant varieties, could be found in more abundance than elsewhere (**Figures 2A–H**, **4** and **Supplementary Figure 1**). We extensively analyzed flower coloring with respect to: (*i*) their diversity; (*ii*) the mechanisms underlying color differences; and (*iii*) the implications of color diversity on the way flowers are perceived by pollinators. As a representative pollinator we consider the honeybee (*Apis mellifera*), an insect whose vision has been investigated in detail (Vorobyev and de Ibarra, 2012). Since the set of photoreceptors of most hymenopterans evaluated so far are similar to that of *Apis mellifera* (Peitsch et al., 1992), our analysis can be extended to other hymenopterans, which represents a guild of pollinators that have been observed visiting flowers of *C. longiscapa* (**Supplementary Figure 2**). We discuss our findings in the context of the ecological and evolutionary consequences of species inhabiting one of the driest places on earth.

Materials and methods

Plant material

Plant material was collected in the field and kept fresh until measurements and histological procedures were carried out. The flowers of *C. longiscapa* were studied at the peak of the flowering season in October 2021 in a location near the city of Caldera (−27.0667°S; −70.8178°W), where a massive flowering event of this species took place (**Figure 1**).

Photographic characterization of ultraviolet-patterns

A digital camera modified for increased ultraviolet (UV) sensitivity (EOS 10D, Canon USA Inc., Lake Success, NY, USA) equipped with a quartz lens (105 mm, UV-Nikkor, Nikon, Tokyo, Japan) was used to take images of flowers. For UV exposures, a narrow band-pass filter was used (Baader U-Venus-Filter, Baader Planetarium, Mammendorf, Germany), which consists of a Schott UG11 substrate with dielectric coating that blocks wavelengths in the visible and infrared range while transmitting between 320 and 380 nm with a half-band-width of 60 nm. For exposures in the visible spectrum, we used a bandpass filter that transmits light between 400 and 700 nm.

Anatomical study

To investigate petal structures, petal thickness and pigment distribution in flowers, cross sections of fresh plant material were examined. In order to provide mechanical support to floral components, petal pieces were embedded in a 10% agarose solution close to its solidification temperature (approximately 55°C). The agarose-embedded petal pieces were cut with a razor blade and the sections were observed and photographed under a microscope (Zeiss, Axioskop) (Stavenga and van der Kooi, 2016; van der Kooi and Stavenga, 2019).

Spectrophotometry

Reflectance and transmittance spectra of flowers were measured using an integrating sphere (Avantes Avasphere-50-REFL). For the reflectance measurements, petal pieces were illuminated from within the sphere by positioning them at the aperture of the integrating sphere and illuminating them with an optical fiber connected to a deuterium-halogen lamp (Avantes AvaLight-DH-S). The reflected light was collected by a second optical fiber connected to a spectrometer (Avantes Avaspec-ULS2048CL-EVO). Reflectance spectra of both the adaxial (R_a) and the abaxial (R_b) side were measured. For the transmittance measurements, petal pieces were illuminated from outside the sphere by an optical fiber connected to the deuterium-halogen lamp. Furthermore, in a survey of 110 flowers, reflectance spectra of petals were measured with a setup where an optical fiber connected to the deuterium-halogen lamp was used to illuminate patches of petals, while a second optic fiber connected to another spectrometer (Ocean Optics USD2000, Dunedin, FL, USA) collected the light reflected by the petal. The spectrometers were calibrated using a white reference standard (Avantes WS-2).

Kubelka-Munk analysis of the absorption and scattering characteristics of the flowers

The reflectance and transmittance of a flower petal depend on its structural components and their absorption and reflection/scattering characteristics. If the flower can be considered to consist of a stack of layers with specific absorption

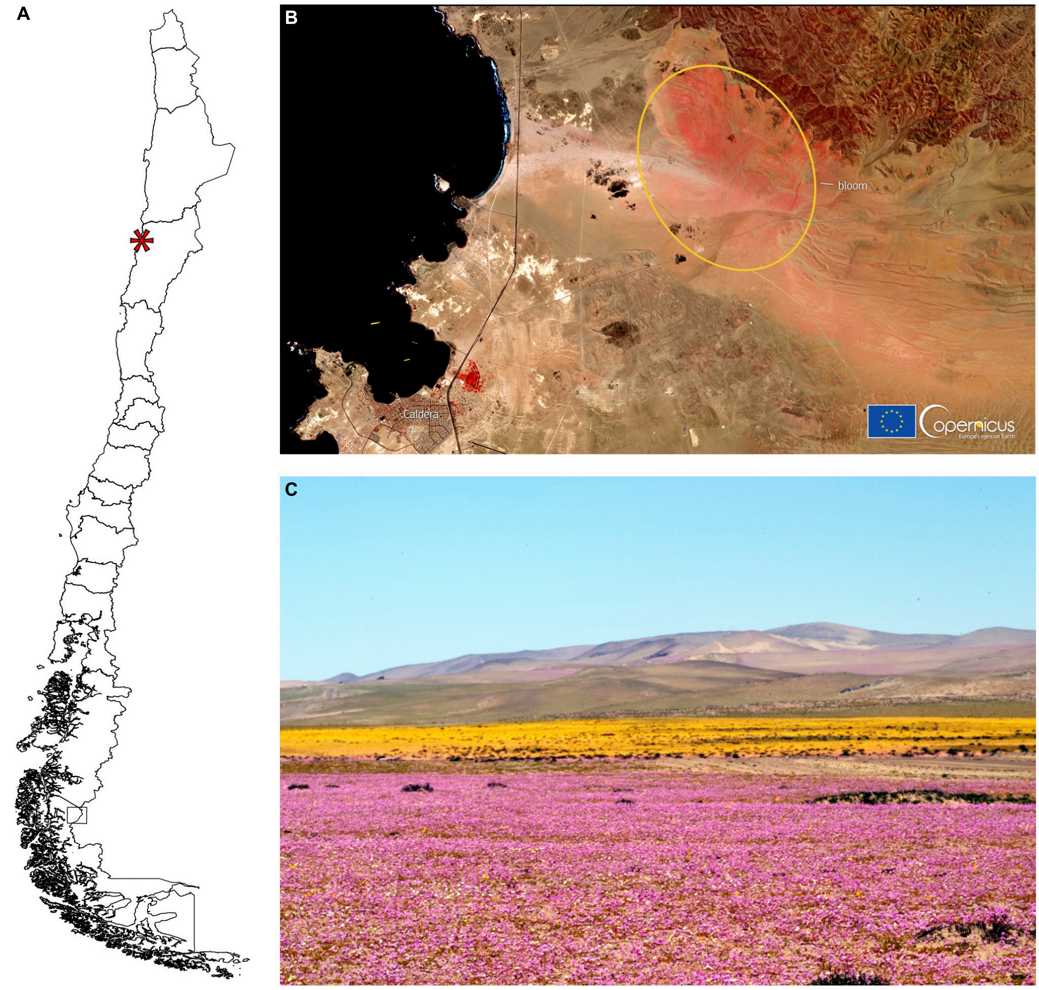

FIGURE 1
Blooming in the Atacama Desert. **(A)** Map of Chile indicating with an asterisk the location of where the studied blooming event took place. **(B)** Copernicus satellite image from 13th October 2021 of the desert near the city Caldera (lower left) and the area with the abundant flower bloom (yellow ellipse) (Credit: European Union, Copernicus Sentinel-2 imagery). **(C)** Photograph of the area showing the dominant purple and yellow flower colors of *Cistanthe longiscapa* (photo by J. Martínez-Harms).

and scattering parameters for each layer, the stack transfer matrix is defined by (Stavenga and van der Kooi, 2016):

$$M_s = \frac{1}{T}\begin{bmatrix} T^2 - R_a R_b & R_b \\ -R_a & 1 \end{bmatrix} \quad (1)$$

with T the transmittance and R_a and R_b the adaxial and abaxial reflectance, respectively. Here we treat the *Cistanthe* petals as a single layer with equal reflectances for illumination from above and below, $R_a = R_b = R$. The petal's scattering parameter S^* then follows from

$$S^* = \{ln(1 - (p - q)\, R)/T\}/q \quad (2a)$$

with

$$p = (1 + R^2 - T^2)/(2R), \quad \text{and } q = \sqrt{p^2 - 1} \quad (2b)$$

and the absorption parameter then is

$$K^* = (p - 1)\, S^* \quad (2c)$$

The scattering and absorption coefficient, i.e., the scattering and absorption per unit length of the petal medium, follow with the petal thickness, d: $S = S^*/d$ and $K = K^*/d$ (Stavenga and van der Kooi, 2016).

