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Applications of conservation physiology to wildlife fitness and population health

Edited by
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Applications of conservation physiology to wildlife fitness and population health

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Editorial: Applications of conservation physiology to wildlife fitness and population health

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Editorial on the Research Topic
Applications of conservation physiology to wildlife fitness and population health

Connecting physiological measurements in wild animals with individual fitness and consequently, the resilience of populations poses a significant challenge in conservation physiology. Physiological variables that indicate nutritional state, stress, disease, or injury are used extensively in veterinary practice and captive settings to assess the health and likelihood of reproductive success of many animals. However, it remains difficult to reliably assess the health and resilience of wildlife, especially with the variety of stressors they can encounter in their environment. The development and refinement of sampling methods that limit disturbance of animals, coupled with advancements in analytical methods have allowed researchers to begin to examine the relevance of these physiological parameters in wild animals for predicting population trends and responses to environmental perturbations.

This Research Topic includes nine articles that improve our understanding of the connections between physiological indicators in wild, freely roaming animals and their individual health and fitness, which in turn influences population health. Manuscripts in this Research Topic link environmental drivers, physiological responses, and individual fitness or population health for a variety of taxa, including insects (Herbst), amphibians (Awkerman et al.), birds (Jodice et al.; Maness et al.; Marciau et al.; McCloy and Grace), sea turtles (Stacy et al.), and large mammals (Laliberte et al.; Payne et al.).

Wild animals face a variety of stressors including food availability (Laliberte et al.; Maness et al.), disease (Payne et al.), weather and climate (Herbst; McCloy and Grace), human disturbance (Marciau et al.), and contaminant exposure (Jodice et al.). These stressors often occur simultaneously, with synergistic and/or cryptic sublethal effects on organisms (Awkerman et al.). To understand and predict the effects of such stressors on wild animal individuals and populations, we must first develop baseline knowledge of both physiological health measures for wild species and the diversity of potential stressors that they face. Two articles in this Research Topic address these first steps, by developing blood

analyte reference intervals for the critically endangered hawksbill sea turtle (*Eretmochelys imbricata*) (Stacy et al.) and assessing viral diversity in wild felids (Payne et al.).

Second, we must determine which physiological markers indicate responses to stressors, a step that four articles in this Research Topic address. Marciau et al. evaluates basal and stress-induced corticosterone and body condition as potential indicators of human disturbance in penguins, and finds no relationship (although chicks may be more sensitive than adults). McCloy and Grace correlate passerine bird body condition to temperature and rainfall and find highly species-specific responses, with many species exhibiting threshold effects. Jodice et al. finds that exposure to polycyclic aromatic hydrocarbons is correlated with hematological and biochemical biomarkers suggestive of poor health. Lastly, Awkerman et al. evaluates a suite of physiological measures indicative of anuran health and development that change in response to stressor exposure, while discussing how differences in ontogeny and ecology can limit the interpretation of these biomarkers across amphibian species.

Finally, we must link environmental stressors and physiological indicators with measures of evolutionary fitness. Three papers in this Research Topic address this step. Regarding environmental stressors and fitness, Herbst investigates effects of osmoregulatory stress on growth rates, emergence success, and fecundity in the aquatic alkali fly (*Cirrula hians*), Regarding physiological predictors of fitness, Maness et al. finds that heterophil/lymphocyte ratios predict long-term, while corticosterone concentrations predict short-term, fitness in a seabird; and Laliberte et al. correlates maternal adult serum beta-hydroxybutyric acid with juvenile survival in bighorn sheep (*Ovis canadensis*).

In conclusion, this Research Topic examines how practical physiological measures, suitable for field conditions, can be used to analyze demographic patterns in wildlife populations, assess their reactions to disturbances, and monitor the health of individuals, populations, and ecosystems. These insights can illuminate underlying ecological and evolutionary processes driving responses to stressors, and refine predictions of wild animal responses to environmental change at both individual and population levels. Human, wildlife, and ecosystem health are inextricably intertwined, where the well-being of each component is vital for the overall balance and sustainability of our planet. Considering that environmental health and protection of vulnerable species is of great interest to management professionals, research scientists, and conservationists, we recommended the following areas for increased research:

1) Baseline health information for wild animal species including reference intervals for healthy populations. Without basic knowledge of what healthy physiological parameters look like, we cannot evaluate what unhealthy physiological parameters are or how they may change with stressor exposure.
2) Studies that connect stressor experience with physiological change and fitness effects. For many species, this will require increased investment in long-term monitoring of physiology, survival, and reproductive success.
3) Evaluation of the synergistic effects of multiple stressors on wild animal physiological and behavioral responses, and downstream effects on fitness and population health. Laboratory studies of contaminants and other stressors typically involve isolating a stressor to determine its effect, but this is largely unrealistic in our current world of rapid environmental change on multiple fronts (e.g., disease, habitat degradation, contaminant exposure, changing weather/climate).
4) Meta-analyses that examine the utility of various field-relevant physiological measures for reflecting stressor experience and predicting fitness in diverse taxa.

Author contributions

JG: Conceptualization, Writing – original draft, Writing – review & editing. MO: Conceptualization, Writing – original draft, Writing – review & editing. TM: Conceptualization, Writing – original draft, Writing – review & editing.

Conflict of interest

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DNA virome composition of two sympatric wild felids, bobcat (*Lynx rufus)* and puma (*Puma concolor*) in Sonora, Mexico

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With viruses often having devastating effects on wildlife population fitness and wild mammals serving as pathogen reservoirs for potentially zoonotic diseases, determining the viral diversity present in wild mammals is both a conservation and One Health priority. Additionally, transmission from more abundant hosts could increase the extinction risk of threatened sympatric species. We leveraged an existing circular DNA enriched metagenomic dataset generated from bobcat (*Lynx rufus*, *n*=9) and puma (*Puma concolor*, *n*=13) scat samples non-invasively collected from Sonora, Mexico, to characterize fecal DNA viromes of each species and determine the extent that viruses are shared between them. Using the metaWRAP pipeline to co-assemble viral genomes for comparative metagenomic analysis, we observed diverse circular DNA viruses in both species, including circoviruses, genomoviruses, and anelloviruses. We found that differences in DNA virome composition were partly attributed to host species, although there was overlap between viruses in bobcats and pumas. Pumas exhibited greater levels of alpha diversity, possibly due to bioaccumulation of pathogens in apex predators. Shared viral taxa may reflect dietary overlap, shared environmental resources, or transmission through host interactions, although we cannot rule out species-specific host-virus coevolution for the taxa detected through co-assembly. However, our detection of integrated feline foamy virus (FFV) suggests Sonoran pumas may interact with domestic cats. Our results contribute to the growing baseline knowledge of wild felid viral diversity. Future research including samples from additional sources (e.g., prey items, tissues) may help to clarify host associations and determine the pathogenicity of detected viruses.

KEYWORDS

viromes, comparative metagenomics, wildlife, bobcat (*Lynx rufus*), puma (*Puma concolor*), virus, Sonoran desert

1. Introduction

Infectious diseases are playing a critical role in wildlife population conservation (Lewis et al., 2017). Through reducing the survival and reproduction of individuals (Woodroffe, 1999; Deem et al., 2001), parasites and infectious diseases can generate trophic cascades (Frainer et al., 2018; Baruzzi et al., 2022), contributing to significant wildlife population declines, affecting multiple species in a community (Pedersen et al., 2007). Furthermore, the transmission of generalist parasites or infectious agents to threatened species can increase the extinction risk of wild animals (Daszak et al., 1999; Woodroffe, 1999; Lafferty and Gerber, 2002; Pedersen et al., 2007). This is evident in carnivores (Woodroffe, 1999; Lafferty and Gerber, 2002; Pedersen et al., 2007), which appear to be particularly susceptible to long term impacts of epizootic diseases at the population-level (Malmberg et al., 2021).

Pathogen surveillance and understanding the viral diversity and potential disease threats associated with more abundant host species may reveal emerging infectious diseases and help prevent future outbreaks and subsequent potential loss of sympatric threatened species. More than just a conservation concern, an increased understanding of the viral diversity in wildlife and the potential for spillover to other species is essential for effectively managing future outbreaks (Olival et al., 2017; Carroll et al., 2018) and is a One Health priority (at the nexus of human, animal and environmental health; Mazzamuto et al., 2022).For example, a study on juvenile and adult red foxes (*Vulpes vulpes*) in peri-urban areas in Croatia noted the dominant presence of fox picobirnavirus and parvovirus in fecal samples, as well as a novel fox circovirus (Lojkić et al., 2016). With red foxes being the most abundant carnivore in the Northern Hemisphere and the novel fox circovirus being very similar to circoviruses isolated from diseased dogs in USA and Italy, it seems possible to posit that red foxes could serve as wildlife virus reservoirs (Lojkić et al., 2016). Such virome characterizations of carnivores are particularly relevant to advancing One Health priorities, with the order Carnivora being ranked among the top five mammalian orders as a source of zoonotic pathogens (Keesing and Ostfeld, 2021). Monitoring wildlife diseases (and particularly wildlife viromes) using non-invasive fecal sample collection is a nascent field (Pannoni et al., 2022; Mazzamuto et al., 2022; Schilling et al., 2022) and could bridge the gap between passive (e.g., voluntary disease reporting) and active wildlife disease surveillance (e.g., submission of samples from hunted game; Cardoso et al., 2022).

The sociality of a species can also affect the spread of infectious diseases (Sah et al., 2018). In group living or social species, group size was thought to influence disease transmission dynamics (Kappeler et al., 2015; Sah et al., 2018), with larger groups and animals living at higher population densities having higher parasite prevalence and burden (Patterson and Ruckstuhl, 2013; Albery et al., 2020). However, recent research suggests that animals can spatially organize their groups to minimize infections (Albery et al., 2020) and that the interactions within a social group and not only its size drive the spread of infectious diseases (Sah et al., 2018).

Social interactions are not limited to group living, as some relatively solitary species have been shown to exhibit complex social networks with a variety of social partners and interactions (Sah et al., 2018). Thus, despite many felids being solitary, the use of shared environmental resources, as well as occasional conflict or predation within and among felid species, creates opportunities for inter- and intra-specific pathogen transmission.

The Sonoran desert is a unique ecoregion home to four species of wild solitary felids: two being more common, bobcat (*Lynx rufus*) and puma (*Puma concolor*), and two listed as endangered, the ocelot (*Leopardus pardalis*) and jaguar (*Panthera onca*). Currently, little is known about the exact disease threats and viral diversity associated with these felids. As such, Sonoran desert felids provide both the conservation need and a unique opportunity to assess levels of viral diversity present within and shared between these populations of closely related sympatric host carnivore species. Bobcats and pumas, as the more abundant felids, are easier to sample, and surveys of viral diversity in these species may serve as a proxy for virome characterization or indication of potential viral spillover for the rarer felids.

In this paper, we leveraged a collection of fecal samples of wild bobcat and puma from Sonora, Mexico to determine (1) what DNA viruses are present in wild felids in Sonora and (2) the similarity of fecal DNA viromes between these sympatric species. These data will contribute to the growing field of wildlife viromics and to our understanding of the viral diversity present in wild mammalian species.

2. Methods

2.1. Sample collection and processing

Bobcat and puma scat samples were collected from Sonora, Mexico, between 2012 and 2014 (Figure 1). Scats possibly of felid origin, were collected only if determined to be fresh, based on color, moisture, smell, and texture. Host DNA was extracted using Qiagen's DNeasy Blood and Tissue Kit, and species identification was performed through Sanger sequencing of a region of the mitochondrial cytochrome B gene (Verma and Singh, 2002; Naidu et al., 2011; Cassaigne et al., 2016), as previously described for these samples (Payne et al., 2020). Thirteen puma and nine bobcat scat samples were randomly selected for DNA virome analysis (Payne et al., 2020). Separate viral DNA extraction from scat cross-sections, circular viral DNA amplification, and library preparation followed the protocol in Payne et al. (2020). Sequencing libraries were generated using the TruSeq Nano DNA Sample Preparation kit and sequenced on an Illumina HiSeq 4,000 (2×100 bp) at Macrogen Inc. (Korea) in 2018.

2.2. Bioinformatics and analyses

The metaWRAP pipeline v. 1.3.2 (Uritskiy et al., 2018) was used to process raw sequencing reads for comparative metagenomic analysis. Within the metaWRAP read_qc module, reads were trimmed using default parameters with Trim Galore v. 0.5.0 (Krueger, 2022) as a wrapper around Cutadapt v. 1.18 (Martin, 2011), human contamination was removed with BMTagger v. 3.101 (Rotmistrovsky and Agarwala, 2011) using the hg38 human genome assembly (GCA_000001405.15), and read quality was assessed with FastQC v. 0.11.8 (Andrews, 2010). Untrimmed reads with human contamination removed were deposited in the Sequence Read Archive. Reads from all samples were co-assembled using metaSPAdes v. 3.14.1 (Bankevich et al., 2012; Nurk et al., 2017) with default parameters outside of metaWRAP, and the scaffolds were then used in the metaWRAP assembly module, allowing for assembly of unused reads with MEGAHIT v. 1.1.3 (Li et al., 2015). We elected to perform one co-assembly across all samples to achieve

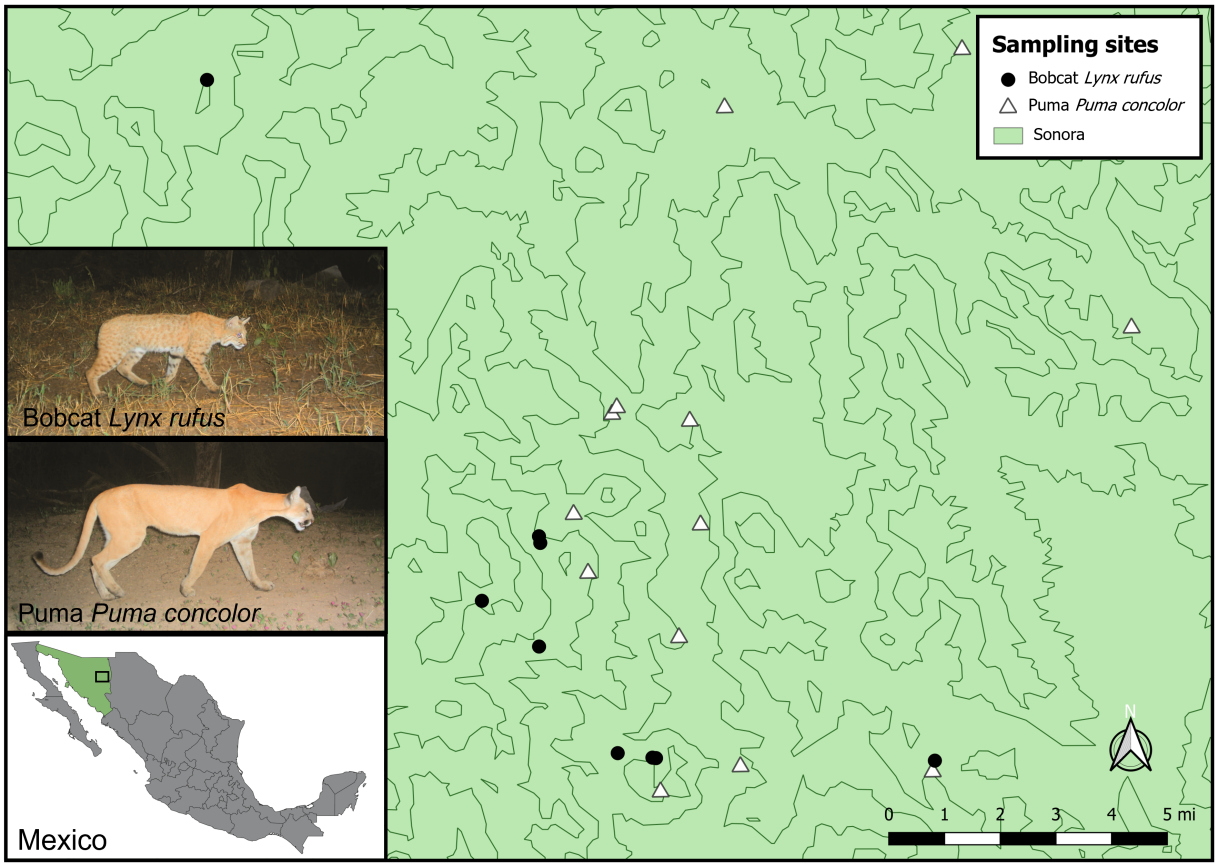

FIGURE 1
Map of bobcat and puma scat sampling sites in Sonora, Mexico. Camera trap photo insets show a bobcat and puma photographed at the same site in Sonora in 2011 (Photos: Primero Conservation and Jesús Moreno from the Management Unit for the Conservation of Wildlife (UMA) "Refugio Privado de Jaguares Silvestres"). Shapefiles: "States of Mexico". Downloaded from http://tapiquen-sig.jimdo.com. Carlos Efraín Porto Tapiquén. Orogénesis Soluciones Geográficas. Porlamar, Venezuela 2015.

