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RESEARCH ARTICLE

Does nocturnal light pollution impair immune function in a wild-living amphibian?

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Abstract

1. Light pollution is among the most rapidly growing anthropogenic stressors on Earth. As it spreads far beyond its original source, it affects natural protected areas playing a key role in protecting biodiversity. While physiological impacts of light are extensively studied in laboratory animals and livestock, it is yet unknown what are the physiological effects of low levels of light at night on animals—such as those experienced by wildlife in natural areas exposed to light pollution.
2. Experimental studies suggest that light pollution have deleterious effects on metabolism and immunity (among others) mainly caused by melatonin suppression. Within the One Health framework, alterations of wild animals' immunity and their capacity to combat infections due to light pollution could have far-reaching implications for species conservation, pathogen dynamics and potential for epidemic outbreaks.
3. The aim of this study was to assess, for the first time, whether the effects of light pollution on immunity previously reported in experimental studies, are also observable in a wild species. To do so, we measured a large set of body condition and immune traits, to obtain a comprehensive picture of individuals' health, in 15 natural populations of common toads (*Bufo bufo*) distributed along a gradient of exposure to light pollution.
4. Although our results reveal a strong negative association between light pollution values of our sites and toads' body mass, associations with immune traits did not fully match our predictions. Toads from the most illuminated sites presented lower levels of inflammatory marker haptoglobin, a decreased albumin to globulin ratio and tended to have more eosinophils than those from the darkest sites. However, these latter associations with immune traits were not specific to light pollution only, but also related to the global urbanisation of the sites.
5. Our study emphasises the need for more studies on the effect of light pollution on physiology in natural conditions of exposure, and its interactions with other anthropogenic perturbations, to be able to truly understand its effects on

wildlife. This is critical to identify health risks for wildlife and for prioritizing conservation efforts of menaced taxa.

KEYWORDS
amphibian, anthropogenic pollution, artificial light at night, eco-immunology, immune system, urban

1 | INTRODUCTION

Over the past century, human activities have profoundly altered the environment, exposing wild organisms to a large range of stressful stimuli such as air pollutants (Thomson et al., 2019) or noise pollution (Troïanowski et al., 2017). This has been accompanied by a massive decline in biodiversity (Singh, 2002) and a parallel increase in emerging infectious diseases (Jones et al., 2008). One of these profound changes is the increasing use of artificial lighting at night, which has drastically transformed nocturnal conditions in large regions of our planet. Light pollution is among the most rapidly growing anthropogenic stressors on the planet: light emissions have increased by at least 49% between 1992 and 2017, and this trend continues to escalate (Sánchez de Miguel et al., 2021). Light pollution, or artificial light at night (ALAN), currently affects around 23% of the Earth's terrestrial surface and 99% of the European territory (Falchi et al., 2016). As light pollution spreads far beyond its original source (Jechow et al., 2020; Kyba et al., 2011), it also affects natural protected areas that are playing a key role in protecting biodiversity from a wide range of other anthropogenic pressures (Gaston et al., 2015). Aquatic ecosystems, recognised as key biodiversity hotspots, are particularly exposed since lit road networks, urban expansion and industrial infrastructures are often concentrated along rivers, lakes and sea shores (Hänel et al., 2018; Jechow & Hölker, 2019).

This profound change in the nocturnal environment has many consequences for both diurnal and nocturnal organisms. Light is indeed a major environmental cue for virtually all living organisms—from bacteria, plants to animals and humans, regulating a wide range of behaviour and physiological pathways that are synchronised by the daily and seasonal light cycles (Walton et al., 2011). A large body of literature now reports that exposure to light pollution, by altering the natural alternation between light and darkness, strongly affects behaviour such as movement, migration, foraging activity, predation risk and communication in wild populations (Bumgarner & Nelson, 2021; Hölker et al., 2010; Longcore & Rich, 2004; Sanders et al., 2021). However, the physiological consequences of light pollution in wild animals living in natural areas remain largely unknown (Dominoni et al., 2016; Hölker et al., 2021). Studies conducted on laboratory animal models and humans consistently report that exposure to artificial light during the nocturnal period particularly disrupts melatonin biosynthesis in a large range of taxa (Grubisic et al., 2019; Yang et al., 2024). Melatonin is a pleiotropic nocturnally peaking hormone, involved in a multitude of physiological processes including the regulation of circadian rhythms, metabolism (Korkmaz et al., 2009), endocrine function (Ouyang et al., 2018), oxidative stress responses (Reiter, Tan, et al., 2000) and immunity (Ziegler et al., 2021). If confirmed in animals living in natural conditions, deleterious effects of exposure to light pollution on immune function could have serious consequences for individual health and performance. Immunity is indeed a crucial physiological function directly related to survival, as it confers protection against the constant threat of parasites and pathogens that are ubiquitous in the wild (Møller & Saino, 2004; Schmid-Hempel, 2003). While allowing animals to survive and thus having a key role in population dynamics, immunity is also a driving evolutionary force that exerts selection on pathogens carried by animals (Grenfell et al., 2004). Within the One Health framework, alterations of wildlife immunity and their capacity to combat infections due to light pollution could have far-reaching implications for species conservation, pathogen dynamics and potential for epidemic outbreaks (Kernbach et al., 2019; Navara & Nelson, 2007).

To this date, although experimental exposures to ALAN have been reported to affect immunity in several species, these consequences remain unproven under natural conditions. Experimental studies reported that constant light exposure alters leukocyte proportions, either as an increase or a decrease depending on the species (e.g. Bowden, 2008; Gastón et al., 2019). In birds, two studies showed that exposure to 3 lux during the night altered nestling innate immune responses after an experimental immune challenge, specifically affecting haptoglobin and nitric oxide levels, but in opposite directions (Raap et al., 2016; Ziegler et al., 2021). Kernbach et al. (2019) also reported that the dysregulations of immunity caused by exposure to high levels of ALAN (8 lux) were accompanied by an increase in the transmission risk of a zoonotic pathogen. It is important to note, however, that all the studies linking exposure to ALAN with immune alterations are experimental and involve constant and/or high lighting intensities. These conditions greatly limit our understanding of the real processes occurring under natural exposure scenarios. In natural settings, light pollution is composed of various lighting types and fluctuating intensities, which most of the time are very low. Being often the result of the sole diffusion of the sky glow, light pollution is often imperceptible to the human eye and occurs at intensities that are almost never tested in experimental studies (sky glow illuminance <1 lux, Jechow et al., 2020). Finally, the diversity of the immune traits measured across studies makes it difficult to draw general patterns, as these traits often reflect different components of the immune system, a common issue in eco-immunology studies.