Plotting loci of flowers in insect color space

Flower colors were plotted in a chromaticity diagram using the receptor noise limited (RNL) color opponent model, which is one of several diagrams used for displaying colors, most

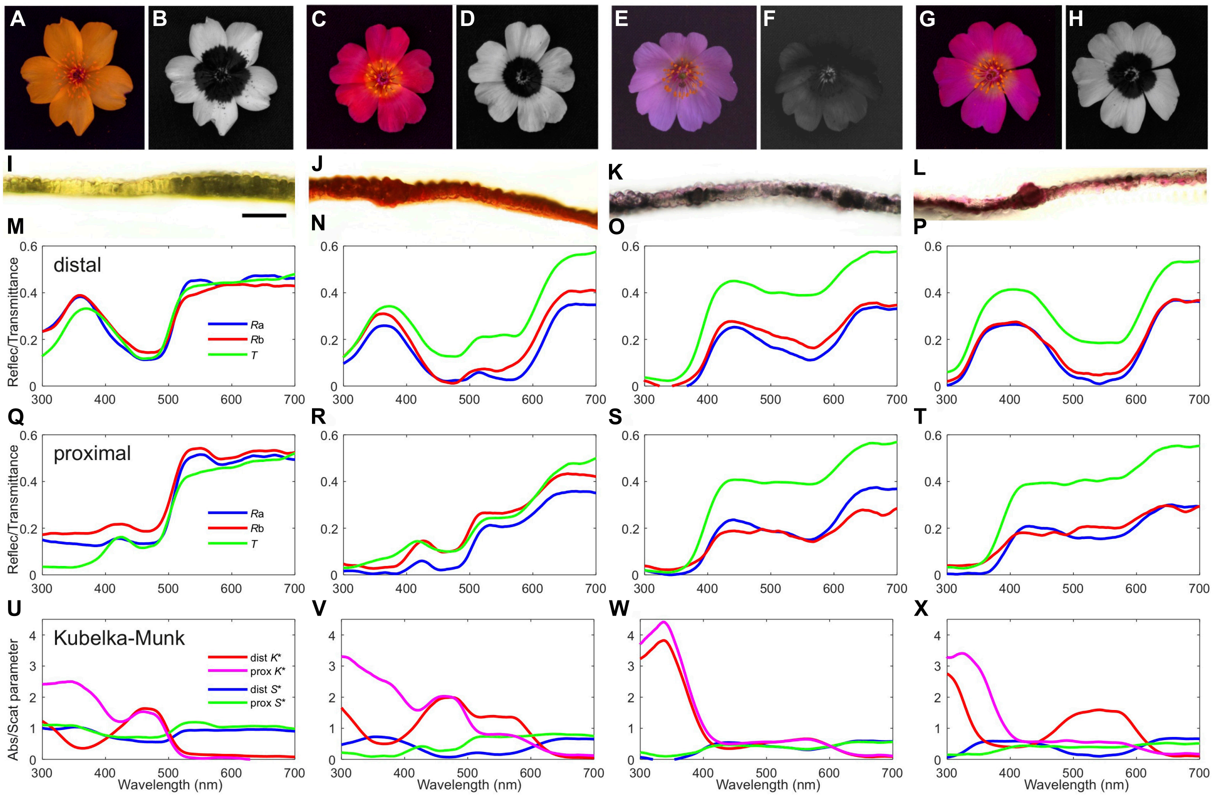

FIGURE 2
Variously colored *Cistanthe longiscapa* flowers, petal anatomy and spectral characteristics. **(A, C, E, G)** RGB photographs. **(B, D, F, H)** UV photographs. **(I–L)** Transversal sections of the petals of the respective flowers above (scale bar, in **I**, 100 μm). **(M–P and Q–T)** Reflectance (*R*) and transmittance (*T*) spectra measured with an integrating sphere from distal and proximal petal areas, respectively; R_a – adaxial, R_b – abaxial. **(U–X)** Absorption (*K**) and scattering (*S**) parameters calculated with a Kubelka-Munk procedure; dist – distal, prox – proximal.

notably the Maxwell triangle (Neumeyer, 1980), the COC model (Backhaus, 1991), the color hexagon (Chittka, 1992) and several other models based on behavioral results of von Helversen (1972) (Brandt and Vorobyev, 1997; Vorobyev and Brandt, 1997; Garcia et al., 2017). According to the RNL model, colors can be depicted as points in a chromaticity diagram where the discriminability between colors is given by the Euclidean distance between points (**Figure 3D**). The greater the distance, the more reliable is the discrimination (Vorobyev and Osorio, 1998; Vorobyev et al., 2001a). This model has been used to describe color discrimination in the honeybee (Vorobyev and Osorio, 1998; Vorobyev et al., 2001a) and in a number of other animals (Goldsmith and Butler, 2003; Koshitaka et al., 2008; Lind et al., 2013). It is important to note that the RNL model predicts the lower limit for discrimination threshold that is set by the noise, which is achieved only after intensive training under controlled laboratory conditions. In more naturalistic conditions, color discrimination may significantly deviate from the predictions of the RNL model because the behavior of animals depend, among other factors, on their previous experience, physiological state or the amount and type of reinforcement associated with stimuli (e.g., Giurfa, 2004; Avarguès-Weber et al., 2010; Reser et al., 2012; Avarguès-Weber and Giurfa, 2014; Martínez-Harms et al., 2014; Sommerlandt et al., 2016). Furthermore, the RNL model would often predict discrimination between the natural variability of the same flower type, indicating the need of behavioral validation of such predictions (Dyer et al., 2012; Garcia et al., 2018). For details of modeling see **Supplementary materials**. The analyses were performed using the PAVO package (Maia et al., 2019).

To describe the difference between flowers belonging to different groups, we used the Fisher Discriminant Analysis (Fisher, 1936). This method finds a direction in a multidimensional space that provides a best separation between the groups. The best separation is postulated to correspond to the maximum of the signal-to noise ratio, which is defined as the square root of the squared difference between the groups to their variance. This maximal signal-to noise ratio can be considered as a distance between clusters. Let μ_1 and μ_2 be the vector means of the two sets of multidimensional data, and Σ_1

and Σ_2 be the covariance matrices of these data, then the maximal separation is achieved along the direction $(\Sigma_1 + \Sigma_2)^{-1}(\mu_1 + \mu_2)$. The ratio of the square of the difference between the means to the variance along this direction is given by:

$$d = \sqrt{(\mu_1 + \mu_2)^T (\Sigma_1 + \Sigma_2)^{-1} (\mu_1 + \mu_2)} \qquad (3)$$

Note that the derivation of Eq. (3) is neither based on the assumption of normality of the distributions nor on the assumption of equality of variances of the groups (both these assumptions are used in a conventional linear discriminant analysis).

Modeling flowers as seen through the eyes of an insect pollinator

The honeybee has three spectral types of photoreceptors, maximally sensitive in the UV-, blue- and green-wavelength range, which are referred to as S, M, and L, for the short-, middle-, and long-wavelength-sensitive photoreceptor, respectively (Menzel and Backhaus, 1991). To create images of flowers as they are seen through the eyes of a honeybee, we applied the procedure described in Vorobyev et al. (2001b). Briefly, we made UV- and RGB-photographs of the flowers and also measured their reflectance spectra. We then calculated the quantum catch values for the L, M and S photoreceptors (LMS values) for the different flower regions assuming that the illumination was D65 daylight and substituted the RGB values in the images with the LMS values (Wyszecki and Stiles, 1982; see **Supplementary materials** for more details). The software for image processing written using Wolfram Mathematica 10, is available on request.