better detection of rare taxa and allow for direct comparison of bobcat and puma virome composition. The metaWRAP Kraken 2 module was used to run Kraken 2 (Wood et al., 2019) and generate Krona plots (Ondov et al., 2011) to assess taxonomic composition of contigs in the final assembly, reads from individual samples (subset to 1,000,000 reads), and all pooled reads for each host species, using the premade Kraken 2 viral database (v. 9/8/2022 available at https://benlangmead.github.io/aws-indexes/k2). Contig abundances (in genome copies per million reads) were estimated with Salmon v. 0.13.1 (Patro et al., 2017) using the metaWRAP quant_bins module, and contig taxonomy was assigned using a megablast search against the NCBI nucleotide database (available at https://ftp.ncbi.nlm.nih.gov/blast/db/, downloaded 10/20/2022) using blast v. 2.13.0 (Altschul et al., 1990) outside of metaWRAP for v5 database compatibility, with the output further processed by the metaWRAP classify_bins module for pruning blast hits and assigning taxonomy with Taxator-tk (Dröge et al., 2015). CheckV v. 1.0.1 (Nayfach et al., 2020) was used to assess quality and completeness of viral contigs. Contigs assigned as viral (and not designated as phages) by classify_bins and which were determined to be complete or high-quality (>90% complete) viral genomes with CheckV were retained for community analyses in R. All downstream analyses were repeated with a second set of viral contigs (>66.7% genome completeness), referred to as our "lower-completeness" set (resulting figures in Supplementary Figures S31–S37). The CheckV quality summary, final taxnomic assignment, top blast hit, and abundance per sample of each contig in the high and lower completeness set can be found in Supplementary Table 1.

The R package vegan v. 2.5-7 (Oksanen et al., 2020) was used to conduct viral community ecology analyses on the contigs representing high-quality viral genomes. Alpha diversity metrics (species richness, Simpson Diversity Index, and Shannon Diversity Index) were calculated for each sample, and Wilcoxon rank sum tests were used to determine if significant differences in alpha diversity exist between bobcats and pumas. Beta diversity metrics were calculated among all pairs of samples, both considering contig abundances (Bray–Curtis Dissimilarity Index; abundances were in genome copies per million reads, as estimated by quant_bins) and considering contig presence/absence (Jaccard distance). Kruskal–Wallis rank sum tests were used to determine if beta diversity differed significantly between host species pairs, and Dunn's test was used *post hoc* to determine which comparisons differed significantly using the FSA R package v. 0.9.3 (Ogle et al., 2022).

Vegan was also used to conduct ordination analyses non-metric multidimensional scaling (NMDS) and principal coordinates analysis (PCoA) with both Bray–Curtis and Jaccard distance matrices, and visualizations were generated using ggplot2 (Wickham, 2016) and ggord (Beck, 2022). To further assess differences in virome composition due to host species, permutational multivariate analyses of variance (PERMANOVA) and analyses of similarity (ANOSIM) were conducted

using Bray–Curtis and Jaccard distance matrices with vegan's adonis2 function and anosim function, respectively. To assess correlations between geographic Euclidean distance and beta diversity, separate Mantel tests were performed using Bray–Curtis and Jaccard distance matrices.

3. Results

3.1. MetaWRAP

We obtained 4,044 contigs of 1,000 to 164,510 nts in length after co-assembly (prior to identification of viral sequences), and Krona plots generated by the Kraken 2 module show the viral taxa represented in the assembly (Supplementary Figure S1), bobcat reads (Supplementary Figure S2), puma reads (Supplementary Figure S3), and individual sample reads (Supplementary Figures S4–S25). Of the portion of the final assembly matching the Kraken 2 viral database (with contig taxonomy weighted by contig length and coverage), 48% represented viruses within *Monodnaviria*, with 28% identified as belonging to circoviruses (Figure 2). Viruses within the family *Anelloviridae* comprised 16% of the viral portion of the assembly, and phages within the class *Caudoviricetes* comprised 33%, reflecting viruses likely associated with enteric bacteria of the felids. When reads from each sample of the same host species were pooled (Figure 2), 96% of bobcat viral reads matched to those of the viruses in the family *Circoviridae*, while puma viral reads largely represented viruses in the families *Genomoviridae* (51%), *Retroviridae* (felispumavirus, 14%), *Anelloviridae* (8%), and class *Caudoviricetes* (25%). The computational analysis identified 38 complete genomes and 16 additional high-quality viral contigs (using CheckV and classify_bins taxonomy results, not including phages), which were used for downstream analyses. We included an additional 34 medium-quality contigs in our "lower-completeness" set.

3.2. Alpha and beta diversity

Species richness, Shannon Diversity Index, and Simpson Diversity Index were the alpha diversity metrics calculated for all samples.

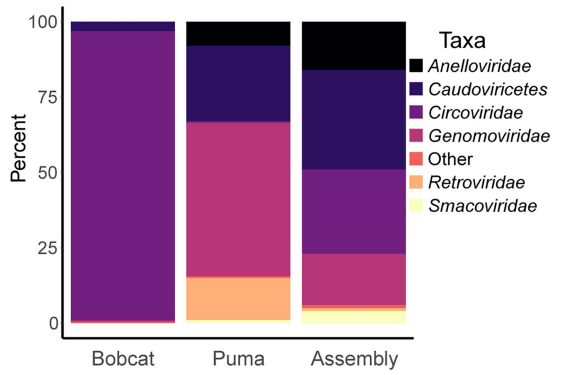

FIGURE 2
Bar plots showing taxonomy of reads matching viral database from bobcat and puma samples, as well as taxonomy of contigs matching viral database. Contigs are from the final assembly generated by metaSPAdes and MEGAHIT, and metaWRAP weights taxonomy results of contigs based on length and coverage.

We observed a wider range of species richness values for pumas (Figure 3A), and pumas had higher median values for each of these metrics (Supplementary Figures S26, S27), although differences in alpha diversity metrics among bobcats and pumas were not significant (richness: $p = 0.1677$; Shannon: $p = 0.1264$; Simpson: $p = 0.1264$). However, using our "lower-completeness" set of viral contigs, we found richness differed significantly between pumas and bobcats ($p < 0.05$; Figure 3C). Median beta diversity values were greatest between pumas and bobcats and lowest among pumas (Figure 3B and Supplementary Figure S28), and both Bray–Curtis and Jaccard distances were significantly different among different host species pairings ($p < 0.01$ for both distances), with significant differences among puma–puma pairings and other host species pairings (p-adj < 0.05 and p-adj < 0.01 with Bray–Curtis and Jaccard distances, respectively, for puma–bobcat pairings and p-adj < 0.05 with both distances for bobcat–bobcat pairings). However, using our "lower-completeness" set of contigs and Jaccard distance (Figure 3D), significant differences among host species pairs were explained by puma–bobcat pairings having significantly higher beta diversity than puma–puma (p-adj < 0.01) and bobcat–bobcat pairings (p-adj < 0.05).

3.3. Effect of host species and geographic distance

The NMDS and PCoA plots reveal an extensive overlap between bobcat and puma viral communities (Figure 4 and Supplementary Figures S29, S30), although pumas and bobcats with the highest richness levels tended to cluster separately in the NMDS based on Bray–Curtis distances (stress = 0.155), and both PCoA and NMDS (stress = 0.211) based on Jaccard distances revealed puma samples outlying the region of overlap between the two species clusters. The PERMANOVAs revealed a significant effect of host species on community composition using Jaccard distances ($p < 0.05$, $R^2 = 0.09418$), but not Bray–Curtis distances ($p = 0.497$, $R^2 = 0.04395$). ANOSIM revealed significant differences between host species using both Jaccard ($p < 0.05$, $R = 0.1901$) and Bray–Curtis distances ($p < 0.05$, $R = 0.1441$). Differences between host species remained significant using the more complex "lower-completeness" set using Jaccard distances (PERMANOVA: $p < 0.01$, $R^2 = 0.12081$; ANOSIM: $p < 0.01$, $R = 0.2639$) and ANOSIM with Bray–Curtis distances ($p < 0.05$, $R = 0.1513$; PERMANOVA: $p = 0.649$, $R^2 = 0.0398$). Mantel tests did not reveal significant correlations between geographic distance and beta diversity for either Bray–Curtis ($p = 0.813$, $r = -0.1011$) or Jaccard distances ($p = 0.165$, $r = 0.1191$).

4. Discussion

Determining the viral diversity present in wildlife is essential for the management and control of emerging infectious diseases. Owing to the large potential for zoonotic spillover, characterizing mammalian viromes is vital to achieving One Health priorities. In non-invasively collected scat samples from wild pumas and bobcats, we observed diverse circular DNA viruses, including circoviruses, genomoviruses, and anelloviruses. Given that rolling circle amplification (RCA) was performed prior to sequencing, we expected to find a high proportion of circular DNA viruses present (although non-circular DNA is not

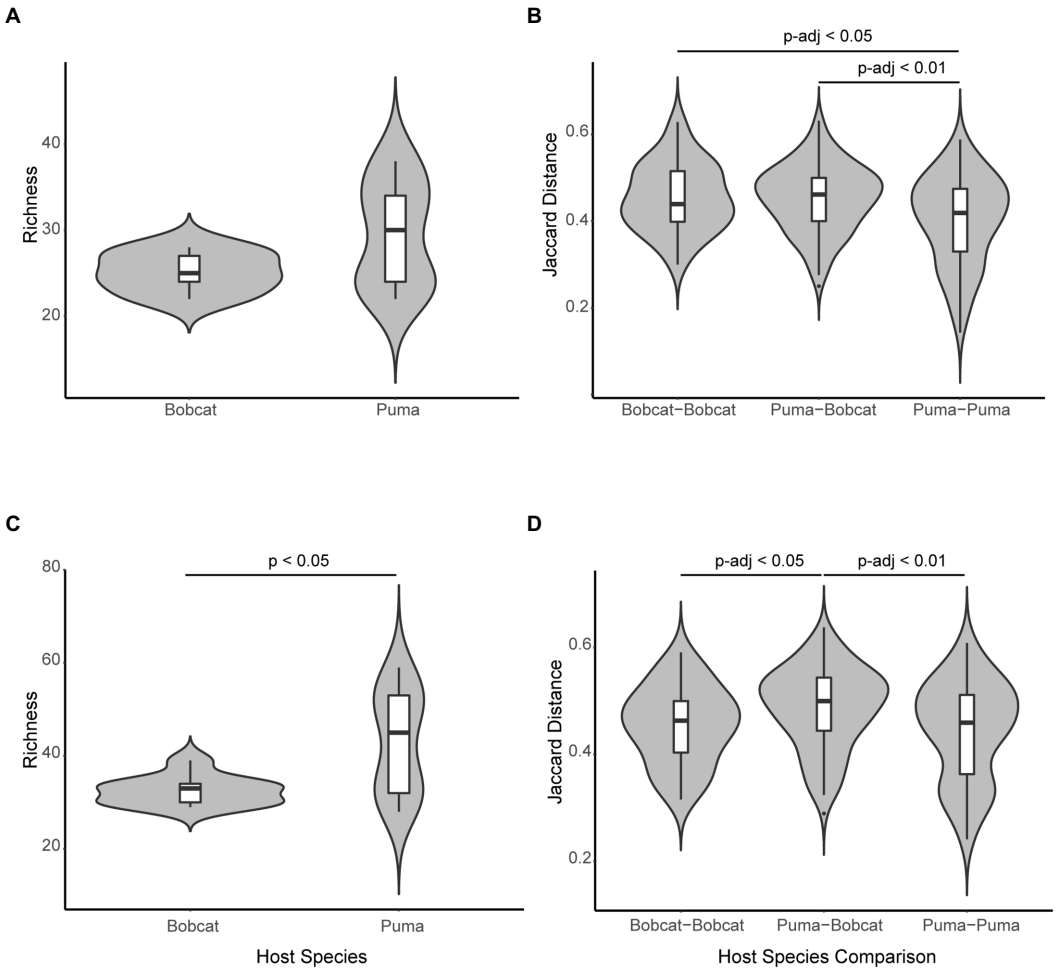

FIGURE 3
Violin plots showing alpha and beta diversity. **(A)** Species richness of bobcats and pumas, using contigs representing high-quality or complete viral genomes. **(B)** Jaccard distances between bobcats, between pumas and bobcats, and between pumas, using contigs representing high-quality or complete viral genomes. Mean Jaccard distance differed significantly between puma–puma pairings and other groups (p-adj<0.01 for puma–bobcat pairings and p-adj<0.05 for bobcat–bobcat pairings). **(C)** Species richness of bobcats and pumas, using viral contigs in the "lower-completeness" set. Mean species richness differed significantly between bobcats and pumas (p<0.05). **(D)** Jaccard distance between bobcats, between pumas and bobcats, and between pumas, using viral contigs in the "lower-completeness" set. Mean Jaccard distance differed significantly between puma and bobcat pairings and other groups (p-adj<0.01 for puma–puma pairings and p-adj<0.05 for bobcat–bobcat pairings).

excluded from sequencing). Specifically, one of the complete viral genomes, with particularly high prevalence in two bobcat samples, was identified as Sonfela circovirus 1, the genome of which was originally identified in these two samples (Payne et al., 2020). Additionally, the high proportion of contigs with homology to anelloviruses was also expected, as the diversity of anellovirus genomes isolated from these samples has been previously characterized (Kraberger et al., 2021).

Interestingly, we documented the presence of feline foamy virus (FFV) within our dataset as a 4.4 kb contig primarily derived from one puma sample (72% of the reads). This suggests that this is an integrated FFV and was detected through carry-over of host DNA. FFV, a contact-dependent, multi-host adapted retrovirus, is known to cause lifelong infection in both domestic and wild felid species (Linial, 2000; Dannemiller et al., 2020), including pumas and domestic cats (*Felis catus*; Kechejian et al., 2019; Kraberger et al., 2020). Most studies document prevalence in domestic cats, with comparatively few detecting the presence of FFV in wild felids (Dannemiller et al., 2020). Pumas have shown a high prevalence of FFV and a high frequency of intraspecies transmission in other studies (Kechejian et al., 2019; Dannemiller et al., 2020; Kraberger et al., 2020). Additionally, frequent cross-species spillover of FFV has been documented from domestic cats to pumas due to depredation events (Kraberger et al., 2020), and the presence of the virus in a Sonoran puma may indicate that interactions between wild and domestic felids have occurred. However, it is also possible that this was a result of social spillover from another puma. While FFV is generally considered apathogenic, clinically silent infection has been associated with histopathological changes in domestic cats (German et al., 2008; Ledesma-Feliciano et al., 2019), and further research is needed to clarify implications for feline health.