The aim of this study was to assess, for the first time, whether the effects of light pollution on immunity previously reported in experimental studies are also observable in a vertebrate species in the wild. We tested this in natural populations of the common toad (*Bufo bufo*). Amphibians are one of the most threatened vertebrate taxa worldwide (Bishop et al., 2012; Bolochio et al., 2020), making it urgent to evaluate the risks of light pollution exposure on their immunity and health—especially given the impact of emerging viral and fungal diseases that are a source of mass mortality in this taxon (Rachowicz et al., 2006; Scheele et al., 2019). The common toad is a commonly encountered species in wetlands, ranging from highly urbanised areas to the natural environments. Their nocturnal activity frequently exposes them to light pollution. Previous experimental studies have reported that exposure to artificial light at night impairs adult toads' activity levels, reproduction and energy metabolism (Touzot et al., 2019, 2020). During the larval stages, it impacts the expression of immune genes, notably through the downregulation of genes related to innate immunity and the upregulation of genes related to inflammation (Touzot et al., 2022). These results suggest that immune function in this species may be particularly susceptible to disruption by light pollution. Here, we thus measured a large set of immune markers, encompassing both innate and adaptive components of the immune response, to obtain a comprehensive picture of the immune system alteration in common toads from 15 natural populations distributed along a gradient of exposure to light pollution. We also assessed toads' body mass and two physiological markers related to condition—as studies reported effects of exposure to light pollution on metabolism (Dominoni et al., 2016), and because immunity, as an energetically costly physiological function, is often related to body condition (Lochmiller & Deerenberg, 2000). Finally, we also characterised the landscape structure of the sites of our study to take into account other environmental variables (altitude, urbanisation, forest cover...) that can affect toads' body mass and physiology. Based on the previous experimental studies on this species cited above, we predicted lower innate immunity and higher levels of inflammation in toads from populations most exposed to light pollution (Touzot et al., 2022), as well as a lower body mass due to disrupted metabolic processes (Touzot et al., 2019) compared to individuals from the darkest sites.

2 | MATERIALS AND METHODS

2.1 | Ethics

The protocols of capture and blood sampling of toads were approved by the French Government via prefectural decrees n° 69-2023-01-20-00005 for the Rhône department; n° DDPP01-23-044 for the Ain department; n° 38-2023-02-21-00003 for the Isère department. All experiments were performed in accordance with guidelines and regulations of the Ethical Committee of Lyon 1 University (project APAFIS #41025-2023021713471183, 20th February, 2023).

2.2 | Sites

We selected 15 populations of toads in sites around a 50-km radius from the Lyon Metropole in France (Figure 1, precise locations in Supporting Information S1). This area is characterised by its variety of environments, from dense urban areas (Lyon metropolis, 1.4 million inhabitants), residential areas/villages to agricultural zones and isolated natural areas. The sites have been chosen in this area to be located on a gradient of exposure to light pollution. Exposure of the sites to light pollution has been measured with a sensitive lightmeter SQM (Sky Quality Meter, Unihedron—following the methodology of Spoelstra, 2014; Kyba et al., 2015): the higher the value obtained is, the darker the site is. As an example, an urban night sky will display a value around 16–17 and a dark site with a starry sky will display a value around 21–22. For this, we used a tripod with a fixed structure to attach the SQM at a similar height (1 m above the ground) and position (at 90° or zenith, 45° and 0° or horizon; at North/West/East/South directions) in each site—giving us a total of 12 standardised measures in each site. These measures have been recorded on a moonless sky to ensure that only the illuminance from light pollution was captured. Recordings were also conducted during periods of dense cloud cover to obtain the maximal value of light pollution exposure in a site, as cloud cover amplifies light pollution (Kyba et al., 2011). All measures are provided in Supporting Information S1. Because we expected that other landscape features would covary with light pollution, additional landscape variables associated with urbanisation and climate were extracted: altitude (as a proxy of ambient temperature), the percentage of forest, urban, agricultural and wetlands areas in a buffer of 500 m around the pond of toads' capture, calculated using QGIS software (see Table 1).

2.3 | Captures and sampling

Toads were captured in spring during the short period of time when they are migrating from their hibernation sites in the woods to ponds to breed. In this study, they were captured between the 8th and the 16th of March 2023, but on the same night for all individuals of a given site. As the sex ratio is strongly biased towards males in this species, only male toads were captured here. Ten toads per site were captured, that is 150 toads in total. After capture, toads were weighed to the nearest g using a digital scale and a blood sample was taken by cardiocentesis (25G heparinised and sterile needles) after local anaesthesia with a lidocaine-prilocaine cream (EMLA 5%). The volume of blood samples taken represented a maximum of 1% of the individual body mass. After sampling, toads were immediately released on site. A drop of fresh blood was immediately used for a blood smear of each individual,