Results

Floral coloration

To characterize the differences in coloration between varieties of *Cistanthe longiscapa* flowers, we made RGB and UV photographs. **Figure 2** shows exemplary cases of flowers with yellow (**Figures 2A,B**), reddish (**Figures 2C,D**), purple (**Figures 2E,F**) and pink (**Figures 2G,H**) colored petals, revealing that flowers differ in their reflectance in the visible and UV range of the spectrum. Whereas the color in the (for humans) visible wavelength range was rather uniform (**Figures 2A,C,E,G**), the UV photographs show that some flowers have distinct differences between the distal and proximal areas, thus forming a UV reflection pattern usually referred to as a bullseye (**Figures 2B,D,H**).

Floral anatomy and pigment distribution

In order to visualize the petal structures and pigment distribution and to estimate petal thickness, cross sections of petals were carried out. This revealed that the thin petals of *C. longiscapa* are composed of a single cell layer and that pigments are distributed homogeneously in the petal cells (**Figures 2G–I**). The petal thickness was found to be $d = 80 \pm 20$ μm.

Flower spectral characteristics

To characterize the differences between the distal and proximal petal areas, we measured reflectance and transmittance spectra of the set of flowers of **Figures 2A–H**. The reflectance spectra of the adaxial and abaxial sides appeared to be very similar for both the distal (**Figures 2M–P**) and proximal (**Figures 2Q–T**) petal areas. This allows the application of a single layer Kubelka-Munk type analysis, which yielded the petal's absorption and scattering parameters (**Figures 2U–X**).

The absorption in three wavelength ranges, i.e., the UV (<400 nm), the blue (400–500 nm) and the orange-red (500–650 nm) wavelength ranges, shows specific variations. The distal and proximal areas of the yellow flower of **Figures 2A,B** contain a pigment with peak absorption ∼465 nm. The proximal area contains additionally a UV-absorbing pigment. The pigmentation of the distal and proximal areas of the reddish flower of **Figures 2C,D** is very similar, but it contains additionally a pigment with peak absorption in the orange wavelength range (∼570 nm). The purple flower of **Figures 2E,F** contains the same pigments in a much lower concentration, but the UV-absorbing pigment is dominant throughout the petal. The pink flower of **Figures 2G,H** instead has distally a large amount of the orange-absorbing pigment, which is less present proximally, but there the UV-absorbing pigment is dominant again.

Flower color diversity

Reflectance spectra measurements were further performed on flowers of 110 different plant individuals along a transect. In order to visualize the spectral differences between flowers, a principal component analysis (PCA) was performed, using the reflectance between 300 and 700 nm at intervals of 1 nm. The analysis revealed that two principal components (PCs) explained 84.5% of the variance in flower color. The analysis highlights the variability in the floral spectral reflectance of individual flowers blooming simultaneously in a geographically restricted area. PC1 accounted for 47% of the total variance and separated flowers with high reflectance between 400 and

600 nm from flowers with high reflectance in the UV range and above 600 nm (**Figure 3A**). PC2 accounted for 38% of the total variance and separated flowers with high reflectance in the UV and long wavelength range (above 500 nm) from flowers with high reflectance in the middle wavelength range (between 400 and 500 nm) (**Figure 3B**). Plotting PC1 versus PC2 scores for each flower results in a reflectance scatterplot that clusters flowers according to their spectral reflectance in different sections of the scatterplot in 4 clusters.

Flower color appearance for pollinators

Color loci of the 110 flowers mentioned above were plotted in the chromaticity diagram of the most common hymenopteran pollinator, the honeybee *Apis mellifera,* using the RNL model. Most hymenopterans investigated so far possess trichromatic color vision, with photoreceptors maximally sensitive in the UV, blue and green regions of the spectrum. Because trichromaticity represents the ancestral condition among arthropods (Briscoe and Chittka, 2001; van der Kooi et al., 2021), modeling a perceptual space using the spectral sensitivity of honeybees provides a good approximation about the way flowers are perceived by a wide variety of hymenopterans, pollinator guild that has been observed visiting flowers of *C. longiscapa* (**Supplementary Figure 2**). Our analysis revealed that the loci of *C. longiscapa* flowers distribute widely in the chromaticity diagram of the honeybee (**Figure 3D**). The flower colors belonging to human red, purple, white and yellow categories form clearly distinguishable clusters in the RNL diagram. The colors corresponding to pink and red human color categories slightly overlap in the RNL diagram. The separation between the clusters has been quantified using Fisher Discriminant Analysis and assessed as the ratio of the distance between the means to the standard deviation of the spread of the data along the line of the best separation between the clusters (Eq. 3). The results are presented in the **Table 1**. All separations, with the exception of that for the red and pink categories, significantly exceed 1, indicating that these clusters can be easily discriminated based on receptor signals of a honeybee, given that a suitable algorithm of discrimination between categories is used.

The fact that flower colors cluster in different sections of the chromaticity diagram indicates that for trichromatic hymenopterans, flowers from the different clusters appear different in color, thus falling in different color categories. For instance, flowers that appear yellow to humans, i.e., those with high reflectance above 500 nm, which vary in UV reflectance will have a different appearance for hymenopterans. Similarly, purplish flowers with low reflectance in the UV, appear different in color to those purplish flowers with appreciable UV reflectance. It is important to note that empirical evidence of color categories in bees is lacking (e.g., Benard and Giurfa, 2008). A matrix with the color perceptual distance between all flowers of *C. longiscapa* considered in this study is provided in the **Supplementary materials** (**Supplementary Figure 3**).

Since color vision of bees differ substantially from that of humans, the latter gives little insight into the appearance of flowers to bees (Vorobyev and de Ibarra, 2012). The reconstruction of flower images as they are seen through the eyes of bees illustrates the differences in appearance of flowers with similar spectral reflectance in the visible spectrum range but that differ in their UV-reflecting properties (**Figure 4**). This is the case when comparing yellow flowers with appreciable UV (**Figures 4A–C**) to yellow flowers having negligible levels of UV reflectance (**Figures 4D–F**). The same holds when comparing purple and pink flowers with different levels of UV reflectance (flowers of **Figures 4G–I** vs. flowers of **Figures 4J–L**). An interesting result is the similarity for honeybees of flowers that for humans appear quite distinguishable, as is the case for UV-reflecting pink flowers (**Figures 4J–L**) and the reddish flowers (**Figures 4M–O**). The color similarity of these flowers for bees is due to the lack of photoreceptors sensitive to red in the color vision system of honeybees, which in turn relies on UV photoreceptors. In case of flowers with low UV reflectance that appear white to humans, bees will not see these flowers as white given that they do not stimulate the three spectral types of bee photoreceptors (**Figure 4R**). Finally, flowers that appear uniform in color to humans with UV-bullseye patterns have a center-surround color pattern for bees (**Figures 4C,L,O**). Although the differences in flower appearance revealed by our results are not surprising considering the distinct UV sensitivity of insects, the analysis highlights that *C. longiscapa* flower color varieties occurring simultaneously in the Atacama Desert are highly variable in their color appearance for insect pollinators.