Our analyses also indicate extensive overlap between bobcat and puma DNA viral communities. The broader diversity of viruses observed in pumas may result from exposure to a wider variety of prey species. Pumas have been observed to predate a broad range of taxa, including ungulates, mesocarnivores, and small mammals (Cassaigne

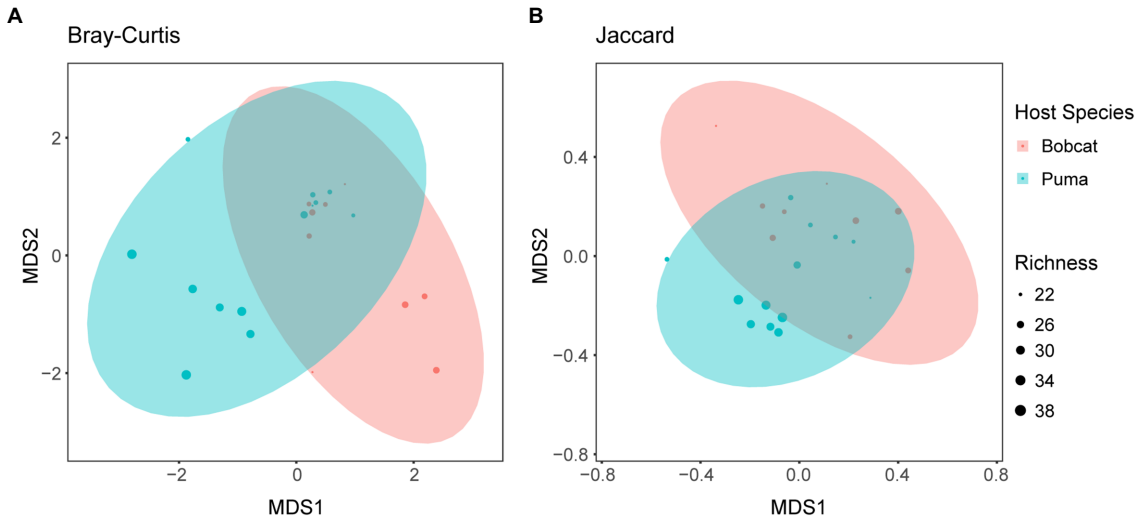

FIGURE 4
NMDS plots generated using **(A)** Bray–Curtis dissimilarity (stress=0.155) and **(B)** Jaccard distance (stress=0.211), using contigs representing high-quality or complete viral genomes. Each point represents the viral community composition within a specific sample. Points are colored by host species, and point size is proportional to species richness of each sample. Ellipses corresponding to the two host species groups are shown at the 95% confidence level. Axes (MDS1 and MDS2) correspond to the two axes of variation.

et al., 2016; Meyer et al., 2020), whereas bobcats are known to primarily specialize on rodents and lagomorphs (Hass, 2009; López-Vidal et al., 2014; Meyer et al., 2020). Furthermore, apex predators are known to experience greater bioaccumulation of viruses (Malmberg et al., 2021). Pumas are also known to occasionally prey upon smaller felids such as bobcats (Hass, 2009; Prude and Cain III, 2021), and previous studies have documented pathogen transmission from bobcat to puma, putatively through competitive contact or depredation (Franklin et al., 2007; Lee et al., 2017; Malmberg et al., 2021). While such interactions may facilitate viral transmission to Sonoran pumas, the presence of shared viral taxa in bobcats and pumas does not necessarily indicate cross-species transmission. As both pumas and bobcats are known to prey on small mammals, and bobcats have been known to predate deer on occasion (Leopold and Krausman, 1986; McKinney and Smith, 2007), shared viral taxa may instead reflect dietary overlap or shared environmental resources, such as water sources. For example, we identified complete viral genomes matching the rodent anelloviruses Neotofec virus NeonRodL2_5 and Neotofec virus NeonRodL2_6 in bobcats and a partial genome matching Dipodfec virus NeonRodF1_131 (within the phylum *Cressdnaviricota*) in pumas. These viruses were first isolated from white-throated woodrats (*Neotoma albigula*) and Merriam's kangaroo rat (*Dipodomys merriami*), respectively, which could be suitable prey items for both felid species (Meyer et al., 2020). Alternatively, shared taxa that infect these felids may be co-evolved within each species. However, we were unable to determine the strains present within each sample (and species) since contigs were generated by co-assembly. Future research including samples from other sources (e.g., prey items, tissue samples) might help to clarify such host associations.

We found that geographic distance among scat samples did not have a significant effect on DNA virome composition at this spatial scale. Our results support previous findings of low levels of spatial autocorrelation of pathogen exposure in pumas and bobcats in Florida, Colorado, and California (Gilbertson et al., 2016). Instead, DNA virome composition appears to be shaped by a combination of host species dependent and independent factors, with extensive virome composition overlap observed between host species using ordination analyses, while PERMANOVA and ANOSIM revealed small yet significant effects of host species. Although pseudoreplication is not suspected, the possibility of some individuals being represented by more than one sample may contribute to the observed effect of host species on DNA virome composition.

Despite providing insight into the possible interactions between host and viral communities, further research is needed to clarify the implications of these results for Sonoran felid health. Fecal virome characterization of non-invasively collected scat samples includes many novel and known viruses derived from the scat depositors as well as prey species and environmental contacts, so the host associations and pathogenicity of each virus in the metagenome is unknown. Of the major viral taxa identified here, viruses in the family *Circoviridae* may be of most interest in terms of bobcat and puma health. Circoviruses are known as the smallest animal pathogens that replicate autonomously (Fisher et al., 2020). They are found in a number of species [freshwater fish, birds, bats, chimpanzees, minks, elk, and humans (Rosario et al., 2017; Fisher et al., 2020)], although their presence is often subclinical (Fisher et al., 2020). In some birds, circoviruses are considered potentially immunosuppressive (Todd, 2000), suggesting that concurrent co-infections could increase the symptoms and severity of disease (Fisher et al., 2020). Some circoviruses are known to cause clinical disease such as hemorrhagic gastroenteritis in dogs (Anderson et al., 2017; Kotsias et al., 2019), often fatal postweaning multisystemic wasting syndrome in pigs (Chae, 2005; Segalés et al., 2005) and virus (BFDV) in birds [beak and feather disease virus (Todd, 2000)]. The identification of potentially disease-causing circoviruses in bobcats and pumas is of concern for both wild felid population health and conservation. With their

propensity for displaying tissue tropism (Todd, 2000), transmission of these circoviruses from more abundant/common species to threatened wild felids could result in catastrophic population declines, particularly if these felids are already immunocompromised from co-infections. Furthermore, the high abundance of Sonfela circovirus 1 reads in two bobcat samples may suggest an active infection. However, with these viruses being identified from non-invasively collected scat samples, further study is needed to clarify host associations and consequences for feline health. Although important feline pathogens present in the scats may have been missed through analysis of the circular viral DNA enriched dataset, these results contribute to the documentation of viral diversity in wild felids. Future studies coupling the characterization of broader virome composition and disease dynamics across sympatric populations of wild mammals could help with the identification of viral threats to wildlife, as well as potentially to humans and domestic animals.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found at: NCBI; PRJNA922235.

Author contributions

NP: conceptualization, data analysis, writing, and manuscript preparation. LC: conceptualization, writing, manuscript preparation, and supervision. SK, RF, and KS: data collection. IC: sample collection. MC: supervision. AV and KD: data collection and supervision. 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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Developmental and reproductive costs of osmoregulation to an aquatic insect that is a key food resource to shorebirds at salt lakes threatened by rising salinity and desiccation

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Saline lakes worldwide are undergoing drying, and as lake levels fall and areas contract, salinities increase. There is a critical need for data on salinity impacts to guide conservation for recovery of the aquatic productivity that supports migratory and breeding birds that depend on these habitats. Brine flies are key sources of food to these birds and are adapted for life in saline waters owing to their capacity for osmotic regulation. The sublethal effects on growth, development and reproduction were determined in experiments and field observations with the alkali fly *Cirrula hians* from alkaline lakes of differing salinity. The cost of osmoregulation to fitness from rising salinity was exhibited in slower growth rates of larvae, smaller size at maturity of pupae, reduced adult emergence success, and lower fecundity. The results identify a salinity management range of 25 to 100 g L^{-1} that would optimize life history traits and productivity of this insect as a food source for birds.

KEYWORDS

osmoregulation, salinity, *Ephydra*, Mono Lake, salt lakes, *Cirrula hians*, Lake Abert

Introduction

Saline lakes worldwide are in crisis as lake levels and habitat areas have declined owing to histories of diversion of inflowing rivers and streams that otherwise would offset the evaporation of water from these closed-basin ecosystems (Wurtsbaugh et al., 2017). As salinities rise at the same time, the aquatic invertebrate life of these lakes that serve as key food resources to myriad migratory and breeding waterbirds are increasingly threatened (Jehl, 1994; Rubega and Robinson, 1996; Andrei et al., 2009). Adult brine flies congregate along shorelines of saline lakes in super-abundance (tens of thousands per square meter), while larvae and pupae stages are aggregated in shallow water where they often secure themselves on rocky substrata or feed on mats of algae (Herbst, 1988; Herbst and Bradley, 1993). Their contagious distribution thus allows easy foraging on all life stages by shorebirds in particular (Elphick and Rubega, 1995; Strauss et al., 2002; Senner et al., 2018; Frank and Conover, 2021). Along with the abstraction of stream inflows, and exacerbated by ongoing drought related to climate change (Seager et al., 2007), these rapidly desiccating habitats are in critical need of clear criteria for multiple-use management of salinity levels to maintain productivity and

ecological integrity in balance with consumptive uses (e.g., Wurtsbaugh and Sima, 2022). Salinization may also be a contributing factor to the long-term decline noted for North American migratory shorebirds (37% loss, Rosenberg et al., 2019).

Halophilic organisms often do not so much as love salt as they have evolved means to tolerate it. In seawater where there is usually little variation in external salinity (around 34 g L^{-1}, excepting estuaries and some tidal habitats), marine invertebrates simply live with it, that is, their internal solute content conforms to the environment (osmoconformers, Prosser, 1973). Inland saline water environments, however, can vary considerably in chemical composition and dissolved ion concentration, and can reach salinities well in excess of 200 g L^{-1}, to saturation and in anionic content of chloride, carbonates and sulfate (Hardie and Eugster, 1970). Common halophilic invertebrates such as brine flies and brine shrimp usually cope with varied salinity through osmoregulation (Bradley, 2009). Keeping metabolic homeostasis requires regulation of a stable internal osmotic concentration. There can be no question that osmotic homeostasis of intra- and extracellular fluids requires energy as ATP generated from mitochondria amassed in cells that power active transport in trans-membrane enzyme ion pumps that remove solutes. The extent to which this limits other physiological processes is critical to the success of these organisms in inhabiting saline environments. Why live in such extremes? If freshwater habitats are full of a diversity of other aquatic life, salt tolerance may provide an extremophile escape from the rigors of coping with competition and predation and often enables achieving high levels of productivity—but at what cost is the subject of the present study. The energy demands of osmoregulation relative to the supply of food over a range of external salinity may be the essential determinants of population productivity where ecological limitations are absent.

The aquatic larvae of *Cirrula hians* Say (Diptera: Ephydridae, formerly placed in the genus *Ephydra*) osmoregulate over a wide range of salinity (Herbst et al., 1988), and must therefore vary in expenditure of energy in maintenance of internal osmotic homeostasis. Although the physiological tolerance and cellular mechanisms associated with ion and water balance have been investigated in this and other species of saline water insects (e.g., Barnby, 1987; Bradley, 1987), only a few studies have investigated the net cost of these processes to the growth and development of such organisms. For example, increased salinity has been found to limit growth, prolong development, and reduce size at maturity in other osmoregulators such as brine shrimp and brine mosquitoes (Nayar, 1969; Dana and Lenz, 1986).

Also known by the common name, the alkali fly, *C. hians* inhabits alkaline salt lakes in western North America (Wirth, 1971) that contain high concentrations of bicarbonate and carbonate anions at high pH (Herbst, 1988). Larvae use a specialization of the Malpighian tubules to concentrate and excrete carbonate in the form of calcium carbonate microspheres (Herbst and Bradley, 1989a). Development occurs through three larval instars that feed on benthic algae and cyanobacteria in the shallow littoral zone (Herbst and Bradley, 1993; Bradley and Herbst, 1994) and attach to hard calcareous substrates as pupae, emerging as adults to gather in dense bands along lake shorelines. The alkali fly is often the most abundant occupant of these habitats along with the related species *Ephydra gracilis*, found primarily in chloride-dominated water chemistry (Aldrich, 1912; Collins, 1980a; Herbst, 1999).

The objectives of this study were to investigate both the developmental and reproductive costs of increasing salinity and how these may be further altered by varied food supply. Over a wide range of experimental salinity treatments, growth of larvae from Mono Lake (California) was measured as was the duration to and size at maturity of resulting pupae and success of emergence of adult flies from these pupae. Experiments at varied salinities with flies raised from the egg stage were also conducted with and without algal food limitations. In addition, field studies contrasting life stages of *C. hians* populations coming from Mono Lake with those from the less saline Lake Abert (Oregon) allowed contrast of life history traits (adult size and fecundity), and relative biochemical food values, under natural conditions of differing salinity.

Methods

Environmental setting

Mono Lake, in east-central California, and Lake Abert, in south-central Oregon are two of the largest closed-basin salt lakes of the western Great Basin high-altitude desert (Herbst, 1988). These are alkaline lakes (mainly CO_3^{-2} and HCO_3^{-} anions, also Cl^{-} and SO_4^{-2}), pH near 10, and salinity of Mono at ~90 g L^{-1} (grams per liter total dissolved solutes) and Abert at ~25 g L^{-1} at the time of these studies (field and lab work were all conducted 1983–1987). Being a shallow lake of smaller volume, the water levels and salinity of Abert are more prone to fluctuate than the deeper Mono Lake, but both have histories of diversions of inflowing streams that have concentrated salts because of evaporative losses exceeding the inflow of freshwater to these terminal lakes (Figure 1).