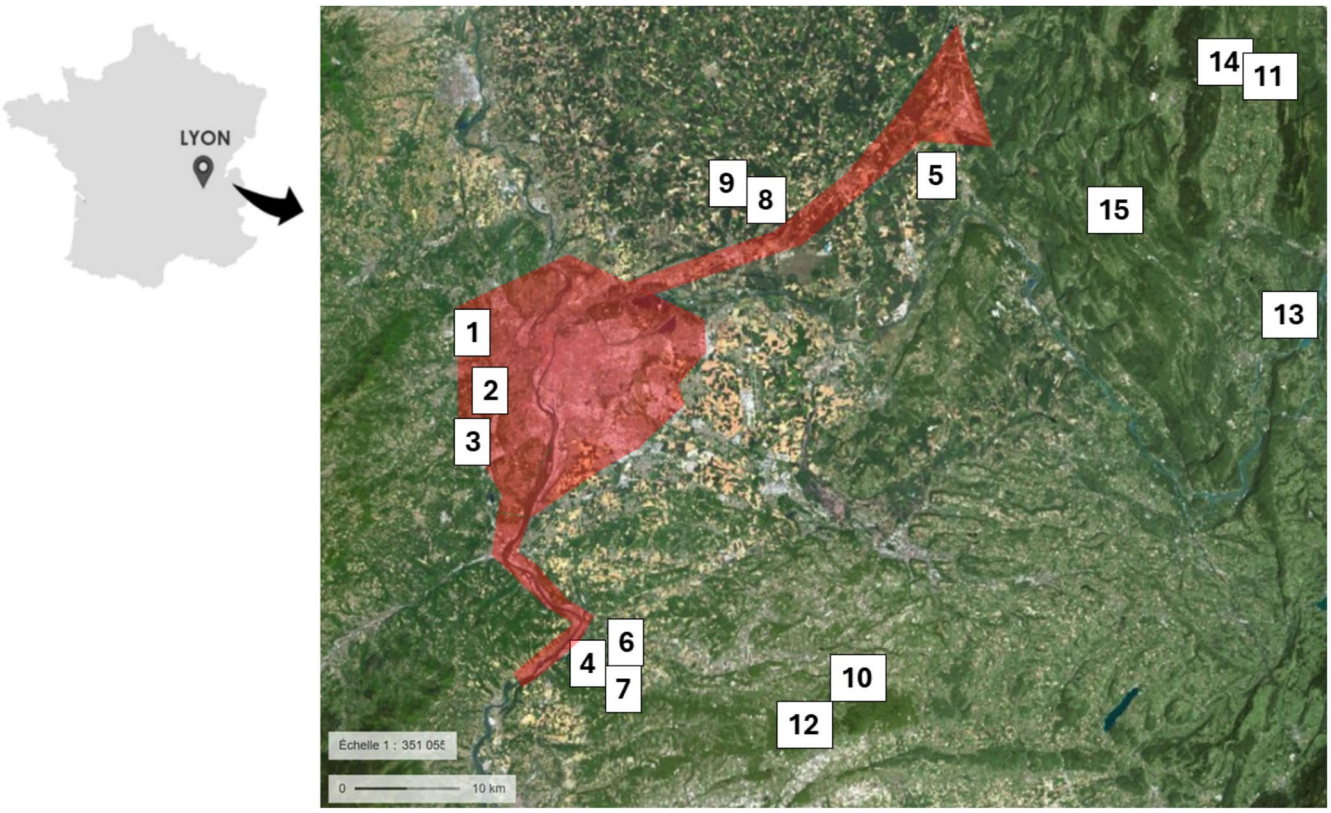

FIGURE 1 Sites where common toads were sampled across Eastern France, around Lyon metropolis. The 15 sampling sites are numbered and ranked according to their exposure to light pollution (measured with a SQM, Sky Quality Meter), from the most exposed site (1) to the less exposed site (15). Cultivated areas appears in yellow; grassland, forest and other seminatural areas in green; and dense urbanised areas are coloured in red.

TABLE 1 Characteristics of the study sites located in the Rhone-Alpes region in France, measured in a 500 m radius buffer around the reproduction site where the toads were captured.

	Name of the site	Altitude (meters)	Mean SQM value at night (cloudy moonless sky) ± SE	Landscape variables in the 500 m area around the reproduction site (%)			
				Forest	Urban	Agriculture	Wetlands
1	Saint-Genis les Ollières	225	17.19 ± 0.47	36.54	45.33	17.74	0.40
2	Chaponost	300	17.49 ± 0.50	26.65	50.53	22.27	0.55
3	Brignais	300	17.84 ± 1.04	30.52	42.38	26.53	0.56
4	Jardin	410	18.34 ± 0.55	68.61	5.78	23.88	1.73
5	Leyment	250	18.44 ± 0.55	66.70	4.32	28.36	0.61
6	Saint-Sorlin de Vienne	340	18.62 ± 0.55	43.59	3.91	51.31	1.18
7	Estrablin	200	18.62 ± 0.53	30.65	24.12	44.25	0.98
8	Pizay	240	18.84 ± 0.53	34.46	7.81	57.42	0.32
9	Sainte-Croix	276	18.96 ± 0.59	23.01	5.63	68.25	3.11
10	Chatonnay	550	19.79 ± 0.49	67.49	2.49	21.17	8.85
11	Songieu	734	19.99 ± 0.22	14.22	2.81	79.65	3.33
12	Bossieu	507	20.17 ± 0.68	86.61	0.00	0.00	13.39
13	Pollieu	298	20.19 ± 0.55	66.21	6.89	12.05	14.85
14	Hotonnes	665	20.27 ± 0.54	17.55	1.11	80.72	0.62
15	La Burbanche	354	21.00 ± 0.50	78.92	3.19	12.31	5.57

Note: Sites are ranked in descending order of exposure to light pollution (mean SQM value on a cloudy night with no moon).

while a small amount of blood was collected in a heparinised microcapillary tube for haematocrit determination (centrifuged for 5 min at 10,000*g*). Haematocrit (%) is a haematological parameter that reflects changes in an animal's oxygen-carrying capacity. The remaining blood sample was centrifuged for 5 min at 1,000*g*, after which the plasma was transferred into an Eppendorf and stored at −80°C.

2.4 | Immune phenotype

We measured a large set of immune traits in order to depict both the innate and the adaptive responses of toads, encompassing humoral and cell-mediated components.

First, we assessed the cellular part of innate and adaptive immunity, by determining the composition of the white blood cell (WBC) populations, that is five different cell types, based on the identification of the first hundred WBC in May-Grunwald/Giemsa-stained blood smears. Among these, neutrophils and monocytes are phagocytes involved in the innate response (Claver & Quaglia, 2009). Eosinophil and basophil function remain poorly understood in amphibians. Eosinophils seem to be associated with defence against internal parasites such as trematode infections but also in response to pollutants (Claver & Quaglia, 2009; Kiesecker, 2002). Basophils play a role in early inflammation and in innate immune responses by releasing proteins such as toxins (Claver & Quaglia, 2009). Cell-mediated adaptive immunity was assessed by lymphocyte counts including both T and B cells (see blood smears above), B cells being particularly involved in the production of antibodies. We also determined the total proportion of white blood cells relative to erythrocytes, based on the identification and count of the first hundred cells in randomly selected areas of the blood smears. This metric was used as a proxy of investment in cellular immunity.