Discussion

Cistanthe longiscapa, due to its massive flowering events, represents a dominant feature across the ephemeral blooming landscape at the Atacama Desert. An interesting aspect of the 2021 blooming event was the co-occurrence of *C. longiscapa* plants displaying different flower colors. We investigated the mechanisms underlying those color differences and found that result from flowers having pigments with different absorption spectra (**Figures 2U–X**). The different pigment composition of petals causes flower color differences in the visible and UV range of the spectrum (**Figure 2**). The variability in *C. longiscapa* flower colors was visualized through a PCA analysis, which separates flowers in 4 clusters, based on their reflectance in different wavelength ranges (**Figures 3A–C**). Using color vision modeling we find that *C. longiscapa* flowers are highly polymorphic in their color appearance for insect pollinators and that their spectral reflectance differences in the visible and UV

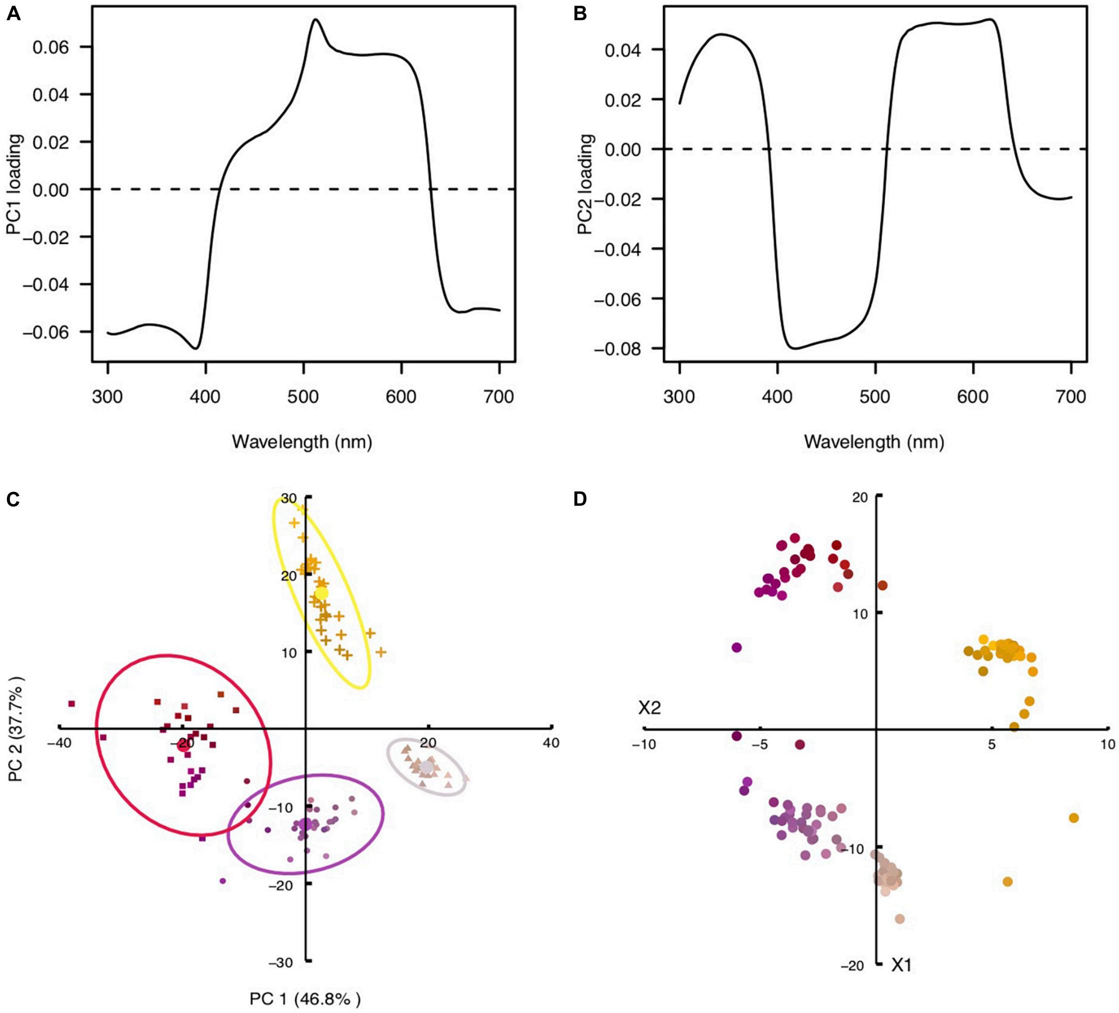

FIGURE 3
Principal component and chromaticity analysis of *Cistanthe longiscapa* flowers. **(A)** Principal component 1 as a function of wavelength, which accounted for 47% of the total variance in flower color. **(B)** Principal component 2 as a function of wavelength, which accounted for 38% of the total variance in flower color. **(C)** Clusters of flowers in the PCA diagram. **(D)** Clusters of flowers in the chromaticity diagram. The colors of the symbols in **(C, D)** resemble the colors of the flowers as seen by humans.

range of the spectrum affect the way they are perceived by floral visitors (**Figures 3D**, **4**). Altogether, our results highlight the variable nature of floral color of *C. longiscapa* forms blooming simultaneously in a geographical restricted area. Given the importance of flower color for attracting floral visitors, the observed color variability could mediate the assurance of cross pollination under highly fluctuating pollinator environment conditions.

The *Cistanthe* are species belonging to Montiaceae that, as members of the Caryophyllales, owe their flower and fruit pigmentation to betalains (Chung et al., 2015) that accumulate in the vacuoles of the cells (Wink, 1997; Sadowska-Bartosz and Bartosz, 2021). The presence of betalains in flowers of *Cistanthe* is further supported by preliminary experiments on extracts from yellow, purple and pink flowers that reveal pigments with different absorption spectra that are stable at pH from 3 to 7 (in preparation). The betalains are water-soluble, tyrosine-derived pigments, containing betalamic acid as their central chromophore (Stafford, 1994; Tanaka et al., 2008). In plants, betalains are taxonomically restricted to most families of the order Caryophyllales and their biosynthetic pathway is mutually exclusive with that of the more common anthocyanins (Stafford, 1994; Timoneda et al., 2019). Betalains comprise yellow-orange betaxanthins and red-violet betacyanins, with absorption peak wavelengths ∼470 nm and ∼536 nm, well corresponding to the pigments identified in the flowers of *C. longiscapa* (**Figures 2U–X**). Our results show that in the thin single cell layered petals of *C. longiscapa,* betalains co-occur with UV-absorbing pigments (**Figures 2U–X**), which most likely correspond to flavonoids (Narbona et al., 2021). On the other hand, the color of the

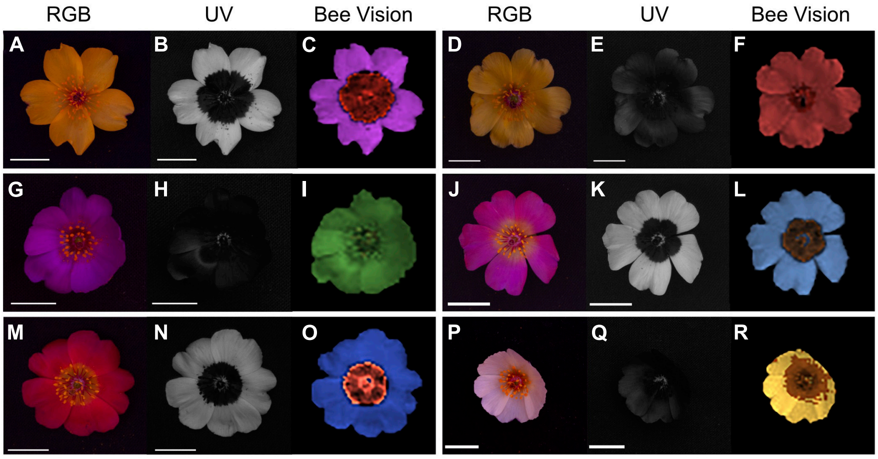

FIGURE 4
Color varieties of *Cistanthe longiscapa* flowers shown in RGB **(A,D,G,J,M,P)**, and UV photographs **(B,E,H,K,N,Q)** and images represented as they would be seen through the eyes of honeybees **(C,F,I,L,O,R)**.

reddish flowers seems to result from two types of betalains, with absorption peaks in the blue and orange wavelength range respectively, accumulating together in the vacuoles of the petal cells (**Figures 2J,V**). Betalains are of special interest regarding their evolutionary origin and homologous functional roles with anthocyanin pigments (Jain and Gould, 2015; Timoneda et al., 2019). While both classes of pigments have comparable spectral properties and have the same histological location, recent evidence indicates that they are effective as photoprotectants, in conferring tolerance to drought and salinity stress and as scavengers of reactive oxygen species in plants facing a variety of abiotic stressors (Jain and Gould, 2015).