Larval growth experiments

The influence of osmotic stress on growth rates, development time, and size at maturity of pupae was examined by exposure of third instar larvae to experimental treatment salinities. Third instars were staged to be of equal age between treatments by rearing field-collected second instar larvae (from shallow near-shore waters using an aquarium net) at the treatment salinity and then isolating newly molted third instars into separate culture dishes to initiate individual trials at salinities of 10, 50, 100, and 150 g L^{-1}. To prepare this salinity range, filtered lake water (0.45 micron membrane filters) was either diluted with distilled water or concentrated by evaporation in large shallow pans over several weeks, with salinities adjusted to a specific gravity corresponding to the desired salinity (as documented in Herbst, 1988). The lower salinity of 10 g L^{-1} is nearly iso-osmotic with the hemolymph (blood) of larvae (Herbst et al., 1988), while the upper salinity of 150 g L^{-1} was chosen based on preliminary scope of response data showing first instar larvae do not survive longer than 72 h at 200 g L^{-1} but some will survive exposures at 150 g L^{-1}. Each larva was contained in a 60 × 15 mm plastic culture dish with lid, in 15 ml of treatment water, held in humidified boxes in an environmental chamber at 20°C on a 14:10 L:D light:dark cycle. Food was provided from fresh lab-cultures consisting of a mix of diatoms and cyanobacteria (mostly *Nitzschia*

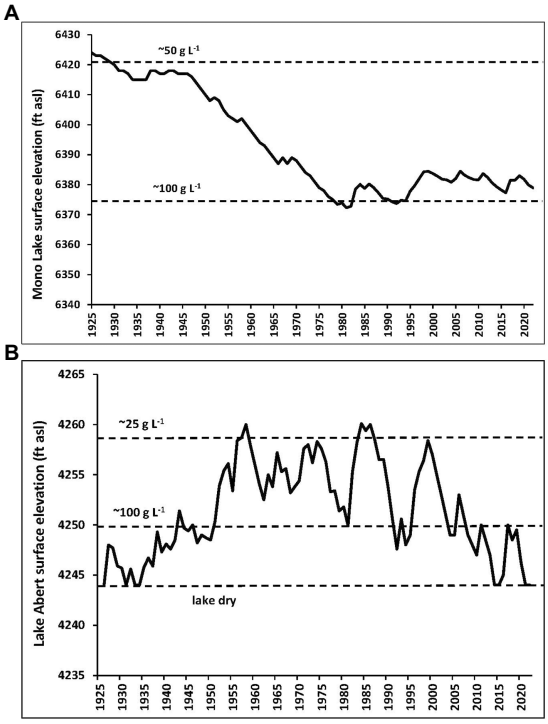

FIGURE 1
Historical trends in the surface elevation of Mono Lake **(A)** and Lake Abert **(B)**, showing key salinity levels associated with lake elevation (feet above sea level, asl). Mono Lake levels and salinity: https://www.monobasinresearch.org/data/levelyearly.php (Mono Basin Clearinghouse), and (Herbst, 1988). Lake Abert levels and salinity: (Larson et al., 2016).

frustulum and *Oscillatoria* spp.) that were grown in nutrient-enriched (Guillard F/2 seawater medium) water only at 50 g L^{-1} so that food quality was held constant. Algae cultures used for feeding were harvested after about 1 week by centrifuging, and the pellet drawn into small syringes and delivered equally into the culture dishes. Food was also supplemented with algae cultured on nutrient-enriched 1% agar pour plates, provided to larvae as small disks. Food was provided regularly as depleted and fecal pellets were removed with a pipette, and treatment water replaced daily to prevent fouling. Third instar growth rates were measured at intervals for each larva over the initial 2–3 weeks after molt (at 3, 6, 9, and 16 days). Each larva was rinsed briefly in distilled water, blotted dry, and weight determined within 10–15 s to ±10 micrograms using a digital Cahn Electrobalance, then returned to culture dishes. As third instars continued to develop, the time from molt to pupa formation (pupariation) was recorded for all maturing to that stage. The size at maturity of pupae was then measured with a microscope eyepiece reticle (to 0.01 mm) at the maximum dorso-ventral width of the puparium (between the 3rd and 4th prolegs).

Interaction of food supply and salinity on growth and development

The effect of food supply on larval survival and development was evaluated in experiments using individuals isolated as newly-hatched first instar larvae. Eggs were obtained from 20 adults reared in the lab (size range 4.8–5.8 mm) from field-collected pupae at Mono Lake (netted from pupae detached from rock in near-shore shallows), harvesting eggs laid into an algae-sediment substrate in screened jars which held the emerged mix of females with male adult flies. Food supply to isolated first instar larvae was provided in the form of algal-microbial mats collected from the field (suctioning off surface sediments in shallows) regularly in protected areas of submerged shallows in Mono Lake. This consisted typically of a mix of diatoms (*Nitzschia* spp.), green algae (*Ctenocladus circinnatus*), cyanobacteria (mostly *Oscillatoria*) and undetermined bacteria. Collected food was first homogenized in a low-speed blender, centrifuged, and the pellet delivered by small syringe into multi-well culture plates with a single larva in 2 mL treatment water. For the first 2 days after hatching, all larvae were given unlimited access to food. Thereafter, larvae were randomly assigned to daily food ration, or a ration of 2 days with food followed by 2 days without. In each case, these were conducted in replicates at three salinity levels—50, 100 and 125 g L^{-1} (larvae could not complete development at 150 g L^{-1}). Water was replaced daily to prevent fouling, and the experiments were again conducted at 20°C and 14:10 L:D photoperiod. Survivorship curves were plotted for each food availability *x* salinity treatment, along with records of the time to pupa formation (pupariation). Adult emergence success as a function of pupa size was determined both from the pupae formed from this experiment and the larval growth study, and supplemented with data from a parallel experiment with Abert larvae, and field-collected pupae from both Mono Lake and Lake Abert.

Adult fecundity and proximate chemical analysis of food value between lakes

To determine the relationship between size of adult females and fecundity, summer field-collections from both Mono and Abert Lake were made in sweep-nets along shores, and the abdomens of preserved female flies dissected and number of eggs in ovaries counted in each. The relative food value to birds of larvae, pupae and adults from both lakes was also determined from field-collections of all these life stages, and proximate chemical analysis performed as follows. Ash was determined by dry weight difference before and after combustion at 550°C for 3 h. Total organic nitrogen was determined by the micro-Kjeldahl method (APHA Standard Methods). Total protein was estimated from nitrogen content by the conversion factor of 6.25. Water soluble protein was determined by the dye-binding assay of Bradford (1976). Water soluble carbohydrate was determined spectrophotometrically by reaction with phenol (Kochert, 1978). Lipid was determined by weight before and after Soxhlet extraction for 20–24 h in 2:1 chloroform:methanol. Caloric content was calculated from protein, carbohydrate, and lipid by standard conversion factors.

Results

Osmotic stress and larval growth and development

The net average growth rate over the initial 16 days from molt for third instar larvae decreased with salinity (Figure 2). Average growth

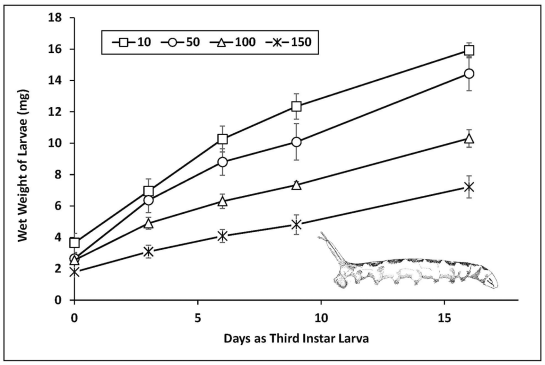

FIGURE 2
Growth rates of third instar alkali fly larvae exposed to a range of salinities of Mono Lake water. The number of larvae tracked through the 16th day of weighing trials were 12, 6, 7 and 4 at 10, 50, 100 and 150 g L^{-1} (standard error for each data point).

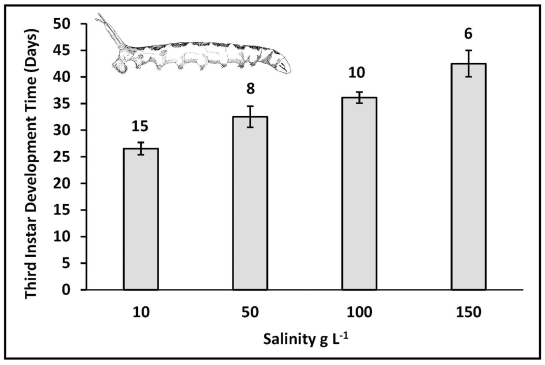

FIGURE 3
Development time of third instar larvae from molt to pupariation at varied salinities of Mono Lake water. Number of larvae tracked to pupariation, and standard errors for each bar.

rate was similar at 10 and 50 g L^{-1} (from 0.77 to 0.74 mg d^{-1}, respectively), but decreased significantly at 100 and 150 g L^{-1} (0.48 to 0.34 mg d^{-1}, two-tailed *t*-tests of pairwise comparisons of slopes). Initial molt weight of third instars from second instars also decreased with salinity (points on the *Y*-intercept).

Duration of third instar development to pupation was prolonged with increased salinity (Figure 3). Development required on average about 3–4 weeks at 10 g L^{-1}, 4–5 weeks at 50 g L^{-1}, 5–6 weeks at 100 g L^{-1}, and more than 6 weeks at 150 g L^{-1}. Even though development time was extended in response to increased salinity, this did not result in achieving equal sizes at maturity. Puparium width decreased with increased salinity (Figure 4; ANOVA with analysis of variance Fisher's least significant difference test of multiple comparisons of means by salinity: 10 = 50 > 100 > 150). Noted also on Figure 4 is the threshold pupa size (1.6–1.7 mm width) at which adult emergence success drops from greater than 75% to less than 50% (Figure 5). Almost no emergence occurs below a size of 1.5 mm width, but when successful took 10–14 days regardless of salinity treatment. In sum then, salinity increase leads to reduced larval growth rates, prolonged development, smaller size at maturity, and reduced success of adult emergence from smaller pupae.

Combined effects of food limitation and salinity on survival and development

Survivorship curves of Mono Lake larvae showed that rearing at reduced algal food availability resulted in substantial mortality, especially among early instars (Figure 6). The detrimental effects of food limitation were more pronounced at elevated salinities of 100 and 125 g L^{-1} where no larvae were capable of surviving unless food was available at all times. Only at 50 g L^{-1} were larvae able to pupate on a reduced food ration but even so, this experiment was terminated beyond 160 days duration. The larvae that had pupated in the reduced food availability level at 50 g L^{-1} were delayed by nearly 3 months relative to those that had been fed full-time and were significantly smaller (average of 1.37 mm, $n = 4$ vs. 1.67 mm, $n = 30$), and most pupae perished if they formed after 3 months. At the full-time food availability level at 50 g L^{-1} 86% of larvae survived to pupate and started forming after 42 days, with all forming before 3 months emerging as adults. When food was reduced only 12% of larvae survived to pupate and these were delayed by over 2 months. At higher salinities and daily food ration, onset of pupa formation was also delayed until after 75 days at 100 g L^{-1} and 90 days at 125 g L^{-1}. Early stage larvae (instars 1 and 2) took 23 days on average to molt to the third instar at 50 g L^{-1} but took twice as long (46 days) when food ration was reduced. At higher salinities with daily food, early instars required 35–37 days on average to reach the third instar stage.

Size and fecundity of adult females and proximate chemical analysis of all life stages

A sampling of field-collected adult flies from both Mono and Abert showed that as body size of adult females increases, so does the number of eggs in the ovaries (Figure 7). Eggs were always of constant size, so this was not affected by field or lab conditions. For this wedge-shaped distribution, it is the upper limits with respect to body size that reflects potential maximum fecundity because many of the field-collected females no doubt had already begun laying some eggs.

Pupae and adults of *C. hians* from Lake Abert were significantly larger than their Mono Lake counterparts, consistent with the lower salinity of Abert, but had similar digestible chemical composition (Table 1, as percent water soluble fractions of protein, carbohydrate, and lipid). Third instar larvae were more variable in composition, most notable being a higher ash content in Mono larvae (24.5% vs. 9.0% at Abert), and lower lipid content (16.3% vs. 28.0% at Abert). For contrast, data from *Artemia* collected at Mono Lake are also reported (Table 1; Enzler et al., 1974) and indicates these have lesser lipid and caloric content than aquatic larvae or pupae of *C. hians*, but similar food value as adult flies.

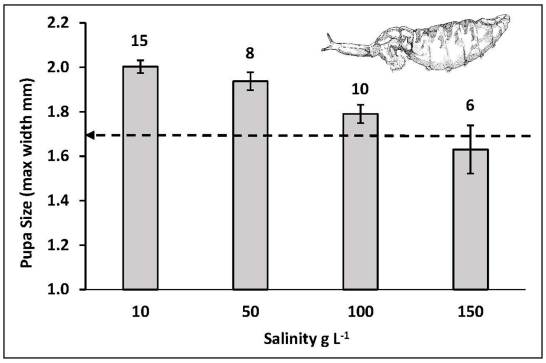

FIGURE 4
Size at maturity of pupae forming from third instar growth experiment at varied salinities of Mono Lake water (dashed line indicates threshold below which emergence success falls below 50%, in Figure 5). Standard error and sample size as in Figure 3 above each bar.

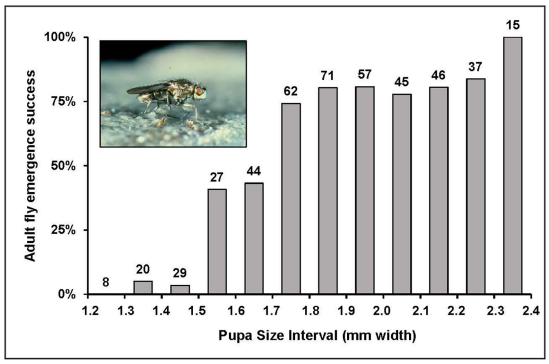

FIGURE 5
Percent adult emergence success from pupae of varied size, with standard error and sample size above each bar. Pupae derived from experimental exposures of larvae to varied salinity along with pupae collected from both Mono Lake and Lake Abert.

Discussion

The important applications of this study are to provide insights to why and where birds are using saline lakes for foraging. The alkali fly can be an abundant inhabitant of saline Great Basin lakes and ponds and this research shows the underlying basis for how population demography, productivity and distribution can be related to the physiological costs of living over gradients of varied salinity. The strength of this research derives from combining lab and field observations along with contrasts between lakes with differing salinity regimes. This approach is further validated because the studies follow salinity effects over the full life cycle of this insect (larvae to pupae and adults) and verifies experimental results with field observations. A limitation of the study is that it applies only to this insect, and other salt lake invertebrates with differing tolerances of salinity and water chemistry may show different optima. However, the general principle of developmental costs associated with osmoregulation will still apply.

Growth and development of larvae and pupae under osmotic stress

Assimilated food is allocated according to differing conditions of quantity and quality of food consumed, and how energetic needs and trade-offs are divided among metabolic regulatory functions, and requirements for growth, storage, and reproduction (the principle of allocation *sensu* Sibly and Calow, 1986). This partitioning determines life history traits such as development time, size at maturity, and fecundity along with the capacity for adaptation to varied environmental conditions. The gradual inhibition of larval growth observed with increased salinity level is consistent with the expectation that increased osmoregulatory costs creates a deficit in resources that can be allocated for growth (Figures 2–4). Larval growth was limited not only by slower rates of weight gain with increasing salinity, but also because second instars acclimated to treatment salinities produced smaller size of third instars at molt. This is a conservative estimate then of how salinity can disrupt growth over all larval stages. Indeed, the experiments using larvae reared from egg hatching on showed how severely survivorship and development rates are curtailed (Figure 6).

At a salinity near iso-osmotic (10 g L^{-1}) where the theoretical cost of osmoregulation should be at a minimum, the highest growth rates, shortest development time and largest size at maturity were achieved. As growth rates are inhibited with increase in salinity it is conceivable that some compensation for lost growth could occur by extending development time. Such prolonged growth at higher salinity does indeed occur (Figure 3) but not enough to maintain a constant body size (Figure 4). At the highest salinity (150 g L^{-1}), this size was actually below a threshold at which emergence success of adult flies from pupae falls below 50% (Figure 5).

Although the lowest salinity tested appears to be the condition under which these salt lake insects would most thrive, in nature these are conditions where other less salt-tolerant insects and other invertebrates (such as amphipods, damselflies, corixids) can also occupy these habitats and restrict population productivity due to competition and predation (Herbst, 1988, 2001, 2006). Given these ecological constraints, the experiments reported here, and observations of comparative population ecology, the salinity optimum for productivity of the alkali fly appears to be in the range of 25–100 g L^{-1}, with a maximum near 50 g L^{-1}.