The humoral part of innate and adaptive immunity was then determined by measuring levels of the following plasma proteins: alpha1-globulins, alpha2-globulins, beta1-globulins, beta2-globulins and gamma-globulins. Alpha- and beta-globulin fractions include acute phase proteins (APPs) of the inflammatory response. Gamma-globulins, or immunoglobulins, represent the majority of circulating antibodies and thus their concentration reflects adaptive immunity. We also calculated the albumin to globulins ratio (A:G), as an altered A:G ratio could indicate changes in physiological status. In particular, a decrease in albumin synthesis and/or an increase in the synthesis of one of the APPs can reflect inflammation (Zaias & Cray, 2002). The total protein content (in mg/mL) was first assessed using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, reference 23,225), followed by automatic agarose gel electrophoresis (HYDRASYS, Sebia, Evry, France) that separates albumin and the five fractions of globulins (α1, α2, β1, β2 and γ). Finally, albumin levels obtained (mg/mL) also reflect the blood level of protein resources of an individual.

In addition, we measured the level of haptoglobin as a proxy of inflammation. These proteins bind free haemoglobin in order to mitigate damages caused by reactive oxygen components produced during inflammation (Quaye, 2008). We measured haptoglobin using 7.5 μL of plasma with a commercially available assay (TP801, Phase Haptoglobin Assay, Tri-Delta Diagnotics, United States) and following recommendations from Matson et al. (2012, in birds) and Lorrain-Solignon et al. (2022, in frogs). This colorimetric assay measures the preservation of the peroxidase activity of bounded haemoglobin, which should be proportional to the concentration of the haemoglobin-binding proteins and thus inflammation in the plasma sample. All samples were run in duplicates.

2.5 | Data analyses

2.5.1 | Effect sizes of light pollution exposure on toads' traits

We calculated the effect sizes of SQM values (i.e. quality of night sky, inversely proportional to light pollution) on toad's body mass, physiological markers and immune traits. Effect sizes were calculated as partial correlation coefficients, which measure the standardised effect of SQM value on toad's traits, while controlling for the potential effects of other variables. To obtain the different effect sizes, for each toad's trait we first fitted a mixed-effect model including SQM value and body mass (at the exception of the model where body mass was the response variable in the model) as fixed factors, and the site as a random effect on the intercept (i.e. additive model) to take into account the genetic proximity of individuals in a given site. We then used the equation provided by Nakagawa and Cuthill (2007, p. 82) for mixed-effect models to calculate effect size. We calculated confidence intervals of effect sizes following Nakagawa and Cuthill's (2007) recommendations. All effect sizes and confidence intervals are provided in Supporting Information S2. Finally, covariation between body mass, physiological markers and immune traits using a PCA analysis is provided in Supporting Information S3. Effect size of SQM values on PCs of body mass, physiological markers and immune traits covariation is also provided in Supporting Information S3.

2.5.2 | Effect sizes of global landscape structure on toads' traits

Because light pollution is not the only environmental factor that can affect toads' physiology, we then tested the effects of landscape structure to compare its effects to those of light pollution alone. To do that, we first performed a principal component analysis (PCA) on

the landscape variables that characterise the 15 sites of our study (see Table 1) to identify the main axes of variation that determined their correlation structure. We expected that, due to probable correlation between light pollution and other landscape features, testing if landscape structure modulates the relationship between light pollution and toad physiology would not be possible. The following 6 variables were used in the PCA: percentage of wetlands, agricultural land, urbanised areas, forest area, altitude and SQM value (i.e. ALAN exposure). PCA results are presented in Supporting Information S4. We then calculated the size effects of the PCs (PC1 and PC2), that is global landscape structure including light pollution, on toads' body mass, physiological markers and immune traits. Effect sizes were calculated as above, with PCs and body mass as fixed factors and site as a random effect. All effect sizes and confidence intervals are provided in Supporting Information S5. Finally, the effect sizes of PCs of global landscape structure on PCs of body mass, physiological markers and immune traits covariation are also provided in Supporting Information S3.

PCA was performed using the R package 'ade4' (Dray et al., 2007). All analyses were carried out in R version 4.3.3 (R Core Team, 2024).

3 | RESULTS

3.1 | Effect sizes of night sky quality on toads' body mass and immune traits

Figure 2 presents the effect sizes of sites SQM value (night sky quality) on body mass, physiological markers of condition and immune traits. A high SQM value, which reflects a dark site or high sky quality, was positively associated with several parameters, that is body mass (effect size and 95% CI, 0.34 [0.05; 0.64]), albumin levels (0.27 [0.06; 0.47]), haptoglobin levels (0.47 [0.20; 0.74]) and A:G ratio (0.35 [0.14; 0.56]) (Figures 2 and 3). Toads in areas with high ALAN values thus displayed a lower body mass, lower levels of albumin and haptoglobin and a lower A:G ratio. On the contrary, the proportion of eosinophils tended to be negatively correlated with SQM values (Figure 2, −0.17 [−0.35; 0.01], Supporting Information S6), which suggests that toads subjected to ALAN tend to display more eosinophils than those from dark sites. All non-statistically significant relationships between immune traits and night sky quality are represented in Supporting Information S6.