Despite their functional similarities with anthocyanins, betalains also have distinctive properties. For instance, unlike anthocyanins, the color of betalains is stable between pH = 3-7 (Strack et al., 2003). This may provide advantages for species like *C. longiscapa*, which upon experiencing water stress switch the mode of carbon assimilation from C3 to Crassulacean acid metabolism (CAM), shifting from a relatively stable vacuolar pH to large diurnal pH fluctuations (Winter and Holtum, 2014; Jain and Gould, 2015; Ossa et al., 2022). Having stable pigments under such fluctuating conditions would ensure their functionality in a harsh, hyperarid environment such as the Atacama Desert (Jain and Gould, 2015). Despite the long interest in betalain pigments, they have been mostly studied in a few model species and much remains to be elucidated with respect to their diversity and biosynthetic and regulatory pathway. Evidence has accumulated about the variability of pigment content in flowers in response to abiotic stress along geoclimatic gradients (Koski and Ashman, 2016; Dudek et al., 2020). The variability in pigment composition revealed by our results points at *Cisthanthe* as suitable models to study betalain biosynthesis, as well as their adaptive role under extreme environments.

Plants native to the Atacama Desert have evolved traits associated not only with tolerance to abiotic stress, but also traits involved in ensuring reproduction during short but massive blooming in the Desert (González and Pérez, 2010). In angiosperms, female reproductive success is often constrained by the supply of pollen, a phenomenon known as pollen limitation, which has been associated with the stochastic nature of pollination (Burd, 1994; Larson and Barrett, 2000; Ashman et al., 2004). Pollen limitation is expected to be particularly strong in habitats such as the Atacama Desert, where unpredictable rainfall drives large interannual variations in plant cover and pollinator abundance (González and Pérez, 2010). Variable pollinator environments could also favor the evolution of strategies to cope with pollen limitation. For instance, the evolution of autonomous selfing (i.e., the ability of flowers to self-fertilize) could provide reproductive assurance in the absence of appropriate pollen vectors (Baker, 1955; Goodwillie et al., 2005). While empirical evidence for increased seed set due to autonomous selfing is available for a few annual species (Kalisz and Vogler, 2003; Pérez et al., 2006), its acquisition seems to be more difficult in perennial species, indicating that the adoption of selfing depends on the species' life form (Morgan et al., 1997). Alternatively, the evolution of floral attractive traits would also allow plants to cope with pollen limitation under fluctuating pollinator environments. A high investment in floral display or reward would increase the attraction of floral visitors and the probability of pollination (Billings and Mooney, 1968; Fabbro and Körner, 2004). An evaluation of the Atacama Desert flora showed that strong pollen limitation was not a general trend and that low pollen limitation levels in annual desert plants, such as *C. longiscapa*, result from the adoption of autonomous selfing as strategy to ensure pollination (González and Pérez, 2010). With respect

TABLE 1 Separation between color clusters belonging to different human categories assessed as the ratio of the distance between mean of the clusters to the standard deviation along the line of the best separation (Eq. 3).

	Pink	Purple	Red	White	Yellow
Pink	0	5.9	1.7	18.4	8.6
Purple		0	6.0	3.2	11
Red			0	11.1	5.3
White				0	11.9
Yellow					0

to investment in reward, flowers of *C. longiscapa* do not provide nectar but provide copious amounts of pollen that might attract a variety of pollinators dependent on this type of resource. Although no association between flower size and pollen limitation was detected (González and Pérez, 2010), further studies are needed to evaluate the extent to which other attractive floral traits, such as color and volatiles, contribute to ensure reproduction in plants inhabiting the Atacama Desert.

The specialized floral morphologies observed in some species inhabiting the Atacama Desert suggest that pollinators have played an important role in the evolution of some desert plants. Mechanical and ethological isolation are the two proposed mechanisms by which pollinators would influence the evolution of flowering plants (Grant, 1949). Under processes of ethological isolation, pollinators' selective behaviors would induce the isolation of floral variants, contributing to the formation of reproductive barriers to hybridization, eventually leading to the development of new races or species (Grant, 1994). Two sympatric modes of origin of ethological isolation have been proposed: (*i*) hybrid speciation followed by the segregation of a new flower form that captures a new type of pollinator; (*ii*) the separation of a polymorphic variant for floral characters from other members of its population by flower-constant behavior and assortative mating (Grant, 1994). When seen through the eyes of honeybees, flowers of *C. longiscap*a can appear quite different from each other in color, providing a scenario that could potentially lead to processes of ethological isolation. Although the circumstances under which such polymorphism developed are unclear, the fact that *C. longiscapa* is a self-autonomous species suggests that pollinators may have promoted cross pollination between the two more common yellow and purple varieties, resulting in the more uncommon UV-reflecting pink and red forms. A higher incidence of autonomous selfing may have contributed to the persistence of those more uncommon forms throughout several generations (Buide et al., 2021). Several mechanisms have been proposed for the maintenance of high levels of variability in floral traits. For instance, negative frequency-dependent selection, although unlikely in pollen rewarding species, has been pointed out as a mechanism for maintaining color polymorphism in rewardless flower plants (e.g., Gigord et al., 2001; Schiestl, 2005; Basist et al., 2021). Opposing selection regimes exerted simultaneously by mutualists and antagonists is also thought to contribute to the maintenance of flower color polymorphism (Irwin et al., 2003; Frey, 2004; de Jager and Ellis, 2014). An interesting example is *Claytonia virginica* (Montiaceae), whose color polymorphism is thought to be maintained through positive pollinator-mediated selection for redder flowers, whereas white flowers receive less damage from herbivores and pathogens (Frey, 2004). Spatial or temporal fluctuation in selection can also maintain phenotypic variation (Siepielski et al., 2009, 2013; Tang and Huang, 2010). A long-term study in the Mojave Desert inhabiting species, *Linanthus parryae*, demonstrated that flower color is subject to fluctuating selection exerted by temporal and spatial variation in spring precipitation (Schemske and Bierzychudek, 2001, 2007). We propose that understanding how biotic and abiotic factors affect flower evolution requires knowledge about the influence of those factors on specific traits along with historical insights about how those factors fluctuate in time and space. In the case of *C. longiscapa*, its outstanding flower color polymorphism may represent an ongoing process of divergence under a complex and fluctuating history of interactions with pollinators. Considering the historical nature of plant-pollinator interactions, the diversity of floral forms provided by *C. longiscapa* and other species across the Atacama Desert may also shed light about the sensory and cognitive capacities of the pollinators contributing to shape this unique flowering landscape.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JM-H, NP, and DS collected the data. JM-H, DS, and MV performed the analyses. JM-H drafted the manuscript. All authors revised subsequent versions and contributed to the design of the study.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.957318/full#supplementary-material

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Melanism and color saturation of butterfly assemblages: A comparison between a tropical rainforest and a xeric white forest

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The study of butterfly coloration has helped to identify the ecological pressures involved in the evolution of animal coloration. However, almost all studies that addressed this issue have focused on species that inhabit more temperate environments, leaving the species and ecological factors of tropical regions mostly understudied. Here, our purpose was to evaluate whether butterfly assemblages from two distinct Neotropical biomes (i.e., tropical rainforest and xeric white forest) differ regarding their melanism and/or color saturation. Our hypotheses were that (1) tropical rainforest butterflies should be more melanic and color saturated, and that (2) butterflies from more open/arid tropical environments should be more melanic on their dorsal wing surfaces than on their ventral wings. Therefore, we quantified melanism and color saturation from dorsal and ventral surfaces of 121 different butterfly species. Comparisons show that rainforest butterflies, when contrasted to white forest butterflies, have more melanic dorsal wing surfaces, which might be seen as a form of protection against parasites. Our data also show that rainforest butterflies, but not white forest species, have darker dorsal wing surfaces, when compared to their own ventral surfaces, a trend that was also found for species inhabiting both biomes, which might be associated to thermoregulatory advantages. At last, our results also point that butterflies' dorsal wing sides present a higher variance between species (regardless of Biome), when compared to their own ventral wing side, an indication that some ecological factor (e.g., predation avoidance) might be exerting a strong homogenizing force on ventral wing coloration.

KEYWORDS

photography, ImageJ, sexual selection, sensory ecology, lepidoptera, Caatinga, Atlantic forest, camouflage

1. Introduction

Butterflies are excellent study models for examining melanism and animal coloration because such features can be used as markers to understand which selective forces have been in action (Endler, 1982; Andersson, 1994). For instance, comparable color patterns that are seen in distinct species might result from convergent evolution driven by similar environmental pressures (Hoekstra, 2006). The color patterns of butterfly wings are formed through a set of physical mechanisms and pigments, such as melanin (Watt, 1964), and might play a role in mate recognition (Wiernasz, 1995), thermoregulation (Clusella-Trullas et al., 2007), protection against predators (Kettlewell, 1955) and parasites (True, 2003), as well as in desiccation and UV damage prevention (Majerus, 1998).