Food limitation substantially reduced survival and delayed maturation of larvae, and when combined with increased salinity from 50 to 100 and 125 g L^{-1}, prevented any further development to the pupa stage. The food limits imposed in these experiments were severe—with food provided only half the time larvae were trying to grow. While this may be an unrealistic situation, it does show the importance of food supply in offsetting the effects of salinity stress, but may still represent how salinity can reduce the quantity and quality of algae food resource available. In unialgal cultures of the filamentous green algae *C. circinnatus*, growth rates in lake water from Mono or Abert were reduced by half or more as salinity increased from 50 to 75 or 100 g L^{-1} and also resulted in changes in the growth form of this species, commonly found at low to moderate salinities in saline lakes (Herbst and Castenholz, 1994). In mixed algae growing in experimental field mesocosms, the production of an assemblage of diatoms, *Ctenocladus*, and cyanobacteria decreased by far more than

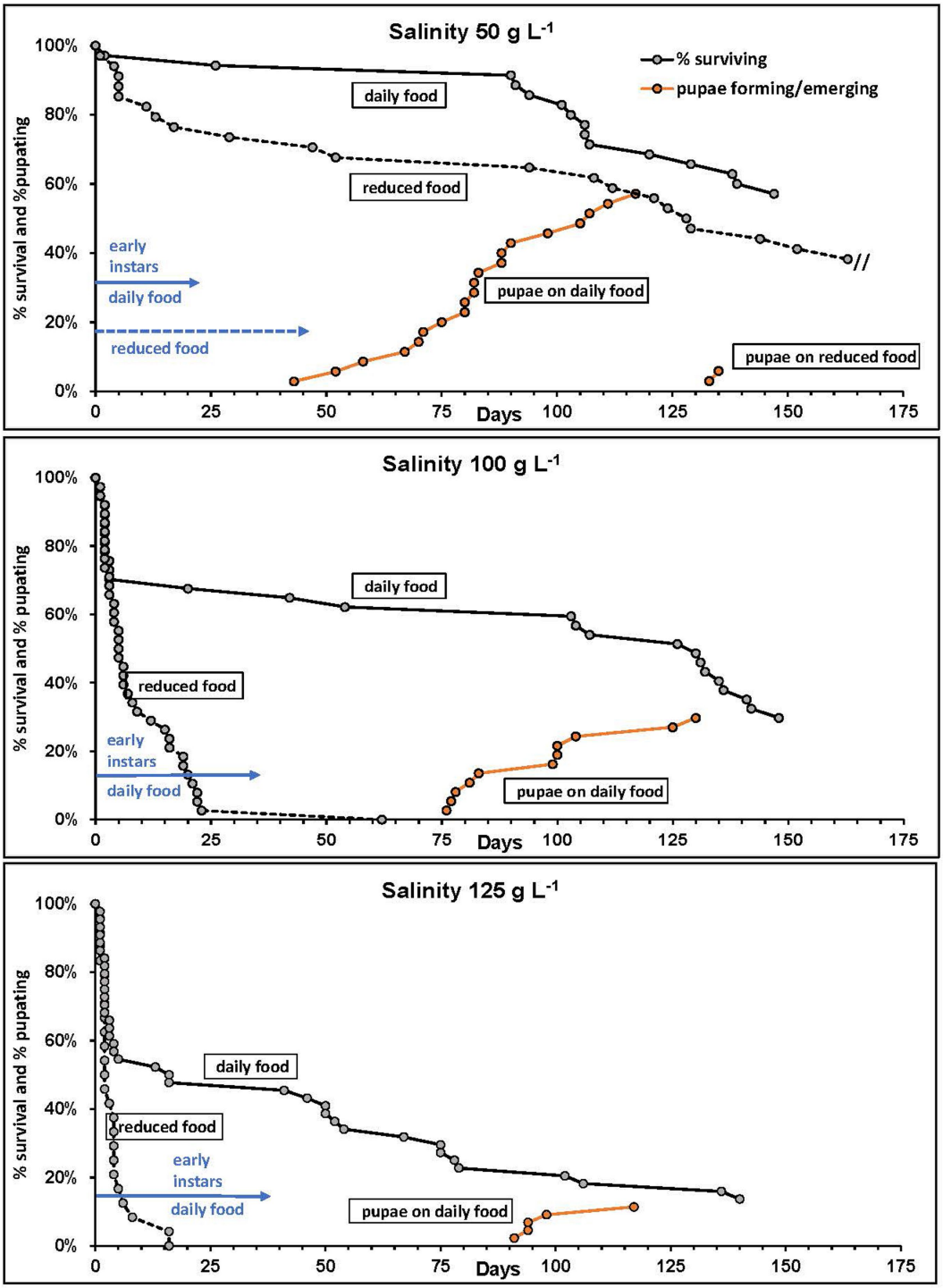

FIGURE 6
Mono Lake alkali fly larval survivorship and development time to pupation at varied levels of salinity and food ration. Curves refer to algal food supplied daily or reduced to 2 of 4 days. Initial sample size of of first instar larvae in each salinity at daily and reduced food ration, respectively: 50 (35,34), 100 (37,38), 125 (44,24). Experiment at reduced food level in 50 g L^{-1} was terminated after 160 days due to slow growth.

half and diatom diversity reduced as salinities of Mono Lake water were increased from 50 to 75 or 100 g L^{-1} (Herbst and Blinn, 1998). Lab studies of mixed algae and microbial cultures from both lakes indicated somewhat broader salinity tolerance (Herbst and Bradley, 1989b), possibly due to greater salt tolerance of the cyanobacteria present. Altogether, these studies suggest algae food resource restrictions may not be an unreasonable expectation as salinities increase. The primarily diatom-based cultures provided as food in growth experiments has been determined to provide for the best larval growth (Bradley and Herbst, 1994), but even when fed *ad libitum* still does not compensate for salinity limitations on survival and development (Figure 6).

The life history correlation of decreasing survivorship of larvae with slower growth rates and smaller size at maturity conforms to how fruit flies (*Drosophila*) respond to crowding or poor food conditions, and is a predictable "reaction norm" to stress (Stearns and Koella, 1986). This variable growth pattern whereby size at maturity is flexible can be interpreted as an adaptation for survival in the face of osmotic stress in brine flies. The brine fly *E. gracilis* (nee *Ephydra cinerea*) living in Great Salt Lake (Utah) also shows plasticity in development and smaller size at maturity when stressed by low food availability (Collins, 1980b). If direct effects of salinity on development depend on osmoregulatory costs, then high hemolyph osmotic concentration would lower the gradient between internal and external fluids and should confer greater resistance to the stress of increased salinity on growth and survival. This appears to be exactly the case with *E. gracilis* larvae, having a hemolymph osmolality approximately three-fold higher than in *C. hians*, and are capable of living in much higher salinities, up to and beyond 200 g L^{-1} salinity of the sodium chloride-rich waters of Great Salt Lake (Nemenz, 1960; Herbst, 1999). Although they have a greater salt tolerance, *E. gracilis* also show reduced size at maturity with increased salinity (Herbst, 2006), but do not occur in the alkaline water chemistry of salt lakes such as Mono or Abert. Plasticity in growth and development then may permit some degree of adaptability to salinity stress, but not without a cost that would result in demographic limitations on population productivity.

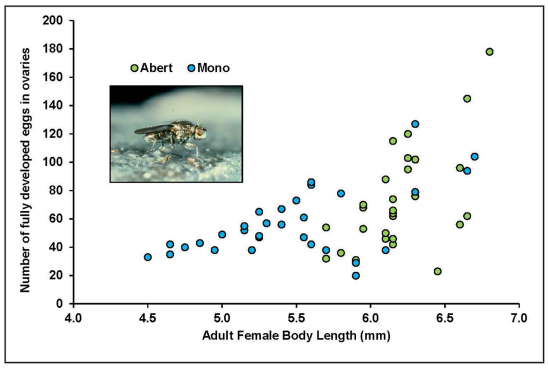

FIGURE 7
Fecundity of field-collected adult females from both Mono and Abert, from dissections of abdomen and counts of eggs present. Abert green points, Mono blue points.

Corroborating field observations

The altered life history traits produced under experimental treatments of increased salinity are consistent with observed differences in these characteristics from collections at Mono Lake and Lake Abert (Table 1). Adults and pupae from the less saline Abert (~25 g L^{-1}) were significantly larger than specimens collected from Mono Lake at the same time (~90 g L^{-1}). Adult flies collected from both lakes covered a broad range of body sizes and egg-laying capacity was obviously dependent on the general relationship to body size (Honěk, 1993). This important life history attribute then is related to how both salinity and food limitations can delay the time

TABLE 1 Potential food value to birds foraging at lakes of differing salinity. Indicated here by proximate chemical analysis comparing life stages of *Ephydra hians* collected from Mono Lake with same from Lake Abert, and to *Artemia* brine shrimp from Mono Lake.

	MONO			ABERT			Mono
	Larvae	Pupae	Adults	Larvae	Pupae	Adults	Adult *Artemia*
Dry Wt/individual	2.85	1.95	1.31	3.04	3.27	2.70	1.78
	(0.91,37)	(0.44,25)	(0.26,25)	(1.05,28)	(0.70,25)	(0.36,24)	(0.05,43)
Percent ash	24.5	8.4	4.2	9.0	5.3	4.4	20.6
	(5.4,10)	(4.5,8)	(0.8,10)	(2.3,10)	(1.0,10)	(0.9,10)	
% Total protein	36.8	52.6	77.3	51.3	50.9	59.4	58.5
	(5.0,25)	(6.9,25)	(3.8,25)	(5.0,25)	(8.1,25)	(3.8,24)	
% Water soluble protein	9.9	11.2	9.0	11.5	13.4	10.3	--
	(1.3,12)	(1.4,12)	(2.3,23)	(2.2,12)	(2.2,12)	(4.0,24)	
% Water soluble carbohydrate	20.9	22.9	11.8	19.2	16.5	11.5	--
	(5.5,12)	(5.6,12)	(3.3,24)	(3.4,12)	(2.0,12)	(2.1,24)	
% Lipid	16.3	22.2	11.6	28.0	20.3	13.6	10.6
	(3.0,12)	(5.6,12)	(5.5,12)	(3.8,12)	(6.4,12)	(4.0,12)	
Caloric content/ individual	12.38	11.15	7.23	18.42	17.94	14.18	7.86
	Numbers in parentheses are standard deviation and sample size						

of reproduction through prolonged development and maturation to the pupa stage, and lower fecundity owing to reduced pupa size from which smaller adult size will result. Smaller adults will emerge from smaller pupae, so these limitations on reproduction extend the influence of rising salinity in demographic constraints on productivity.

Although the proximate analysis of chemical composition of field-collected specimens showed percent digestible (water soluble) fractions of tissue to be similar in pupae and adults from both lakes, third instar larvae differed. Abert larvae stored significantly more lipid, important both in growth and later vitellogenesis in adults, but also giving them a higher caloric content and fat storage value to birds consuming them. Mono larvae had not only lower lipid content but higher indigestible ash fraction, likely related to the greater inorganic fraction of $CaCO_3$ concretions stored in the lime glands of larvae from more saline waters. To the extent that prey type and capture by birds is similar between lakes, foraging on any life stage of the flies would be less productive at Mono than Abert under these conditions (as in Herbst, 2006).

Conclusion: Conservation, or not

Since 1994, partial protection of stream inflows to Mono have been in place intended to raise lake elevation to 6,292 ft. (about 75 g L^{-1}). With intervening drought, however, this has not been achieved. Without these protections ordered by the State Water Board of California the lake level would by now have dropped to around 6,350 ft. (in excess of 150 g L^{-1}). Abert has no such protections in place and recently has been near dry, all but for inches of water near freshwater seeps at the northeast edge of the lakebed. With a shallower profile and smaller volume than Mono, restoring inflows of the Chewaucan River to Abert could raise lake levels and reduce salinity to viable conditions (Figure 1B), with flies re-inhabiting the lake from refuge habitats created by salinity gradients within lakebed margin seeps and springs. At Mono, where some stream diversions to the Los Angeles aqueduct are still permitted, leaving those to flow to the lake would achieve the mandated water level in about 30 years (Mono Lake Committee, 2022).

Other saline lakes of the Great Basin are in similarly dire condition, with lake levels falling to historic lows at the Great Salt Lake and Walker Lake in Nevada (Herbst et al., 2013). Impaired survival of the benthic littoral assemblage of midges and damselfly nymphs at Walker Lake over lower ranges of salt concentration than are optimal for brine flies illustrates the fact that salinity management is not "one size fits all." Conservation of saline lake ecosystem networks should consider the ranges of water chemistry, native aquatic life in each, and historic conditions with and without diversions of inflowing rivers and streams that sustain productivity, diversity, and habitat value to birds (Wurtsbaugh et al., 2017). Major shifts in the utilization and connections among saline wetlands for migratory waterbirds of the Great Basin appears to be related to reduced availability and salinization of these habitats (Haig et al., 2019). In addition to their value as food resource to birds visiting saline lakes, these halophilic invertebrates are of concern in their own right, often with prolific populations being limited to just the few larger more perennial lakes. Where present in smaller and refuge aquatic habitats they may be subject to the genetic bottlenecks of small populations and temporary availability of optimal environmental conditions. Conservation of large perennial saline lake habitats provides protection for multiple ecosystem types and species.

Although the drying of Owens Lake (California) is sometimes cited as a cautionary tale for the demise of other saline lake ecosystems, it is actually an example of how rapidly the ecological values of these lakes can be recovered. The Owens basin had been regarded as a problematic dry lakebed emitting dust pollution during wind storms, but under the requirements of federal air quality standards, the Los Angeles Department of Water and Power was ordered to control dust emissions from the Owens dry playa. Using a system of flood irrigation, not only was dust pollution abated in large part, but in the process shallow saline water habitat was recreated in which algae and brine flies flourished (Herbst and Prather, 2014). When proper water quality returns so does the life of saline lakes. The lake now hosts an annual bird festival.[1] Add water and they will come.

Data availability statement

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

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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I am grateful to Faye Cummins for her excellent artwork in Figures 2–4 depicting larvae and pupae of the alkali fly. Portions of this work are drawn from the PhD dissertation of the author (Herbst, 1986).

Conflict of interest

The author declares 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

1 https://friendsoftheinyo.org/owens-lake-bird-festival/

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Short-term weather patterns influence avian body condition during the breeding season

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Despite a large body of literature investigating the effects of long-term climate trends on birds, the effects of short-term weather on individual body condition are less established. Poor body condition is associated with declines in individual fitness for many avian species, thus changes to body condition may result in altered population productivity. We utilized a large existing dataset from the Monitoring Avian Productivity and Survivorship program to analyze the effects of daily maximum temperature, daily minimum temperature, and monthly precipitation on avian body condition over a 15-year period across 79 sampling sites in the southeastern United States. We used a model selection approach with generalized additive models at both species and guild levels and found largely nonlinear responses of avian body condition to weather variables. For many species and guilds, a threshold effect was evident, after which the relationship between body condition and weather changed drastically. As extreme weather becomes more common under climate change, species will be pushed further towards or away from these thresholds. Non-linear effects were also highly species-specific and not easily explained by expected effects on food availability. Thus, avian responses to altered weather may be difficult to predict across species. We discuss the implications of these results for individual fitness and population productivity.

KEYWORDS

bird, fitness, songbird, temperature, precipitation, scaled mass index, climate change, physiology

Introduction

Predicting the effects of climate change on wild animals is one of the major challenges facing modern ecologists (Walther et al., 2002; Moritz and Agudo, 2013). Our current rate of climatic warming is already producing changes to climate and weather patterns, including more frequent heatwaves and drought, increased average over-land precipitation, more frequent intense precipitation events, and shifts in storm tracks (IPCC, 2021). The effects of current and future weather patterns on birds are of particular interest because birds are valuable providers of ecosystem services (Sekercioglu, 2006) and perform a diverse array of ecological functions, making them important indicators of ecosystem health (Fischer et al., 2007; Bregman et al., 2016). Moreover, birds are conspicuous to the public, leading to an abundance of available community science data (Neate-Clegg et al., 2020; Binley et al., 2021), and this conspicuousness drives conservation efforts in many regions (Myers et al., 2000; Larsen et al., 2012).