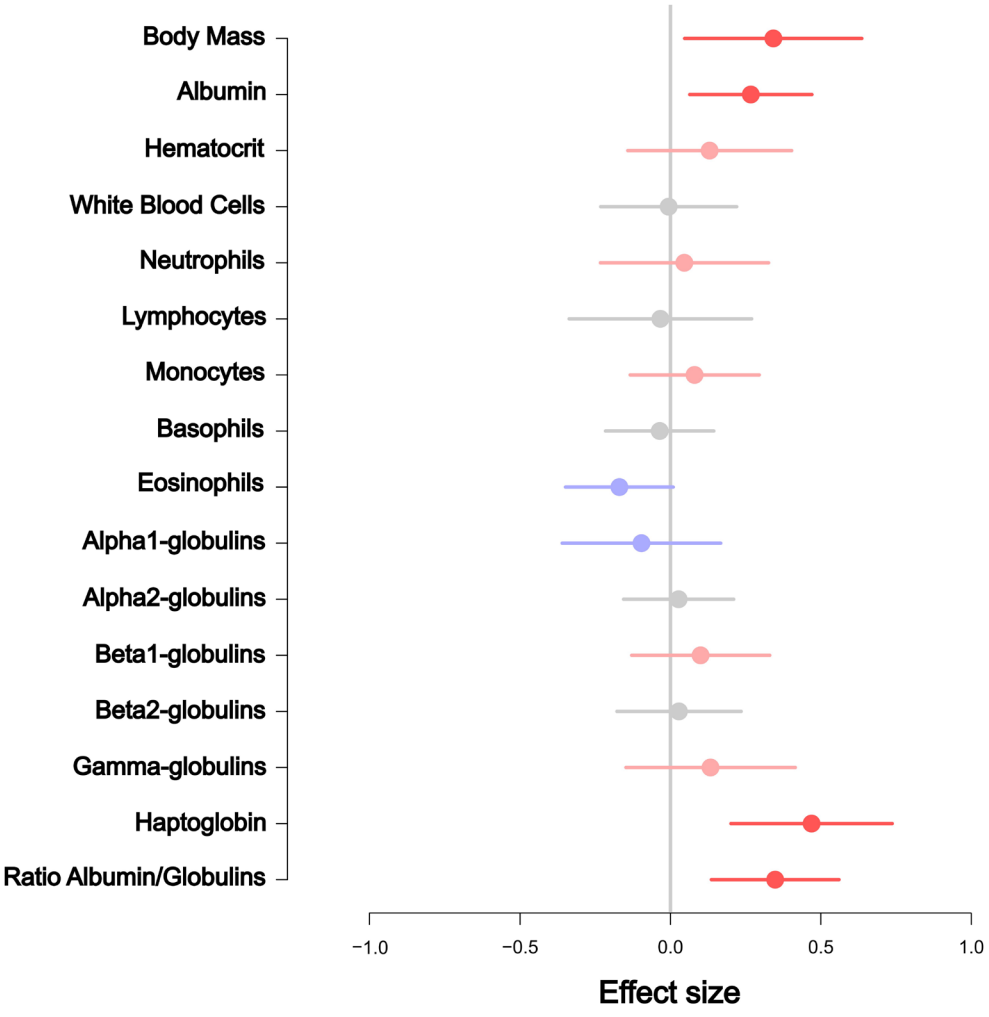

FIGURE 2 Effect sizes of the night sky quality (i.e. SQM values) on body mass, physiological markers of condition and immune traits measured in toads from 15 natural populations. Effect sizes (symbols) are reported together with associated 95% confidence intervals.