Organisms are expected to have darker colors in environments with lower temperatures because the concentration of melanin in the cuticle increases the absorption of thermal energy (Clusella-Trullas et al., 2007). As ectothermic and diurnal individuals, butterflies need heat to carry out their activities, such as foraging and looking for mates (Brakefield and Willmer, 1985; Wiernasz, 1995). So, the presence of darker patches might favor butterflies by quickly increasing their body temperature to carry out their activities (Kingsolver, 1983). Dark melanic butterflies also seem to be associated with humid environments (Stelbrink et al., 2019) as a form of protection against parasites. Alternatively, in warmer, highly sun-exposed, environments, insects are expected to be brighter to avoid overheating (Schmitz, 1994; Heidrich et al., 2018). However, melanism may not always be expressed homogeneously throughout the animal's body, as is the case for melanic differences found between dorsal and ventral surfaces of butterfly wings, a thermoregulatory strategy (Zeuss et al., 2014; Stelbrink et al., 2019) that captures more thermal energy when exposing dark melanic dorsal surfaces and dissipates more heat when exposing less pigmented ventral surfaces (Tsai et al., 2020). In addition to affecting lightness, melanin might also influence other spectral parameters of animal coloration, such as saturation (i.e., the purity of the color of an area judged in proportion to its brightness). For instance, melanic colors mostly result in patterns with lower saturation (i.e., less vivid colors), such as black, gray, and brown (McGraw, 2006), although the opposite relation has also been shown to be true for monarch butterflies (Hanley et al., 2013).

Whereas, most studies of lepidopteran coloration assemblages have addressed species and environmental conditions from the Palearctic (Zeuss et al., 2014; Heidrich et al., 2018; Stelbrink et al., 2019), Nearctic (Zeuss et al., 2014; Stelbrink et al., 2019), or Australian (Xing et al., 2016, 2018; Dalrymple et al., 2018) regions, the Neotropical species have been considerably understudied. In a country of continental dimensions, which holds an astonishing biodiversity and has seven different morphoclimatic and phytogeographic domains (Ab'Saber, 1967), it is surprising that only one study, conducted with Amazonian species, aimed to investigate lepidopteran color assemblages in Brazil (Spaniol et al., 2019).

Considering that environmental conditions can drive behaviors and select different coloration patterns (Endler, 1992) and that the spectral composition of sunlight can be modified by the physical characteristics of the environment (Endler, 1993), the present study aimed to compare the visual patterns displayed by assemblages of butterflies from two distinct Brazilian biomes, namely, the tropical rainforest and the xeric white forest. We expect butterfly assemblages from each biome to differ regarding their melanism and/or color saturation; our hypotheses are as follows: (i) butterflies that occur in the rainforest should be more melanic than those occurring exclusively in the white forest since in warmer, more sun-exposed environments, such as the xeric white forest, insects are expected to be brighter to avoid overheating (Schmitz, 1994; Heidrich et al., 2018), while in moist environments, such as the tropical rainforest, butterflies should tend to be darker (Stelbrink et al., 2019) as a form of protection against parasites; (ii) also due to thermoregulatory advantages (Zeuss et al., 2014; Stelbrink et al., 2019), differences in melanism between dorsal and ventral wing surfaces should be more pronounced in more heat-stressful environments (Tsai et al., 2020), such as the xeric white forest; finally, (iii) in highly shaded environments, such as rainforests, in opposition to considerably leafless ecosystems, such as the xeric white forest, individuals should have more saturated colors to enhance their contrast against the green dappled foliage background and compensate for the greenish illuminant created by sunlight filtering through the treetops (Endler, 1993).

2. Materials and methods

This study was approved by the UFRN's Animal Ethics Committee (CEUA, protocol number 013/2020) and followed all legal requirements of Brazilian law (number 11.794/2008).

2.1. Selection of specimens

We used well-preserved butterfly specimens from the scientific collection of the Laboratory of Ecology and Evolution of Butterflies, of the Federal University of Rio Grande do Norte (LEEB-UFRN), from which we also assembled information regarding the municipality, locality, date, and geographic coordinates regarding each specimen's capture.

We also checked the occurrence of species according to the available literature to identify those species that occurred in both biomes, in opposition to those occurring in only one biome: the Brazilian Atlantic Forest (referred to here as tropical rainforest) and the Brazilian Caatinga (referred to here as a xeric white forest). The taxonomic classification of the species followed Lamas (2004) and Mielke (2005).

2.2. Morphoclimatic and phytogeographic domains

The Brazilian Caatinga (hereafter referred to as xeric white forest), a kind of semi-arid forest/scrubland, is a poorly studied and highly threatened biome (Olson et al., 2001; Leal et al., 2003). This xeric and thorny scrub forest, which consists mostly of cacti and small thorny trees that lose their leaves seasonally, presents a high degree of floristic endemism with extreme meteorological patterns, which make it completely devoid of leaves in the dry season, when only the gray of the trunks and branches are evident, which made it known in the native-American language by the name of Caatinga (i.e., white forest; Prado, 2003). Rainfall in Caatinga (300–1,000 mm/per year) is usually concentrated for about 3–5 months (Sampaio, 1995), with an annual mean temperature of 28°C (Sampaio, 1995; Campos and Andrade, 2021).

The Brazilian Atlantic Forest (hereafter referred to as tropical rainforest), a tropical and subtropical moist broadleaf forest biome (Olson et al., 2001), has a hot and humid climate without a defined dry season, usually occupies regions of lower altitude ($<$1,000 m) and extends from northeast to southeast Brazil. Currently, the Atlantic forest is restricted to $\sim$98,000 km^2, or 7.6% of its original extension, and the last remnants of the forest still suffer intense anthropic pressure and imminent risk of extinction (Ministry of the Environment of Brazil, 1999), showing high levels of endemism (Morellato and Haddad, 2000) that exceed those found on most of the Amazon Forest, in such a way that the Atlantic forest, with its high biodiversity and elevated risk of extinction, is deemed as a hotspot (Sechrest et al., 2002). Precipitation in the tropical rainforest is 1.455 mm/per year, with an annual mean temperature of 26°C.

2.3. Color metrics quantification

We adopted a well-established protocol (Stevens et al., 2007) to obtain standardized photographs using a Canon EOS REBEL T2i camera (Canon 550D), coupled with an EF-S18-55mm lens and mounted on a tripod. Individually, and in a sequential manner, we chose one specimen from 121 different species of butterflies, and positioned each of them on a gray background, next to a color chart (X-Rite ColorChecker Passport), before capturing digital images of their dorsal and ventral surfaces (see Supplementary Figure S1 for a pictorial explanation on how the parameters were obtained). The use of a color chart ensured the maintenance of color information fidelity and allowed us to balance distortions caused by the camera's sensors and ambient lighting variation. After verifying the occurrence and geographic distribution of these 121 species of butterflies, data from those that did not occur in both environments ($N = 46$; Supplementary Table S1) were selected, rejecting those co-occurring in both biomes ($N = 75$; Supplementary Table S2). We evaluated each photo regarding its exposure through RawTherapee 5.4 software, converted the digital files to 8 bits, and obtained gray values for each pixel by using ImageJ software (Schneider et al., 2012). This procedure of 8-bit conversion and scaling of values followed what has been done in previous articles (Zeuss et al., 2014; Stelbrink et al., 2019). After photo calibration in ImageJ, we used a custom-made R package (Mello et al., 2022) to select regions of interest (i.e., wing area, excluding the thoracic and the abdominal region of the specimen) and extracted calibrated RGB values for each pixel. From each scaled image, we determined the mean gray value for each channel (R, G, and B), and then calculated the overall mean gray value by averaging the three channels. In the RGB system, lighter pixels are coded by higher values, while darker pixels lead to lower values, so the lower the mean gray value, the greater the melanism presented by the animal (each channel varies from 0 to 255).