Many bird species are vulnerable to the wide-ranging effects of climate change (Jetz et al., 2007; Langham et al., 2015), through a combination of exposure to changing climatic means and extremes, and intrinsic sensitivity to those changes (McCloy et al., 2022). Although several studies have provided predictions regarding phenological responses of species (Reed et al., 2013) to

changing environmental variables (Crick, 2004; Jetz et al., 2007; Reif, 2013; Scridel et al., 2018), and on trait-specific responses to climate change (Jenouvrier, 2013; McLean et al., 2020), less emphasis has been placed on physiological responses to short-term weather, which can vary at fine (e.g., taxonomic grouping) and broad scales (e.g., local community and population; Parmesan et al., 2000; Maxwell et al., 2019). For example, individual physiological changes such as body size and body condition are widely accepted effects of climate change (Gardner et al., 2011), and can also be affected by short-term weather (Romero et al., 2000; Skagen and Adams, 2012; Gardner et al., 2016) and acute disturbance events. They may also indicate potential shifts in fitness and overall health (Parmesan et al., 2000; van de Pol et al., 2016; Kouba et al., 2021). Existing studies regarding the effects of short-term weather on individual wild birds tend to focus on either (a) a single species (Angelier et al., 2011; English et al., 2018), (b) a particular geographic region (McLean et al., 2018), or (c) within the context of migratory ecology (Danner et al., 2013). However, the effects of weather patterns on individual avian physiology can vary greatly by species and region (McLean et al., 2018; Lindenmayer et al., 2019), and understanding these effects is critical to accurately predict animal responses under climate change. For these reasons, more studies are needed- particularly multi-species studies in regions that are vulnerable to an increase in severe weather events, such as the coast of the Gulf of Mexico (Reece et al., 2018).

While the exact definition of body condition varies within the literature, it is commonly used as a wide-ranging indicator of avian health (Stevenson and Woods, 2006; Peig and Green, 2010; English et al., 2018) and can provide insights to disease prevalence and the health of the individual (Granthon and Williams, 2017), while also indicating a phenotypic response to climatic and environmental variables (McLean et al., 2018, 2020; Kouba et al., 2021). An individuals' body condition can reflect early-life stressor exposure (Grace et al., 2017), and affect survival and intra-specific population dynamics (McLean et al., 2016). Body condition can also indicate phenotypic responses to climatic and environmental variables (McLean et al., 2018, 2020). For example, warmer temperatures are correlated with lower body condition in a variety of songbirds (van Buskirk et al., 2010; Gardner et al., 2016; McLean et al., 2018) an effect that is exacerbated by climate change (van Buskirk et al., 2010).

Body condition is dynamic in birds and can be affected by previous and current environmental conditions and activities (Tonra et al., 2011; Rockwell et al., 2012; Akresh et al., 2019a,b, 2021). Existing literature establishes that temperature and/or precipitation patterns may have lasting, spillover effects from the wintering grounds to breeding grounds in various passerine species (Tonra et al., 2011; Rockwell et al., 2012; Akresh et al., 2019a,b, 2021), with these effects generally being more pronounced in younger birds (Rockwell et al., 2012). The active breeding season may place additional energetic demands on adult birds through incubation and raising young. Adaptive anorexia may occur to cope with these increased energy demands, thus adaptively decreasing body condition (Walsberg, 2003). Additionally, body condition reflects fat storage for thermoregulation as well as energetic storage (Stevenson and Woods, 2006); thus, lower body condition may be adaptive under high temperatures because it results in more rapid cooling (McLean et al., 2020). Temperature and precipitation may vary greatly between exposed and sheltered sites within the same local area and can consequently affect the metabolic rates, and thus body condition, of birds (Weathers, 1979). Microhabitat variation promotes preferential use of microhabitat refugia by a number of taxa (Scheffers et al., 2013), including birds (Walsberg, 1985; Martin et al., 2015). For example, phainopeplas (*Phainopepla nitens*) use of microhabitat in interior woodland reflects an avoidance of more exposed areas (Walsberg, 1993) while African songbirds have been shown to preferentially select trees with a higher shade density on hot days (Martin et al., 2015).

In this study, we utilize the Monitoring Avian Productivity and Survivorship (MAPS) dataset (Institute for Bird Populations) to evaluate how passerine body condition is influenced by breeding-season weather patterns (i.e., temperature and precipitation). We chose to use breeding season data because of their widespread availability at a large scale, recognizing that high energy expenditures during the breeding season may introduce variability into the dataset. We extracted breeding season data from 79 locations of the MAPS project across the Gulf of Mexico region of the southeastern United States, including: Texas, Louisiana, Mississippi, Alabama, and Florida. We used data from these states because of the strong projected impacts of climate change in this region (Savonis et al., 2008; Anthony et al., 2009) coupled with their high ecological importance as migratory stopover locations for Neotropical migrant songbird species (Moore et al., 1990; Hobson et al., 2007). We generated a series of hypotheses predicting differential responses to weather patterns by bird species, habitat and dietary guild, and migratory status of the species (Figure 1). Keeping in mind the complexities posed by thermal microhabitat refugia, we chose broad guild classifications because of our ability to accurately classify each species and our ability to retain an adequate sample size in each category for analysis.

We predicted that: (*H1*) precipitation would have a positive effect on granivorous species until a certain threshold is reached because low precipitation typically decreases seed production, after which flooding and excess precipitation destroys seed-producing herbaceous vegetation (Oram et al., 2021); (*H2*) insectivorous bird species will be positively affected by warmer temperatures and higher precipitation because insects themselves are highly sensitive to temperature and precipitation changes (Rebaudo et al., 2016; Møller, 2019; Ma et al., 2021); and (*H3*) omnivorous species would show little to no effect of any weather parameter on body condition because of their high foraging plasticity. For habitat guilds, we predicted that: (*H4*) the body condition of bottomland species would be negatively affected by precipitation after a certain threshold is reached, because flooding can decrease seed and insect abundances (Beja et al., 2010; Oram et al., 2021). Additionally, we predicted that (*H5*) bottomland species would be less affected by temperature than early-successional species because of the strong dichotomy in overstory cover between these habitats. As a result of the ability of habitat generalists to select a wider range of favorable microhabitats, we also predicted that (*H6*) temperature and precipitation would have minimal effects on this guild. Lastly, we predicted that (*H7*) migratory species would be positively affected by warmer temperatures and higher precipitation because the energetic expenditure of migration may make them more sensitive to changes in food sources.

Materials and methods

Avian data collection

Data were collected for this study during a 15-year period from 2005 to 2019 at 79 avian banding stations in Texas, Louisiana,

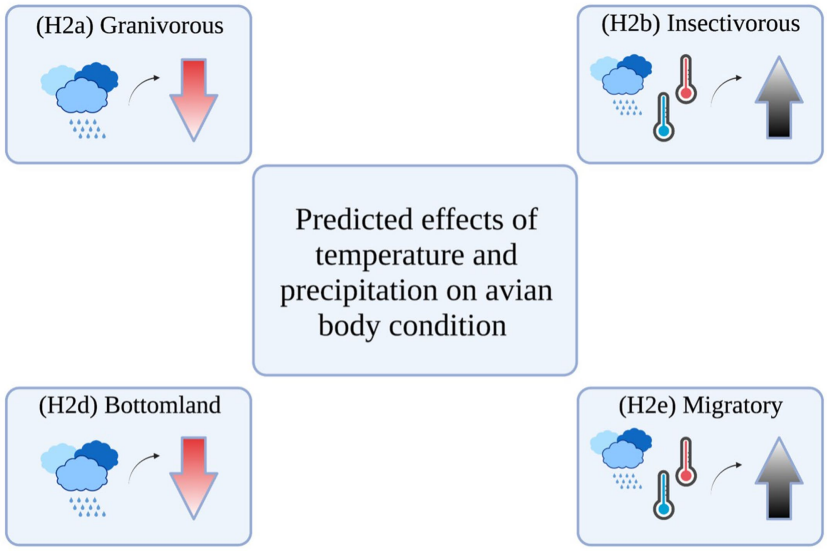

FIGURE 1
Predicted effects of average daily minimum temperature, average daily maximum temperature, and total monthly precipitation on guilds. (H4) was not included as no effects were expected. Created with BioRender.com.

Mississippi, Alabama, and Florida (United States of America) as part of the MAPS program. The MAPS program is a nationwide program operated in adherence to a standardized protocol detailed in DeSante et al. (2015). In brief, ten to twelve 30 mm mesh, 12-m mist nets were operated for one morning in each 10-day period at each station between May and August. Each captured bird was banded with a nine-digit USGS aluminum band, weighed, wing length was measured, and if possible, the species, sex, and age was determined according to Pyle (1997). Not all 79 MAPS stations were operated during the entirety of the 15-year period, but all stations were operated for at least 1 year between 2005 and 2019. Data were downloaded through the Institute for Bird Populations from the MAPSPROG database, formatted in Microsoft Excel, and statistical analyses were performed in program R (v. 3.6.0).

Sampling design

Across all station locations, we analyzed body condition from mass and wing length for 18 avian passerine species in relation to weather data (see "Weather Data" section below). We also categorized each species into three guild classifications (Table 1) using the Birds of the World database species accounts (Billerman et al., 2022). First, we grouped each species by primary habitat: bottomland forest specialist (B), early successional specialist (E), or habitat generalist (G) (Table 1). We also categorized each species by dietary guild: insectivorous (I), granivorous (G), or omnivorous (O) (Table 1). Finally, since many Neotropical migrant species are declining and of conservation concern (Rosenberg et al., 2019) we classified species as either Neotropical migrants (N) or resident (R) species (Table 1). The sample size for each species across all sites and years, after all outliers were removed (see "Data Analysis"), ranged from 300 (Bewick's wren; *Thryomanes bewickii*) to 5931 (Northern cardinal; *Cardinalis cardinalis*), with an average sample size per species of 1963 and total sample size across species of 35326 (Supplementary Table S2). Sample sizes for guilds were: bottomland ($n = 10731$), early successional ($n = 11995$), habitat generalist ($n = 12685$), resident ($n = 13226$), migratory ($n = 22100$), omnivorous ($n = 1703$), granivorous ($n = 11541$), and insectivorous ($n = 22821$). Guild-level analyses have long been established as an effective means of analyzing avian data (Gray et al., 2007), and can provide insights that species-specific analyses oftentimes cannot. We chose these specific guilds because of our hypotheses that habitat preference and dietary habits may influence the degree to which temperature and precipitation effect individual body condition, and because of the steep population declines experienced by many North American Neotropical migrant songbird species (Rosenberg et al., 2019).

Weather data

Weather data was downloaded from DayMet (Thornton et al., 2021) *via* the Single Pixel Extraction Tool, which provides a 1 km^2 resolution of surface weather data from each set of MAPS station coordinates. We analyzed total precipitation, average maximum temperature, and average minimum temperature for the 30 days prior to the capture of each individual bird. Thus, if a bird was captured on 30 May, weather data for that bird was collected for the period between 01 May and 30 May. We chose to analyze temperature and precipitation because the existing literature allowed us to make *a priori* predictions (Gardner et al., 2016; McLean et al., 2018) and there was high availability of data on a fine spatio-temporal scale. To avoid model overfitting, we did not include additional weather variables (e.g., wind, relative humidity, solar radiation) because they were not available for all sites and we had an *a priori* expectation that these variables would not have strong influences on avian body condition. The effects of weather on animals can be temporally heterogeneous (Salewski et al., 2013; English et al., 2018), and we chose to use a 30-day time frame

TABLE 1 Species analyzed and the corresponding guild designations of each species.

Species	Scientific name	Code	Guild		
			Habitat	Dietary	Migratory
Acadian flycatcher	*Empidonax virescens*	ACFL	B	I	M
Bewick's wren	*Thryomanes bewickii*	BEWR	E	I	R
Carolina chickadee	*Poecile carolinensis*	CACH	G	O	R
Carolina wren	*Thryothorus ludovicianus*	CARW	G	I	R
Common yellowthroat	*Geothlypis trichas*	COYE	G	I	R
Field sparrow	*Spizella pusilla*	FISP	E	G	R
Hooded warbler	*Setophaga citrina*	HOWA	B	I	M
Indigo bunting	*Passerina cyanea*	INBU	E	G	M
Kentucky warbler	*Geothlypis formosa*	KEWA	B	I	M
Northern cardinal	*Cardinalis cardinalis*	NOCA	G	G	R
Painted bunting	*Passerina ciris*	PABU	E	G	M
Prothonotary warbler	*Prothonotaria citrea*	PROW	B	I	M
Red-eyed vireo	*Vireo olivaceous*	REVI	B	I	M
Swainson's warbler	*Limnothlypis swainsonii*	SWWA	B	I	M
Tufted titmouse	*Baeolophus bicolor*	TUTI	G	O	R
White-eyed vireo	*Vireo griseus*	WEVI	E	I	M
Wood thrush	*Hylocichla mustelina*	WOTH	B	I	M
Yellow-breasted chat	*Icteria virens*	YBCH	E	I	M

Habitat guilds were comprised of bottomland forest (B), early successional (E), and generalist (G) classifications, dietary guilds were comprised of insectivorous (I), granivorous (G), and omnivorous (O) classifications, and migratory guilds were comprised of either migrants (M) or resident (R) species.

instead of annualized weather data to capture shorter-term effects of extreme weather (e.g., dehydration due to high heat), along with slightly longer-term changes in resource availability.

Scaled mass index

Numerous condition indices exist for quantifying body condition. Multivariate indices are widely considered to provide the most robust and accurate assessment of the body condition of an individual (Freeman and Jackson, 1990). Although there is still ongoing debate as to which method of quantifying body condition is "best" (Labocha et al., 2014; Akresh et al., 2019b), here we chose to use scaled mass index (Peig and Green, 2009), which is an effective measure of condition in birds (Peig and Green, 2009; Danner et al., 2013; English et al., 2018). The scaled mass index approach scales body mass to body size (Danner et al., 2013), while accounting for changes in the relationship between body mass and size in an individual over time (Peig and Green, 2010). In the scaled mass index equation:

$$\hat{M}i = M_i\left[\frac{L_0}{L_i}\right]\wedge\left(b_{SMA}\right)$$

$\hat{M}_i$ represents scaled mass index value, M_i is body mass, L_0 is the mean of a body size measure (in this study: wing length) across the population, L_i is individual wing length, and b_{SMA} is the scaling exponent, calculated as the slope of the Standardized Major Axis (SMA) regression of the log of body mass on the log of wing length (Peig and Green, 2009). We calculated scaled mass index scores separately for each species, and then standardized these scores to a mean of zero through z-scoring for guild analyses.

Data analysis

Exploratory data analysis through linear regression indicated that avian body mass was not strongly affected by time of day, Julian date, amount of body molt, and breeding condition (i.e., cloacal protuberance or brood patch), thus we did not incorporate these variables into our subsequent analyses. To evaluate the effects of temperature, precipitation, and age on avian body condition we used the *'mgcv'* package (Wood, 2011) in the R statistical programming language (v 4.2.1; R Core Team, 2021) to construct a series of nonparametric generalized additive models (GAMs; Gaussian, identify link function) that separately predicted the scaled mass index of each species and guild. GAMs are a popular technique with large ecological datasets for modeling multiple regression functions (Yee and Mitchell, 1991; Wood, 2013; Wood et al., 2015). Moreover, unlike generalized linear models or linear mixed effects models, GAMs avoid making *a priori* assumptions regarding the relationship between the dependent variable and covariates. Effects are additive instead of linear, and smooths are fitted *via* smoothing splines using cubic polynomials.

We used a restricted maximum-likelihood approach to estimate the degree of smoothness of the model terms (Viana and Chase, 2022). Our GAMs included total precipitation, average daily maximum temperature, average daily minimum temperature, year (2005–2019), and age (hatch year or after hatch year, subsequently referred to as "juvenile" or "adult").

The relative strength of the partial effects in each model can be inferred from the y-axis values, in units of scaled mass index (or in the case of guilds, units of z-scored scaled mass index), where higher values represent stronger partial effects of the plotted variable. A positive partial effect which does not bound zero indicates that the given weather variable has a generally positive effect on body condition for that species or guild. We included "station" as a random effect (i.e., the MAPS station from which each bird was captured) to account for potential effects of geographic variation and site-specific human bias on scaled mass index.