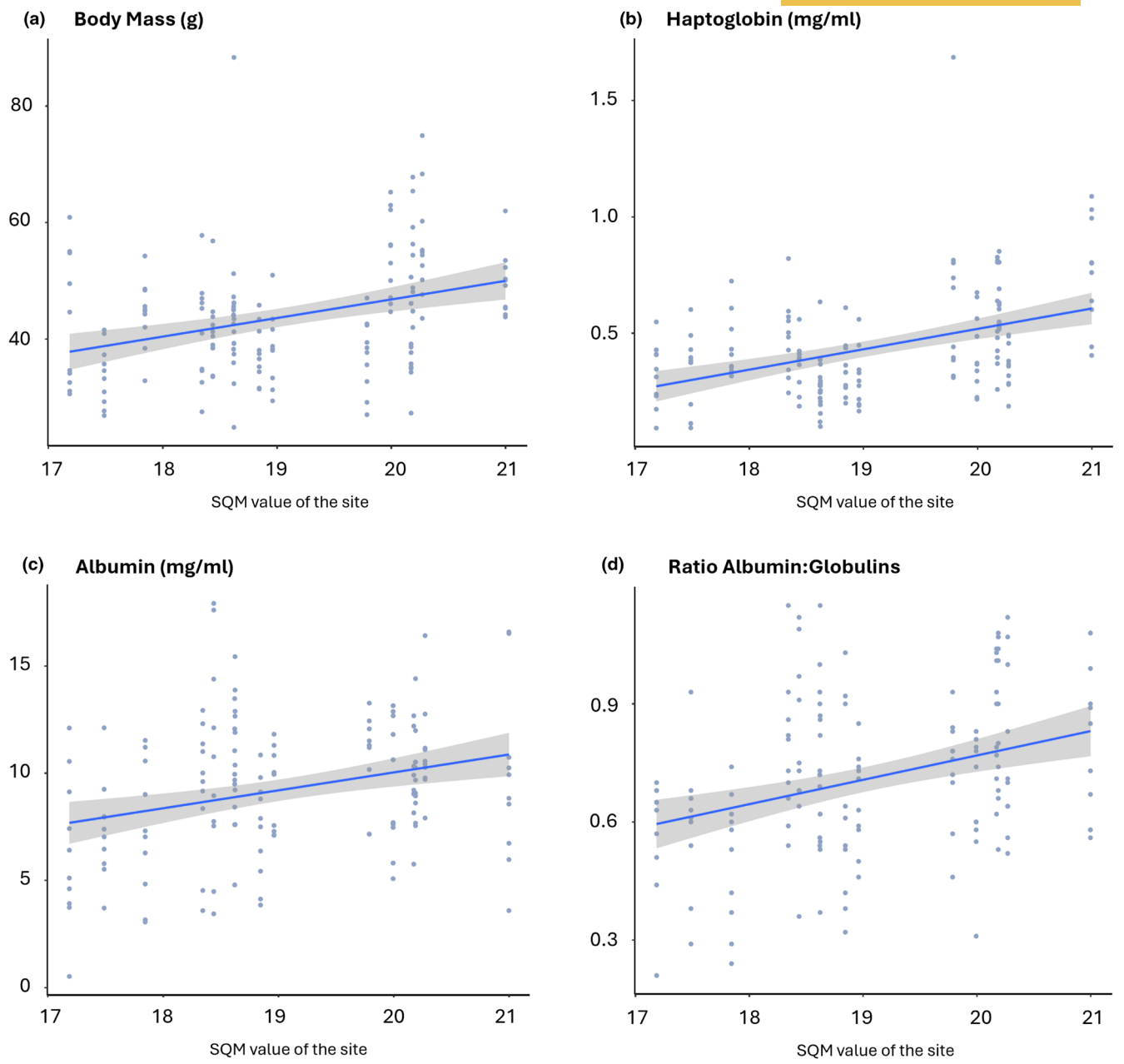

FIGURE 3 Relationships between night sky quality and (a) body mass, (b) haptoglobin-like proteins, (c) albumin and (d) albumin to globulin ratio of wild-living toads. Blue lines represent the linear relationships between toad traits and SQM value of the site with 95% CIs.

3.2 | Effect sizes of landscape structure on toads' body mass and immune traits

As light pollution represents only one part of several environmental variables that may affect toads' body mass, physiological markers of condition and immune traits, we also tested the effects of global landscape structure (which includes ALAN) on toads' traits. Covariation between landscape variables was assessed with a PCA, whose results are presented in Supporting Information S4. The first axis of covariation among the landscape variables (PC1) accounted for 48% of the total inertia. PC1 was positively correlated with urbanisation ($r=0.77$) and negatively correlated with SQM value or site 'darkness' ($r=-0.87$), wetlands surface ($r=-0.84$) and forest surface ($r=-0.71$). PC1 thus reflects a gradient of dark natural areas of forest and wetlands to lighted urbanised areas. PC2 captured 35% of the total inertia. PC2 was positively correlated with altitude ($r=0.66$) and agriculture ($r=0.93$) and negatively correlated with forest cover ($r=-0.63$). The correlation of PC2 with urbanisation and site SQM value was quite low ($r=-0.42$ and $r=0.35$, respectively). PC2 thus reflects a gradient of land use for agriculture with a limited relationship with light pollution. These two axes captured 83% of the total variation of the sites.

Effects sizes of landscape profiles PC1 and PC2 on body mass, physiological markers of condition and immune traits of toads are presented in Figure 4. The effects of PC1 on these traits were very similar to those of SQM values alone presented above: individuals

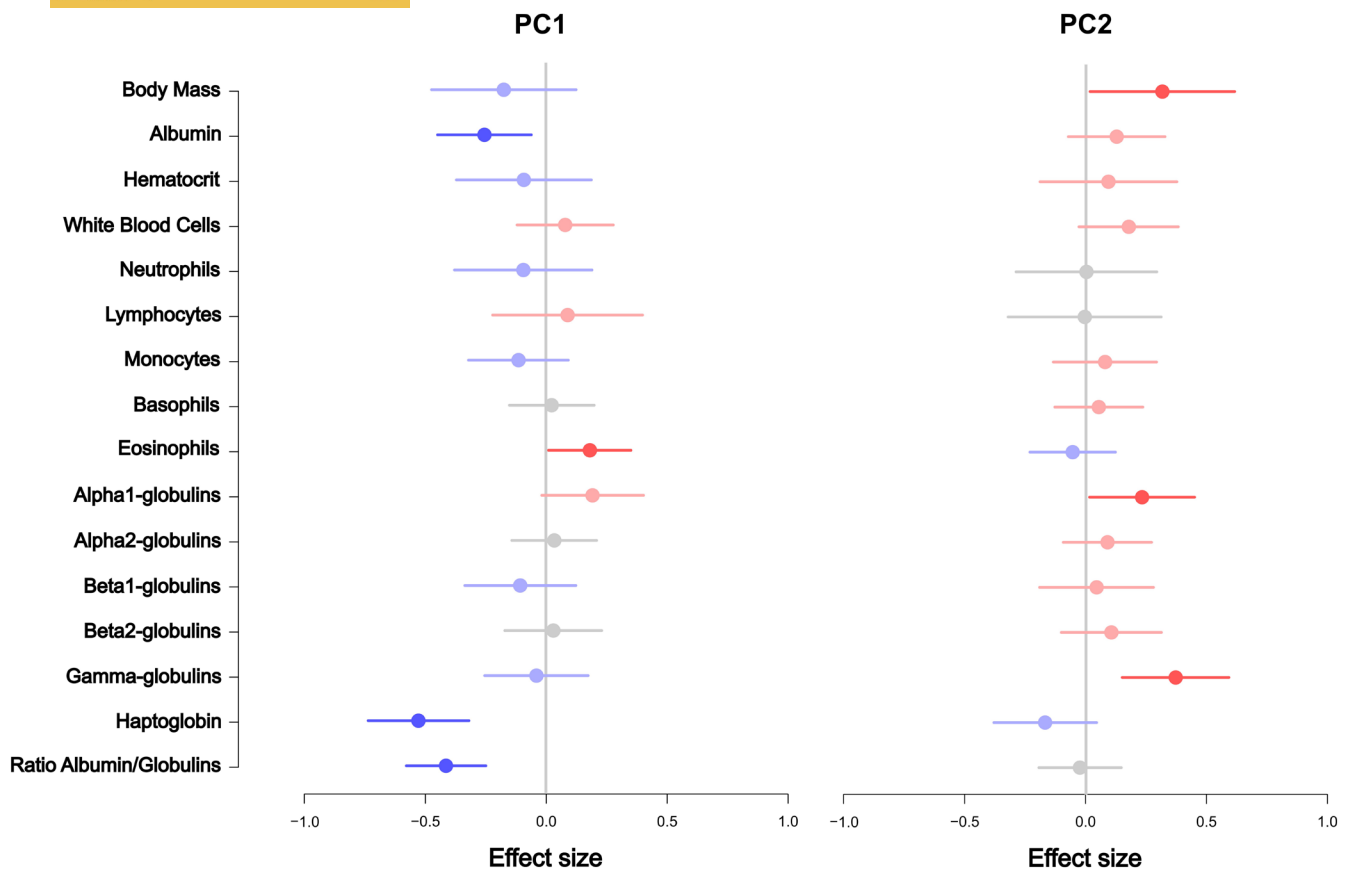

FIGURE 4 Effect sizes of landscape variables on body mass, physiological markers of condition and immune traits measured in toads from 15 natural populations. Effect sizes are reported with their 95% CI. PC1 is a gradient of urbanisation, positively correlated with urbanised areas and negatively correlated with site sky darkness, forest and wetlands areas. PC2 is a gradient of land use for agriculture and altitude, rather negatively correlated with urbanisation.