Furthermore, we used a kernel density estimation method to extract the most common (mode) RGB triplet pixel values (see Mello et al., 2022 for details). Therefore, in cases where wing coloration has many different color patches, this method allows for capturing the RGB values of the most common color patch. We then used these RGB values to estimate the color saturation by calculating the mean absolute difference between channels divided by the overall mean gray value (as calculated earlier). This metric varies from zero to two, with higher values indicating higher color saturation. Finally, we measured the relative contribution of each channel by dividing each value by the sum of all three channels.

2.4. Statistical analysis

We performed Bayesian phylogenetic multilevel statistical analyses. We extracted the phylogeny from Chazot et al. (2019) and obtained a new tree with an "ape" package (Paradis and Schliep, 2019) using species phylogenetically close to ours. We generated a branch size distance matrix which was used to model their phylogenetic dependence in our statistical analyses. We ran one model for each color metric (mean gray value, saturation, R proportion, G proportion, and B proportion). The color metric was entered as the response variable. Wing side (dorsal or ventral), biome (tropical rainforest or xeric white forest), and their interaction entered as population-level effects (fixed effects). The species identification and the variance-covariance matrix of phylogenetic distances were entered as group-level effects (random effects). To fulfill statistical assumptions, we log-transformed mean gray value and square-root transformed saturation before analyses. Furthermore, because there was evidence of a difference in variance between wing sides for saturation, we included sigma as a function of the wing side in the saturation analysis. We ran models in R (v4.2.2; R Core Team, 2022) using the package brms (v2.18.0; Bürkner, 2018), which implements Bayesian models in Stan (Carpenter et al., 2017). For each model, we ran four independent chains for 10,000 iterations, 5,000 as a warm-up, in a total of 20,000 sampled iterations. We set weakly informative priors scaled to our data. We evaluated model fits by checking chain convergence, the presence of transitions with diverging errors, visual posterior predictive checks, and leave-one-out cross-validation. Figures were produced with *ggplot2* and *introdataviz* packages (Wickham, 2016; Nordmann et al., 2021). Statistical estimates are shown in the original, back-transformed, scale as the mode and the 95% high-density credible interval.

3. Results

3.1. Mean gray value

The statistical analysis indicated a lower mean gray value for the dorsal wing surfaces of butterflies from the tropical rainforest ($n = 31$ species) when compared to xeric white forest butterflies ($n = 15$ species) (Figure 1 and Supplementary Figure S2; mode and 95% high-density credible interval: −11 [−28, −1]). For the ventral side, although in the same direction as the dorsal side, the difference between biomes does not exclude the possibility of no difference at 95% CI (−6 [−21, 6]).

Furthermore, while butterflies from the xeric white forest did not show clear differences between their dorsal and ventral wing surfaces (−1 [−11, 7]), we found the wings of tropical rainforest butterflies to have darker dorsal than ventral surfaces (Figure 1 and Supplementary Figure S2; −7 [−15, −3]).

3.2. Color saturation

In general, we found that, on average, butterfly wings have similar saturation values. The statistical analysis did not show any clear difference between biomes (i.e., rainforest vs. white forest), either for the dorsal (mode and 95% CI: 0.02 [−0.12, 0.14]) or ventral wing surfaces (0.07 [−0.02, 0.15]). We also did not find support for differences between wing surfaces (i.e., dorsal vs. ventral) from the rainforest (−0.03 [−0.11, 0.06]) and the white forest (0.02 [−0.09, 0.14]). We found, however, the dorsal wing side to present a higher variance between species than the ventral side (Supplementary Figure S2; dorsal side estimated sigma of 0.15 [0.12, 0.19]; ventral side estimated sigma of 0.09 [0.06, 0.12]).

In addition, we did not find evidence for differences in the proportion of R, G, and B between locations (i.e., rainforest vs. white forest; dorsal R: 0.010 [−0.027, 0.039]; dorsal G: −0.009 [−0.026,

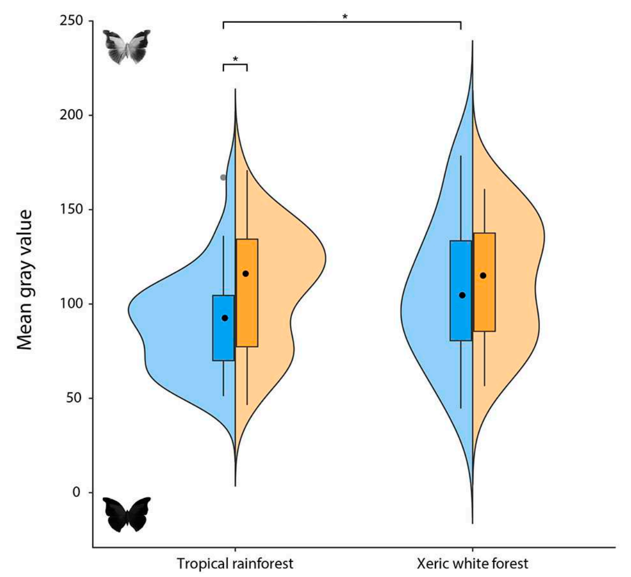

FIGURE 1
Mean gray value of butterflies from two biomes (tropical rainforest and xeric white forest). Blue and orange violin areas represent the frequency distribution of values for the dorsal and the ventral sides of butterflies' wings, respectively. Boxes indicate interquartile ranges, whiskers show 95% confidence intervals, and median values are represented by black dots. Gray dots represent outliers, while asterisks and brackets indicate statistically significant differences. The lower the mean gray value, the greater the melanism exhibited.

0.011]; dorsal B: 0.002 [−0.022, 0.026]; ventral R: 0.019 [−0.014, 0.052]; ventral G: 0.000 [−0.020, 0.017]; ventral B: −0.015 [−0.039, 0.008]) nor wing side (i.e., dorsal vs. ventral; rainforest R: 0.001 [−0.022, 0.025]; rainforest G: −0.001 [−0.020, 0.017]; rainforest B: 0.009 [−0.006, 0.024]; white forest R: 0.013 [−0.020, 0.047]; white forest G: −0.004 [−0.022, 0.017]; white forest B: −0.009 [−0.030, 0.012]).

4. Discussion

The comparison of butterfly assemblages from each biome did show differences regarding the melanism of their wings, confirming our first hypothesis and previous evidence showing that the lightness of butterflies' wings contrasts between different ecosystems, a trait that has been linked to thermoregulation (Tsai et al., 2020). Our expectation was that tropical rainforest butterflies should be more melanic (i.e., to present lower mean gray values) to counteract a higher parasitic rate found in humid environments (Stelbrink et al., 2019). Studies have shown that there is a pleiotropic effect between melanocortins and the immune function of animals (Ducrest et al., 2008) and that more melanic animals could defend themselves against parasites by strengthening their immunocompetence or directly preventing the proliferation of parasites due to the toxicity of melanin molecules (Côte et al., 2018). In addition, we also expected that white forest butterflies should be less melanic to avoid overheating (Schmitz, 1994; Forsman et al., 2002; Heidrich et al., 2018) since the xeric white forest is notoriously known for its high solar radiation, high average annual temperature, low cloud cover, and low relative humidity rates (Prado, 2003).

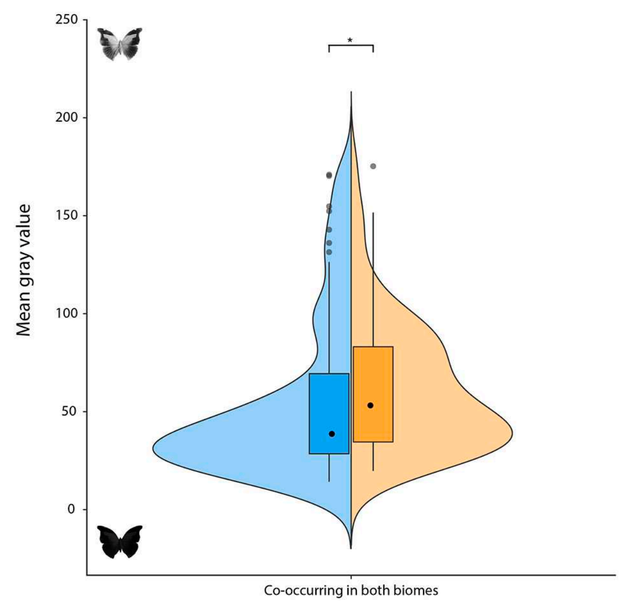

FIGURE 2
Mean gray value of butterflies co-occurring in both biomes (tropical rainforest and xeric white forest). Other conventions as in Figure 1.