We used the '*dredge*' function in the '*MuMIn*' package (Bartoń, 2009) in R to perform model selection using Akaike's Information Criterion (AIC) on all possible models derived from our base model for each species and guild. We composed our global model using our *a priori* hypotheses and examined each derived model for biological relevance. Thus, we feel that the use of this dredging function was appropriate for this analysis and that our risk of *post-hoc* hypotheses and inadvertent inclusion of biologically improbable models was low (Delgado-Rodríguez and Llorca, 2004; Johnson and Omland, 2004). We considered all models with Δ AICc <2 (Supplementary Table S4) and then used the top model of each species and guild for assessment of parameters (Supplementary Table S3). We checked for concurvity of weather variables in all top models.

Only initial captures of each individual were analyzed to prevent potential bias from repeated captures of the same individual, an approach that was possible given the large overall sample size of our dataset. Across all species 54.2% of all records were removed, and 51% of these excluded records were recaptures. The remaining excluded records were due to missing values (48.8% of removed records), or outlying measurements (less than 1% of removed records) attributable to human error in data collection or entry. The total percentage of records removed per species ranged from 21.6% (red-eyed vireo; *Vireo olivaceous*) to 66.3% (prothonotary warbler; *Prothonotaria citrea*) with a mean of 44.7% of records removed for each of the 18 species analyzed (Supplementary Table S2).

Results

Model selection

Model selection through AICc of all possible parameter combinations indicated multiple competing top models with Δ AICc <2 in 14 of 18 species and 4 of 8 guilds, with as many as 14 models with Δ AICc <2 present for wood thrush. However, 9 of 18 species and all 8 of 8 guilds contained fewer than 5 models with Δ AICc <2. For all species and guilds, the null model (i.e., intercept-only) ranked considerably below the Δ AICc <2 set (intercept only, Supplementary Table S4). Selected top models frequently contained age (23 of 26 species and guilds), and weather parameters of precipitation (19 of 26), maximum temperature (18 of 26), and minimum temperature (17 of 26) were also comparably represented (Supplementary Table S3). The random effect of station was present in 16 of the 26 top models (Supplementary Table S3). Top models that included both minimum temperature and maximum temperature exhibited a high level of concurvity in each case (>0.90). Concurvity between all other pairs of predictor variables was significantly lower (typically <0.40).

Our global model was the top model for Carolina chickadee, white-eyed vireo, Carolina wren, hooded warbler, and northern cardinal, which incorporated the linear effect of age and the random effect of station in addition to the three weather variables. The top model for indigo bunting and prothonotary warbler contained spline effects of only precipitation. For Bewick's wren and common yellowthroat, the top model contained the spline effect of maximum temperature alongside the linear effect of age. The top model for red-eyed vireo included the spline effect of precipitation alongside the linear effect of age and random effect of station (Supplementary Table S3).

For Acadian flycatcher and Kentucky warbler, the top model included the spline effect of minimum temperature alongside the linear effect of age and random effect of station. For wood thrush, the top model consisted of the spline effects of maximum temperature and minimum temperature along with the linear effect of age. The top model for field sparrow included the spline effects of minimum temperature and precipitation, alongside the linear effect of age and random effect of station. The top models for painted bunting and yellow-breasted chat included only the spline effects of maximum temperature and precipitation. For tufted titmouse, the top model included the spline effects of maximum temperature and minimum temperature alongside the linear effect of age and random effect of station. Finally, the top model for Swainson's warbler included no weather parameters and only the linear effect of age (Supplementary Table S3).

Our global model containing all three spline effects of maximum temperature, minimum temperature, and precipitation alongside the linear effect of age and random effect of station were the top model for five of the eight guilds: the granivorous and insectivorous dietary guilds, the resident (nonmigratory) guild, the bottomland guild, and the habitat generalist guild. Additionally, the top model for the early successional guild contained all three parameters while dropping the random effect of station. For the migrant and resident guilds, the top model included all three spline effects of maximum temperature, minimum temperature, and precipitation alongside the random effect of station. Finally, the dietary generalist guild had only the spline effect of precipitation along with the linear effect of age in its top model (Supplementary Table S3).

Effects of weather parameters on scaled mass index

Average minimum temperature

We found that average daily minimum temperature had varied effects across species and guilds. For six species (Carolina chickadee, northern cardinal, hooded warbler, tufted titmouse, Carolina wren, and white-eyed vireo), daily minimum temperature had a negative effect on scaled mass index, up to a critical point between 10 and 15°C, after which this negative effect either stabilized (Carolina wren, hooded warbler, and white-eyed vireo) or reversed (Carolina chickadee, northern cardinal, and tufted titmouse; Figure 2). The 95 and 85% CIs for minimum temperature did not overlap zero for any of these six species (Supplementary Table S5). We found a dynamic but generally negative relationship between average daily minimum temperature and scaled mass index for the Acadian flycatcher, field sparrow, and Kentucky warbler (Figure 3). Again, the negative effect was strongest prior to 10–15°C. The 95 and 85% CIs did not overlap zero for the Acadian flycatcher and field sparrow (Supplementary Table S5), but closely approached zero for the Kentucky warbler, indicating a weak effect. The effect of minimum

temperature was negligible for wood thrush (Supplementary Figure S4), where both the lower 95 and 85% CIs closely approached zero (lower 95% CI: 2.23e-47; lower 85% CI: 7.92e-36).

At the guild level, we also found a negative effect of minimum temperature on body condition at very low temperatures (i.e., below 10°C). This inflection point remained relatively even across all guilds. After this there was a generally a weak, positive effect of minimum temperature on body condition (Figures 4, 5). The 95 and 85% CIs for minimum temperature did not overlap zero for any guild analyzed (Supplementary Table S5).

Average maximum temperature

For six of the eighteen species (Carolina chickadee, northern cardinal, tufted titmouse, hooded warbler, Carolina wren, and white-eyed vireo), we found that average daily maximum temperature had an inverse relationship with body condition compared to that of daily minimum temperature, such that its effect on scaled mass index was positive until a certain point (between 20 and 30°C), after which the effect either stabilized or became negative (Figure 6). We found a dynamic relationship between average daily maximum temperature and scaled mass index for common yellowthroat, painted bunting, and yellow-breasted chat, with unclear overall directionality (Figure 7). The effect of maximum temperature on scaled mass index was negligible for wood thrush (Supplementary Figure S4), where both the lower 95% CIs (3.07e-37) and 85% CIs (2.29e-28) very closely approached zero (Supplementary Table S3).

In terms of guilds, we found a generally positive relationship between average daily maximum temperature and scaled mass index until approximately 20°C, after which the relationship appeared to reach a threshold. This relationship remained remarkably similar across all guilds (Figures 8, 9). For all species and guilds analyzed, the 95 and 85% CIs for maximum temperature did not overlap zero (Supplementary Table S5).

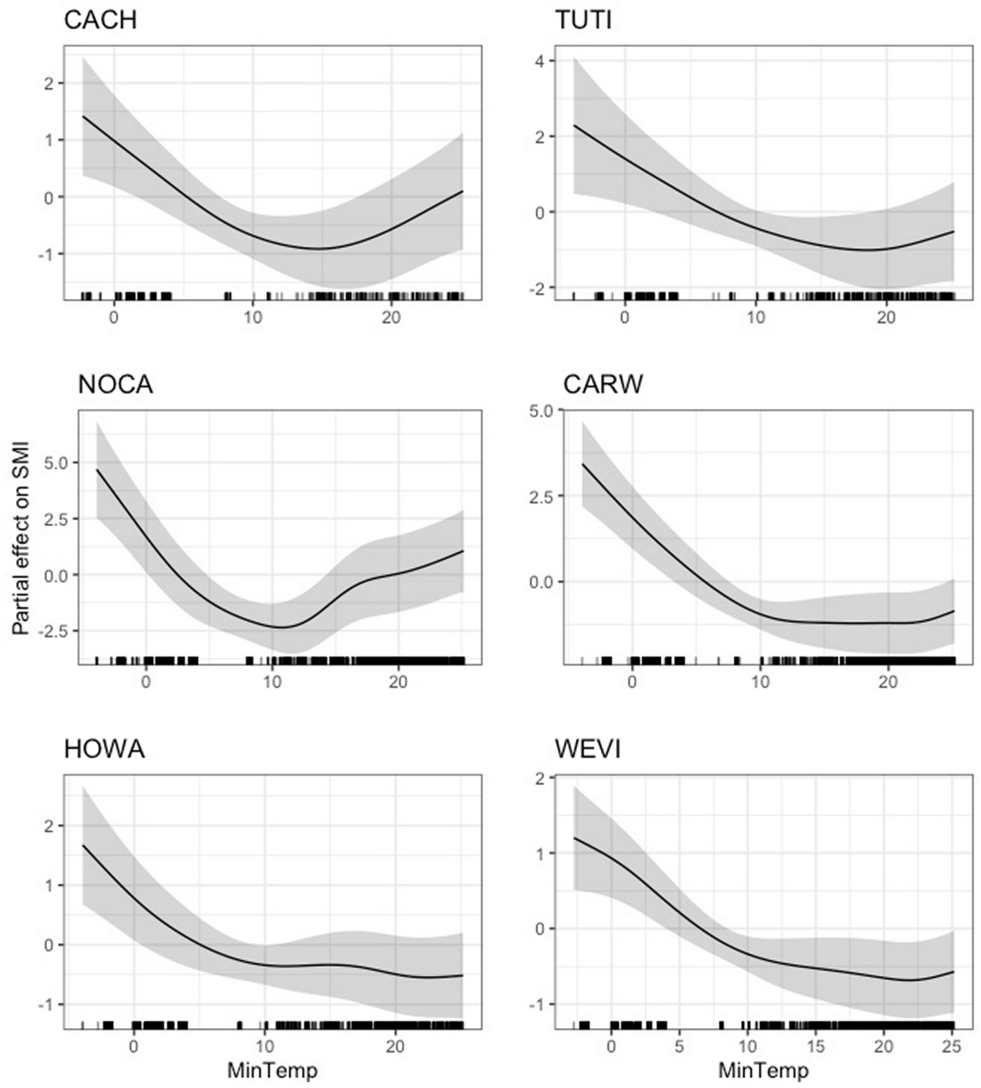

FIGURE 2
Partial effect of average daily minimum temperature (degrees Celsius) on scaled mass index for six species. Left: Carolina chickadee (CACH, top), northern cardinal (NOCA, center), hooded warbler (HOWA, bottom); Right: tufted titmouse (TUTI, top), Carolina wren (CARW, center), white-eyed vireo (WEVI, bottom). Black lines along the x-axis reflect the distribution of individual data points.

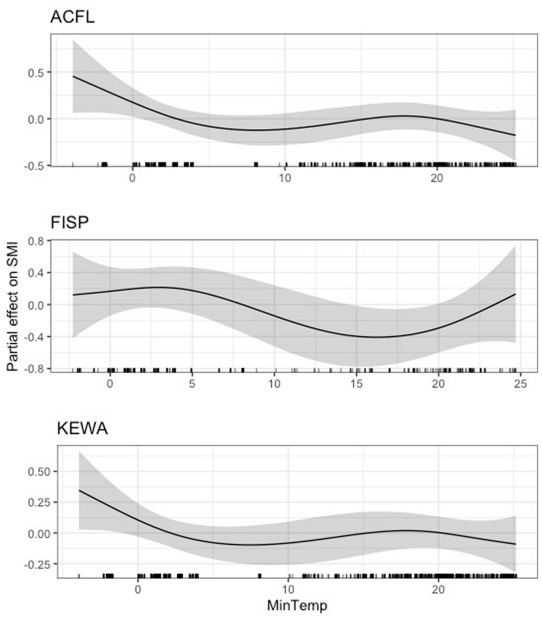

FIGURE 3
Partial effect of average daily minimum temperature (degrees Celsius) on scaled mass index for Acadian flycatcher (ACFL, top), field sparrow (FISP, center), and Kentucky warbler (KEWA, bottom).

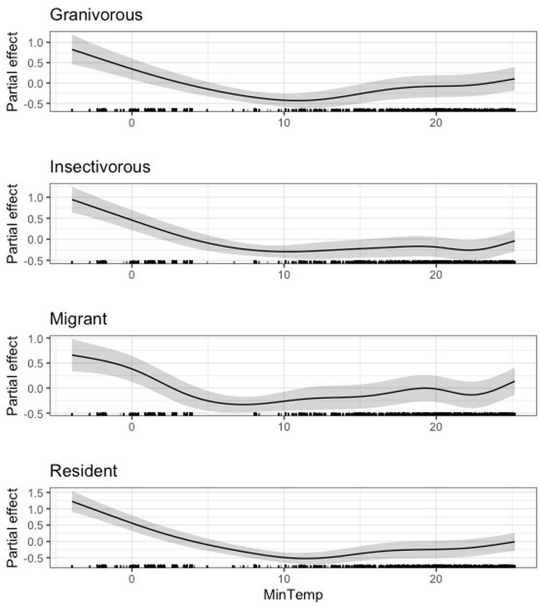

FIGURE 5
Partial effect of average daily minimum temperature (degrees Celsius) on scaled mass index for the granivorous guild (top), insectivorous guild (second from top), Neotropical migrant guild (third from top), and resident guild (bottom).

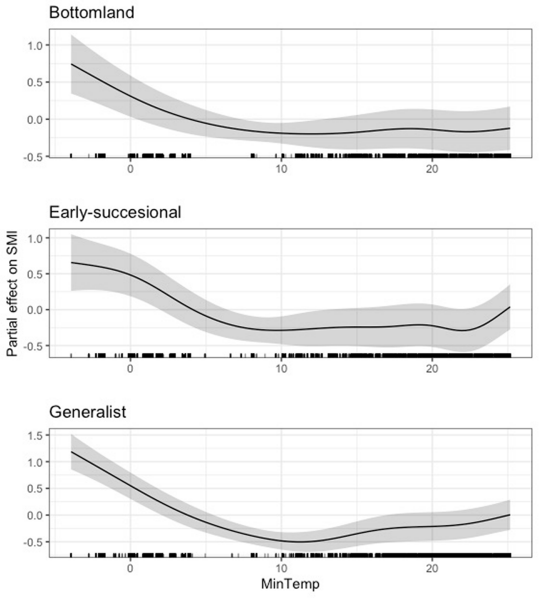

FIGURE 4
Partial effect of average daily minimum temperature (degrees Celsius) on scaled mass index for the bottomland guild (top), early successional guild (center), and habitat generalist guild (bottom).

Precipitation

For four species (Carolina wren, indigo bunting, northern cardinal, and hooded warbler), we found that low levels of precipitation were negligibly or negatively related to body condition until a threshold was reached (between 10 and 17 cm), after which precipitation was positively related to body condition (Figure 10). For painted bunting and yellow-breasted chat, we found a highly dynamic, though generally positive, relationship between precipitation and scaled mass index (Figure 10). Neither the 95% nor 85% CIs overlapped zero for Carolina wren, northern cardinal, painted bunting, yellow-breasted chat, or hooded warbler. However, for indigo bunting the lower 95% CI closely approached zero (4.62e-03), suggesting a potentially weak effect of precipitation (Supplementary Table S5). Precipitation was also positively related to scaled mass index for Carolina chickadee, prothonotary warbler, red-eyed vireo, and white-eyed vireo, although these effects were quite weak (Figure 11). The lower 95% CI for Carolina chickadee (2.76e-35) closely approached zero, and for prothonotary warbler, red-eyed and white-eyed vireos both the lower 95% CI (prothonotary warbler: 5.46e-20, red-eyed vireo: 1.94e-06, white-eyed vireo: 1.10e-21) and lower 85% CI (prothonotary warbler: 9.32e-16, red-eyed vireo: 1.58e-05, white-eyed vireo: 4.20e-17) both closely approached zero (Supplementary Table S3). Precipitation was negatively related to scaled mass index for field sparrow (S.5), however this effect was very weak as the lower 95% CI (2.64e-32) and 85% CI (7.51e-25) both closely approached zero (Supplementary Table S5).