from more urbanised and illuminated sites displayed lower levels of albumin and haptoglobin, a lower A:G ratio and a higher proportion of eosinophils. Contrary to SQM values alone, PC1 showed no strong effect on body mass and tended to show a positive relationship with alpha1-globulins (Figure 4). PC2, which reflects a gradient of agriculture and altitude with no major relationship with light pollution had a positive relationship with body mass (0.32 [0.02; 0.62]) and with the levels of two globulins (alpha1, 0.23 [0.02; 0.45]; and gamma-globulins, 0.37 [0.15; 0.59]) (Figure 4). PC2 also tended to have a negative relationship with the level of haptoglobin of toads (−0.17 [−0.38; 0.04]).

4 | DISCUSSION

Experimental studies have reported that the immune function is disrupted by exposure to artificial light at night in several vertebrate species (Bowden, 2008; Gastón et al., 2019; Navara & Nelson, 2007; Raap et al., 2016; Ziegler et al., 2021). Here, we investigated the associations between light pollution, body mass, physiological markers of condition and immune traits in a wild vertebrate. These results bring new insights to the field of study of the consequences of light pollution on biodiversity in three ways. First, this is the first study of which we are aware where natural, and not experimental, exposure to light pollution is tested on immunity in a vertebrate from wild populations located along an increasing gradient of exposure to light pollution. Second, we characterised physiological traits across multiple populations, a rare achievement in wild species given the field constraints that often limit the number of populations studied. Third, these results are of particular interest because they consider multiple traits to assess immunity, which provide a comprehensive and unique picture of toads' health. However, although our results reveal a strong negative association between the light pollution values of our sites and toads' body mass, associations with immunity did not fully match our predictions and were not specific to ALAN, that covaried with landscape features. As explained below, our results thus highlight that the effects of light pollution on animals' physiology are likely to be different from those reported in experimental studies and that other anthropogenic factors also come into play.

First, we found a negative relationship between increasing exposure to light pollution and toads' body mass, in accordance with our predictions. Toads from the darkest sites were bigger and also presented higher albumin levels than toads from the most ALAN-exposed sites. Higher albumin levels can reflect that individuals from the darkest sites have higher protein resources than toads from

illuminated sites. Interestingly, we did not find the relationship with body mass when testing for the effect of global urbanisation of the sites (through PC1 of environmental variables, representing a gradient of natural to urbanised areas and exposure to light pollution)—which leads us to think that the relationship could be quite specific to ALAN exposure. If this negative relationship between exposure to ALAN and body mass is contrary to the one described in humans and laboratory mice (Dominoni et al., 2016; Fonken & Nelson, 2014), it confirms that the effects of ALAN on physiology, particularly on body mass regulation, may differ between vertebrate groups (as highlighted in Secondi et al., 2021). Melatonin suppression due to ALAN exposure causes metabolic and endocrine alterations, which can affect glucocorticoid hormone regulation such as corticosterone (Fonken & Nelson, 2014; Touzot et al., 2019). Endocrine circadian rhythms vary between day-active and nocturnal animals; their alteration by ALAN could thus lead to opposite effects in these species, such as those found here on body mass (Secondi et al., 2021). Additionally wild animals experiencing limited resources, a high increase in corticosterone levels would likely affect their survival and thus population demography. Studies on the relationship between exposure to light pollution, stress levels and population demographics would bring interesting insights here. Secondly, several studies in nocturnal amphibian species reported that exposure to artificial light at night strongly disrupts activity and foraging patterns (Baker & Richardson, 2006; Buchanan, 1993; Mazerolle et al., 2005; Secondi et al., 2021; Touzot et al., 2019; Wise et al., 2006). More specifically, common toads experimentally exposed to ALAN present a decreased motor activity (−56% to −73% depending on the light intensity used) and energy reallocation (Touzot et al., 2019). This could be explained by the fact that ALAN disrupts the natural alternation between day and night and thus desynchronises activity patterns from the day/night cycle. Additionally, nocturnal amphibians have dark-adapted eyes (Baker & Richardson, 2006), and a recent experimental study conducted on cane toads, *Rhinella marina*, has demonstrated that artificial light at night decreases the pupillary light response of animals and probably alters their visual performance (Secondi et al., 2023). This could explain why increased environmental illuminance results in an increased time for prey detection and capture in some amphibian species (Buchanan, 1993). In addition to a decreased activity and altered foraging success in amphibians from illuminated sites, the quality and quantity of prey available could also be affected in urban sites exposed to high levels of light pollution (Desouhant et al., 2019).

As for immunity, exposure to ALAN was associated with variation in some specific immune traits. Toads from ALAN-exposed sites displayed lower levels of haptoglobin, a decreased albumin/globulin ratio (A:G ratio) and tended to have more eosinophils than toads from the darkest sites. However, these latter associations with immune traits were not specific to light pollution only, but also related to the global urbanisation of the sites (i.e. PC1 of the landscape variables). These results did not match our first prediction of a lower innate immunity in ALAN-exposed individuals, as suggested by a previous experimental study on toad tadpoles (Touzot et al., 2022). This is in accordance with an experimental study that used dim light at night in the aim of being closer to a natural exposure, which resulted in no change in fish innate immunity after a 2-weeks exposure at 0.01 to 1 lux (Kupprat et al., 2021). Secondly, our results only partially matched our prediction for higher inflammation in ALAN-exposed toads, as they displayed lower levels of haptoglobin (a marker of inflammation), while having a lower A:G ratio which can reflect an inflammatory status (Zaias & Cray, 2002). It is to note that this decrease in A:G ratio is largely underpinned by lower levels in albumin discussed above.