Our data also revealed that butterflies from the rainforest have wings that are significantly more melanic on their dorsal faces when compared to their own ventral wing surfaces, but the same was not true for white forest assemblies, which is the opposite of what we would expect according to our second hypothesis. Differences in melanism between dorsal and ventral wing surfaces have been associated with thermoregulatory advantages (Tsai et al., 2020), in such a way that heat-stressful environments, such as the xeric white forest, should have selected for more prominent differences, although it has also been observed that butterflies from regions with less sun exposure tend to be darker (Dalrymple et al., 2018). Here it is worth noting that the studies that proposed the relationship between melanism and thermoregulatory strategies were based on assemblages of butterflies from colder regions (Zeuss et al., 2014; Stelbrink et al., 2019; Tsai et al., 2020), a rule that might not fully apply to tropical environments. Having a darker dorsal surface and a lighter ventral area might also be a thermoregulatory adaptation related to more heterogenous biomes, which consist of different phytophysiognomies, as in the case of the tropical rainforest. The tropical moist broadleaf forests biome (Olson et al., 2001) presents two distinct phytophysiognomies that were considered in this study, the Coastal Atlantic Forest, a true tropical rainforest, with tall trees that form high and closed canopies, and the Atlantic Coast Restinga, a type of coastal tropical forest, characterized by medium-sized trees and shrubs adapted to drier conditions. Those tropical rainforest butterflies that frequently transition from a dryer and open phytophysiognomy (e.g., the Atlantic Coast Restinga) to another that is much more shaded and humid (e.g., Coastal Atlantic Forest) should benefit from the thermoregulatory advantages (Tsai et al., 2020) of having a darker dorsum and a lighter ventral surface. If these interpretations are correct, it would be expected that butterflies inhabiting both biomes (i.e., rainforest and white forest) would also show differences in melanism between their ventral and dorsal wing surfaces. Indeed, a posteriori analysis, following the same method described in this study, but using 75 butterfly species that occur in both biomes (Supplementary Table S2; the statistical analysis used the taxonomy as a proxy for the phylogenetic dependence), showed a significant difference in melanism between their dorsal and ventral wing surfaces (9.89 [4.37, 19.4]), with a darker dorsum and a lighter ventral surface (Figure 2 and Supplementary Figure S2). In contrast, for a considerably dryer, open, and leafless biome, like what is found in the Xeric Shrublands (Olson et al., 2001), known in Brazil as the Caatinga or white forest, a tendency to show a similar degree of melanism on both sides of their wings (Figure 1) could be understood as a strategy to avoid desiccation and UV damage (Majerus, 1998).

An alternative explanation for the difference in melanism between the dorsal and the ventral faces of rainforest butterflies' wings would be that it works as an anti-predatory strategy (Murali, 2018), making it difficult for predators to capture them (Pinheiro et al., 2016). When butterflies present a different pattern of melanism between ventral and dorsal areas they can produce an effect called dynamic flash when flapping their wings during flight, which would help to confuse predators about their exact location (Murali and Kodandaramaiah, 2020). Although literature still lacks studies comparing the predation pressure of butterflies in different tropical environments, we believe is reasonable to guess that the abundance of butterfly predators is greater in the rainforest than in the white forest, due to the greater general diversity of species found in the rainforest, which would be in line with our proposition.

Even though the latitude in which our specimens were captured did not vary much (Supplementary Tables S1, S2), and despite the fact that the solar incidence of different regions of the Brazilian northeast is comparably high (Lopo et al., 2014), differences in solar incidence between localities with diverse kinds of phytophysiognomies were expected (although the solar incidence was not directly accessed in the present study), since vegetation cover reduces the intensity of sunlight and considerably decreases the proportion of short wavelength light from the illuminant (Endler, 1993). However, our third hypothesis, stating that shaded environments (i.e., rainforest) should have butterflies with more saturated colors, was not corroborated.

Since the illuminant spectrum also influences the production of animal coloration (Endler, 1993), changes in the geometry of canopies, resulting from deforestation, could also influence the dynamics and spatial distribution of organisms of different colors (Spaniol et al., 2019). Spaniol et al. (2020) showed that degraded environments might be experiencing a loss in the diversity of butterflies' color patterns since in degraded and more exposed environments butterflies with more conspicuous colors may have been reduced by predator activity. In line with this, we have found that butterflies' dorsal wing sides present a higher variance between species (regardless of the environment) when compared to their ventral wing side. On the one hand, a lower color variance on ventral wing surfaces might be seen as an indication that some ecological factor (e.g., predation avoidance) is exerting a strong homogenizing force on ventral wing coloration. On the other hand, for dorsal wing surfaces, a greater variety of active selective forces (e.g., thermoregulation and socio-sexual signaling) could explain the existence of a higher color variance.

To our knowledge, this is the first study to compare the coloration of butterfly assemblages in humid and semi-arid tropical environments. Due to its occurrence in a variety of ecosystems, future research with Neotropical butterflies must be encouraged and should incorporate data on communities from savannahs, prairies, and floodlands, in addition to comparing butterfly populations from a specific biome (e.g., rainforests from the Northeast and the Southeast regions of Brazil), but that occur in very different latitudes (Zeuss et al., 2014). It would also be interesting to investigate the relationship that other body regions, such as butterflies' thorax and the basal region of their wings (Munro et al., 2019), have with melanism and color saturation, as well as how Neotropical butterflies reflect infrared radiation (see Kang et al., 2021). Furthermore, since most studies that assess macroecological patterns focus on the consequences of population dynamics and changes in coloration patterns (Zeuss et al., 2014; Pinkert et al., 2017; Xing et al., 2018), experiments that relate environmental degradation to the degree of melanism in butterfly populations and communities should also be pursued. Future studies could also include visual modeling techniques to assess how changes in the sensory environment of organisms (e.g., changes in vegetation structure and environmental light) may affect the trade-off between animal communication, predation, and thermoregulation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SS: conceptualization, methodology, software, formal analysis, investigation, data curation, writing—original draft, and visualization. FG: formal analysis and writing—review and editing. MC: conceptualization, resources, and writing—review and editing. DP: conceptualization, methodology, resources, writing—review and editing, visualization, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.932755/full#supplementary-material

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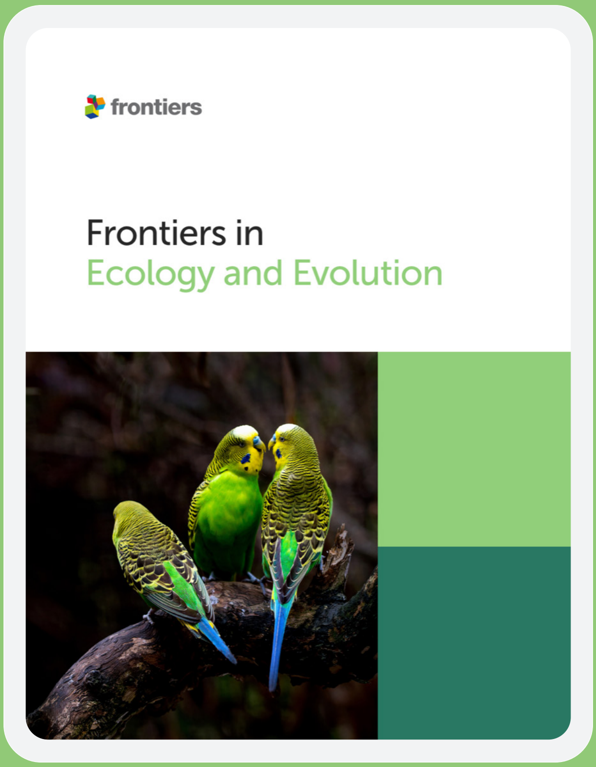
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