Haptoglobin is an acute phase protein highly released in the blood during inflammation, with strong anti-inflammatory and antioxidant properties (Huntoon et al., 2008). To our knowledge, only two studies measured haptoglobin in experimentally ALAN-exposed wild vertebrates, that is in nestlings exposed to an intensity of 3 lux lighting during the night period. After two days of exposure and following an experimental immune challenge, the first study reported an increase in haptoglobin responses in exposed nestlings (Raap et al., 2016), while the other study found a decrease in haptoglobin responses when individuals were exposed for 7 days (Ziegler et al., 2021). In this last study, this effect was likely mediated (directly or indirectly) by decreased melatonin levels, as ALAN-exposed nestlings also displayed on average 49% lower plasma melatonin concentrations than non-exposed nestlings (Ziegler et al., 2021). As melatonin is known to have the ability to entail both anti- and pro-inflammatory properties, its suppression can strongly disrupt inflammatory responses (Carrillo-Vico et al., 2013; Hardeland, 2019; Reiter, Calvo, et al., 2000). This could be one cause of the lower levels of haptoglobin in ALAN-exposed individuals of our study, though we lack evidence on how ALAN exposure affects melatonin synthesis in amphibians (Yang et al., 2024). It is also likely that toads from the most ALAN-exposed sites are subjected to other perturbators due to urban life (noise pollution, human presence) which could increase their release of stress hormones such as glucocorticoids that are known to have more immune-suppressor effects (Isaksson, 2015; Troïanowski et al., 2017). Furthermore, immunity is a physiological function that entails various energetic costs depending on the responses and effectors used that should be considered in a context of fluctuating resources in the wild. This is particularly the case for inflammatory responses that are known to be very costly (Lee & Klasing, 2004). In our study, male toads were captured at the end of winter when arriving at reproduction sites. Common toads are 'explosive' breeders arriving at their breeding sites immediately following emergence from hibernation (Reading et al., 1991). While they have been fasting for several months, they have to mobilise energy for migration to reproduction sites and to mate in a context of intense intrasexual competition (Davies & Halliday, 1979). Toads from the most ALAN-exposed sites were of lower body condition than those from the darkest sites, which could limit their energetic ability to invest and maintain costly immune responses in addition to other costly life-history traits.

Toads from ALAN-exposed sites also displayed lower levels of albumin/globulin ratio, which is here underpinned by their lower

levels of albumin. The other plasma proteins, the five globulins, were not affected by exposure to light pollution. A change in this ratio is used as an indicator of disease in exotic pets vet medicine (Zaias & Cray, 2002). A decreased level of albumin, which can be used as a marker of body condition (see above), can also reflect a state of chronic inflammation and/or the presence of internal parasites (Zaias & Cray, 2002). Toads from the more ALAN-exposed sites also tend to have slightly more eosinophils than toads from dark sites. In amphibians, eosinophils are leukocytes that respond to pollutants or parasites such as trematodes (Claver & Quaglia, 2009; Kiesecker, 2002). Altogether, these results could reflect the presence of pollutants and/or parasites in the most ALAN-exposed and urbanised sites. Studies conducted in rivers and wetlands located in urban or peri-urban areas showed that they usually contain a wide range of pollutants including heavy metals, phosphorus, fertilisers, pesticides, suspended solids, hydrocarbons or salts (Paul & Meyer, 2001). It could also reveal that toads from urban sites are more parasitised, because of a higher prevalence of parasites on urban sites and/or poorer protective immune responses. In amphibian populations, several studies reported that the virulence and density of pathogens such as *Ranaviruses* and trematode parasites are aggravated in urban and peri-urban areas that display disturbed or degraded habitats (Hamer & McDonnell, 2008).

In this study, we found that exposure to artificial light at night is negatively associated with body condition in a nocturnal vertebrate. Additionally, immune traits variation in individuals from the most ALAN-exposed and urbanised sites suggest that they could be subjected to more parasites and/or pollutants. While exposure to light pollution and urbanisation associated with many other sources of pollution is somehow confounded in our study and are likely to be in any study performed in a natural gradient of urbanisation, our study still remains one of the first to look for the effect of ALAN on immunity in natural conditions of exposure. Our results, which do not support our first hypotheses, highlight that the effects of ALAN on animals' physiology are likely to be different from those reported in experimental studies as they probably do not fully match the complexity of light pollution exposure in the wild. Our study emphasises the need for more studies on the effect of light pollution on physiology in natural conditions of exposure, and its interaction with other anthropogenic pollutions, to be able to truly understand their effects on wildlife. This is critical to identify health risks for wildlife and for prioritising conservation efforts of menaced taxa.

AUTHOR CONTRIBUTIONS

Louise Cheynel, Nathalie Mondy and Thierry Lengagne conceived the ideas and designed the methodology; Louise Cheynel, Adeline Dumet, Nathalie Mondy, Thierry Lengagne, Emmanuelle Gilot-Fromont, Benjamin Rey, Corinne Régis, Eve Ramery and Vanessa Gardette collected the data, performed the laboratory work and participated in data interpretation; Louise Cheynel performed the statistical analysis; Louise Cheynel wrote the first draft. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

This study has no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data deposited in the Dryad repository: https://doi.org/10.5061/dryad.7d7wm3888 (Cheynel et al., 2025).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Supporting Information S1. Location and SQM values (i.e. quality of night sky measured by cloudy night with no moon) of the 15 toad populations.

Supporting Information S2. Effect sizes of the night sky quality (i.e. SQM values) on body condition and immune traits measured in toads from 15 natural populations.

Supporting Information S3. Covariation between body condition and immune traits in toads from the 15 natural populations.

Supporting Information S4. PCA analysis, covariation between landscape variables from the 15 sites of our study.

Supporting Information S5. Effect sizes of landscape variables on body condition and immune traits measured in toads from 15 natural populations.

Supporting Information S6. Toads' immune traits that are not statistically significantly affected by the night sky quality of the sites (SQM values).

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