In terms of guilds, we found a generally variable but generally negative effect of precipitation on scaled mass index in the bottomland and granivorous guilds until approximately 17 cm for the bottomland guild and just above 10 cm for the granivorous guild, after which the effect of precipitation was generally positive (Figure 12). Our results for the early successional, insectivorous, and Neotropical migrant guilds largely mirrored those for indigo bunting, Carolina wren, northern cardinal, and hooded warbler in that precipitation was negligibly related to body condition until a certain precipitation threshold was reached (16 - 20cm for these guilds), after which precipitation positively related to body condition (Figure 13). Finally, we noted a strong positive relationship between precipitation and scaled mass index above

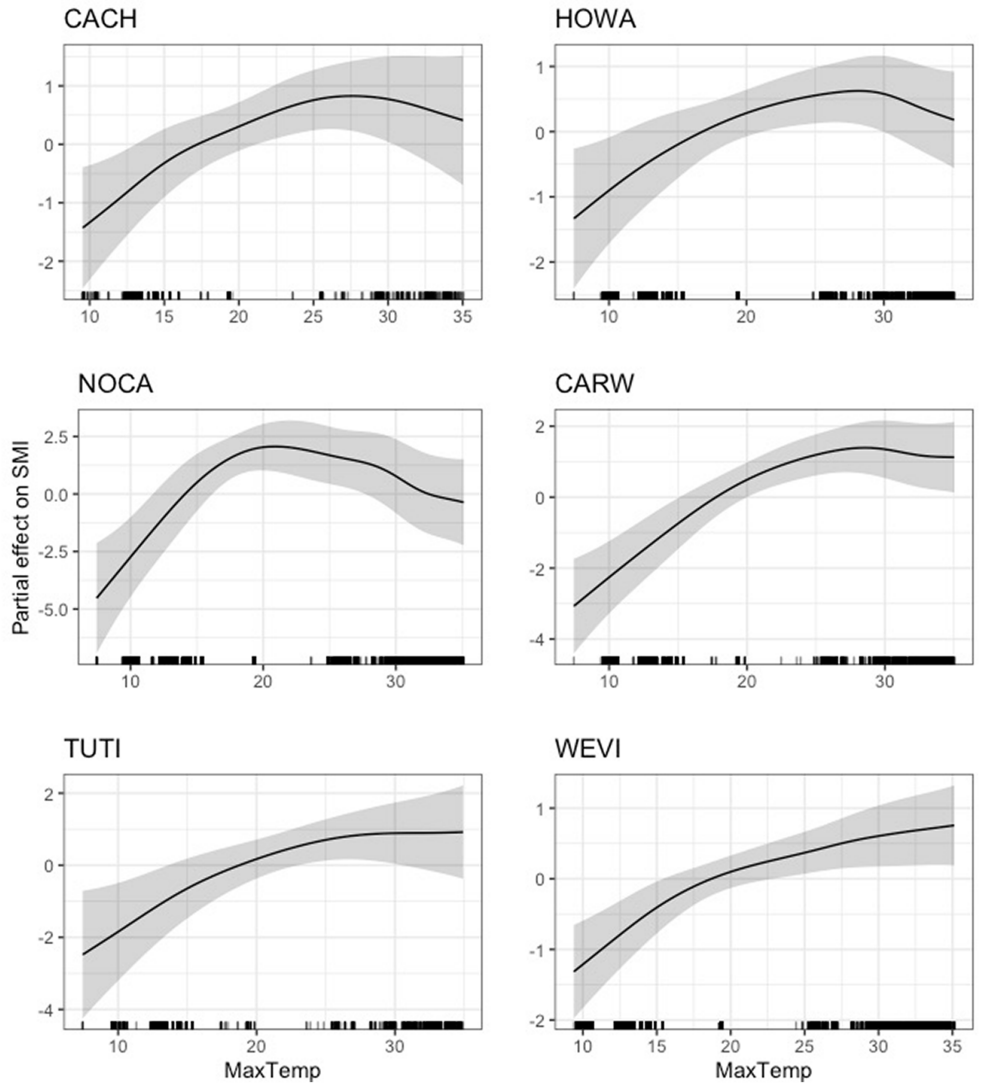

FIGURE 6
Partial effect of average daily maximum temperature (degrees Celsius) on scaled mass index for six species. Left: Carolina chickadee (CACH, top), northern cardinal (NOCA, center), tufted titmouse (TUTI, bottom); right: hooded warbler (HOWA, top), Carolina wren (CARW, center), white-eyed vireo (WEVI, bottom).

10cm for the habitat generalist and resident guilds, while the relationship between precipitation and scaled mass index for the omnivorous guild was weak overall (Figure 14). The 95 and 85% CIs for all guilds analyzed did not overlap zero (Supplementary Table S5).

Effects of age on scaled mass index

Species

The effect of age was present in the top models for 16 out of the 18 species analyzed (Supplementary Table S3). Juveniles had higher scaled mass indices than adults (i.e., a negative effect of age) for Acadian flycatcher ($p < 0.001$), hooded warbler ($p < 0.01$), indigo bunting ($p < 0.001$), Kentucky warbler ($p < 0.001$), northern cardinal ($p < 0.001$), prothonotary warbler ($p < 0.01$), red-eyed vireo ($p < 0.001$), Swainson's warbler ($p < 0.001$), and white-eyed vireo ($p < 0.001$; Supplementary Table S6). However, this directionality was not uniform, and scaled mass index was higher in adults than juveniles for Carolina chickadee ($p < 0.01$), Carolina wren ($p < 0.001$), common yellowthroat ($p < 0.001$), field sparrow ($p < 0.001$), and tufted titmouse ($p < 0.01$; Supplementary Table S6). Although age was included in the top models for Bewick's wren and wood thrush, it did not significantly influence scaled mass index for these species ($p > 0.05$; Supplementary Table S6). The top models for painted bunting and yellow-breasted chat did not include the effect of age (Supplementary Table S3).

Guilds

Age was present in the top models for 7 out of 8 guilds. Scaled mass index was higher in juveniles than adults for the granivorous, bottomland, and early successional guilds ($p < 0.001$), and the insectivorous guild ($p < 0.01$; Supplementary Table S6). Conversely, scaled mass index was higher in adults than juveniles for the omnivorous guild ($p < 0.001$; Supplementary Table S6). Age was in the top models for the resident and habitat generalist guilds, but it did not

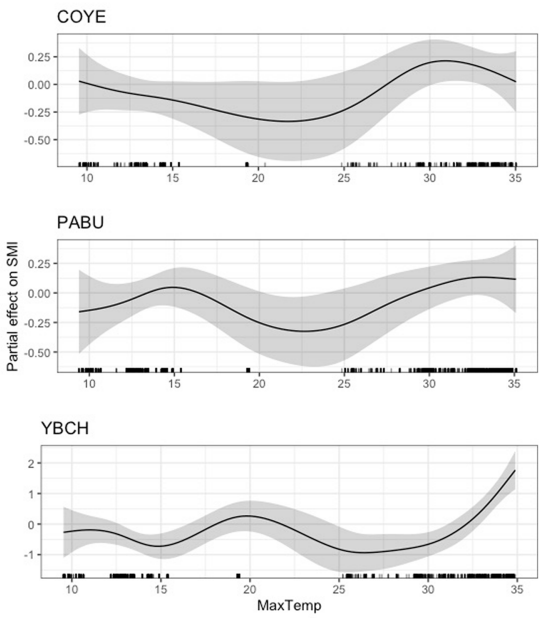

FIGURE 7
Partial effect of average daily maximum temperature (degrees Celsius) on scaled mass index for common yellowthroat (COYE, top), painted bunting (PABU, center), and yellow-breasted chat (YBCH, bottom).

have a significant effect on scaled mass index for either group ($p > 0.05$; Supplementary Table S6). The top model for the migrant guild did not include the effect of age.

Discussion

We observed strong heterogeneity in the relationships between scaled mass index and weather parameters across species and guilds. The response of avian body condition to weather has previously been established as dynamic and complex, driven in part by a balance between competing weather variables (Gardner et al., 2018). Our results are consistent with this observation and highlight the role of weather thresholds in relation to avian body condition during the breeding season. For many species and guilds, the body condition response shifted dramatically after reaching a certain threshold of temperature or precipitation. For temperature this threshold tended to occur around 10°C for minimum and 20–30°C for maximum daily temperature, and for precipitation the threshold typically occurred between 10 and 20 cm. As expected for datasets this large and varied, weather parameters typically explained a low amount of overall variance in our top models ($r^2 < 0.25$ for all models). Thus, our results establish that relatively short-term weather parameters are important external drivers of avian body condition, but body condition is complex and likely influenced by many additional variables including seasonality and the availability of local thermal refugia.

Effects of temperature on body condition

We predicted that warmer temperatures would positively impact body condition for insectivorous and migratory species but would have little effect on omnivorous species. Although we did find positive effects of warmer temperatures on body condition for some species, these effects were more dynamic than the predicted linear relationship. Regarding minimum daily temperatures, body condition tended to decrease with increasing minimum temperature until approximately 10°C, after which body condition either increased or became generally unrelated to minimum temperature. At low minimum temperatures, body condition may be influenced by fat storage for thermoregulation and limited activity (Stevenson and Woods, 2006), and thus not follow simple food-availability trends. The scale of the y-axis values for minimum temperature partial effects plots indicates that the observed effects of minimum temperature were generally stronger than those observed for other weather variables. For maximum temperature, we observed a similar threshold effect at 20–30°C, with many species exhibiting a positive relationship between body condition and increasing maximum temperatures until this point, after which the relationship became slightly negative or negligible. At the guild-level, the threshold effect largely mirrored that of individual species with inflection points for minimum temperature occurring around 10°C, and maximum temperature around 20°C. We also found that early-successional species hit their threshold in positive response to maximum temperature sooner than bottomland or generalist species (18 vs. 20°C), and exhibited stronger effects of temperature (indicated by CIs further from zero), which are consistent with our predictions in (*H6*). High levels of concurvity in top models that included both minimum temperature and maximum temperature indicate large confidence intervals and suggest that detailed interpretations of these results may be limited.

Effects of precipitation on body condition

Like our temperature findings, we observed effects of average daily precipitation on many individual species and some, but not all, guilds. For most of our species and guilds, we observed a threshold effect, where body condition was negatively or negligibly related to body condition until a threshold of precipitation was reached (10–20 cm), after which body condition tended to increase with more precipitation. A smaller proportion of species and guilds exhibited a generally positive relationship between precipitation and body condition, and only one species and guild (i.e., field sparrow and the omnivorous guild, for which the effect was weak) exhibited generally negative relationships between precipitation and body condition. Thus, contrary to our predictions, we found little evidence of high precipitation correlating with a decline in body condition due to flooding for the granivorous (*H1*) or bottomland guilds (*H4*). Instead, precipitation tended to have the strongest positive effects after approximately 17 cm. For some species, such as painted bunting, the effects of precipitation appeared to change quickly within relatively small windows of precipitation totals. This variation in response to precipitation seems biologically improbable, and may be due to confounding, unaccounted for factors (e.g., microhabitat selection) or interactions between weather variables which were not considered in this study. Therefore, interpretation of these results is limited.

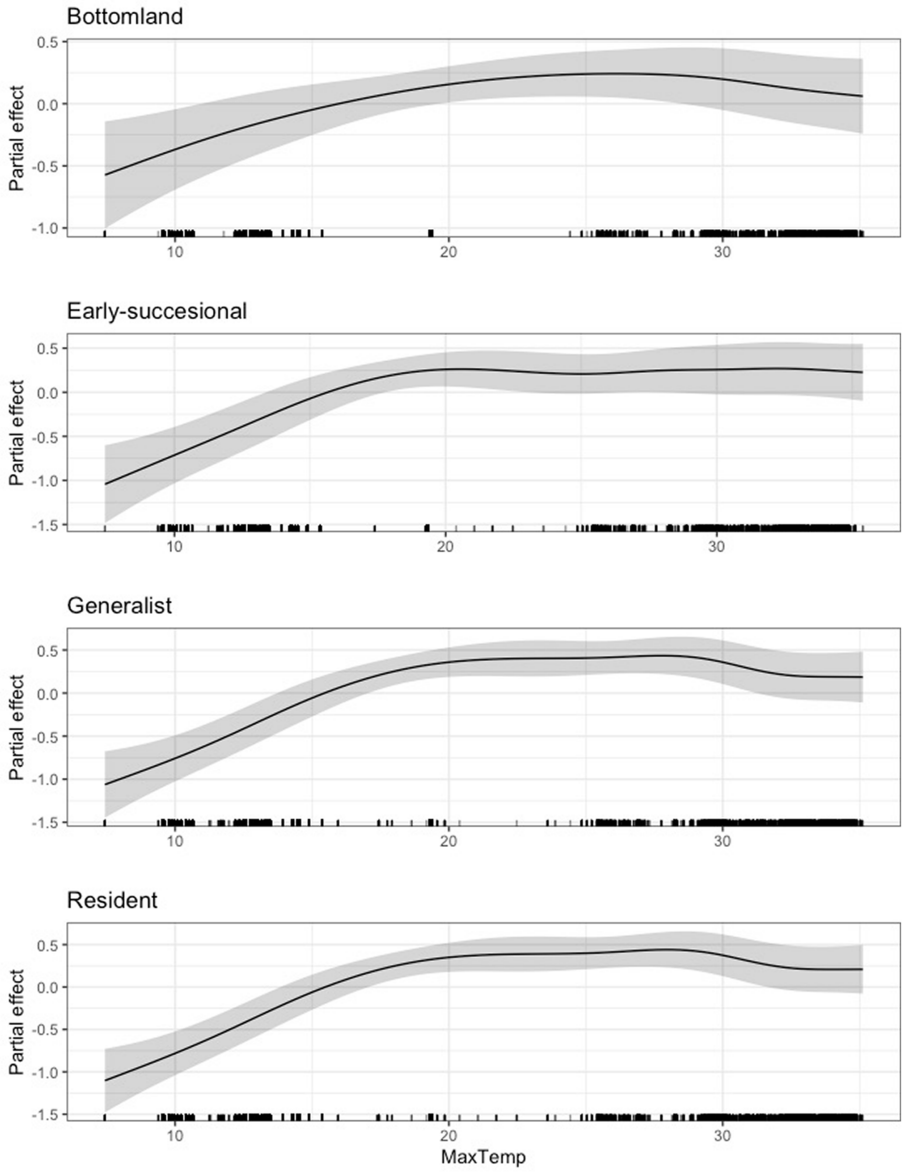

FIGURE 8
Partial effect of average daily maximum temperature (degrees Celsius) on scaled mass index for the bottomland guild (top), early successional guild (second from top), habitat generalist guild (third from top), and resident guild (bottom).

Age effects

We found that for most species analyzed (14 out of 18), age was an important driver of scaled mass index. Age-dependent responses to weather variables have been demonstrated previously *via* numerous pathways including differential migratory phenology (Jarjour et al., 2017), phenological (Bonamour et al., 2020) and phenotypic plasticity (Ward et al., 2021), and existing studies on body condition (Gardner et al., 2016, 2018; McLean et al., 2018). A wide body of existing literature also suggests that body condition fluctuates as birds age (Angelier et al., 2011; Rockwell et al., 2012; Welcker et al., 2015), due in part to the fact that adult birds may outcompete juvenile birds for more limited resources in times of extreme weather (Rockwell et al., 2012). For most species (i.e., nine of the 14 species) juvenile birds exhibited higher body condition than adults. This result may have been influenced by both age-specific adaptation and species-specific factors such as fledging date, clutch size, or quality of parental care, for which we were unable to control. Additionally, this finding could reflect a different mass-wing scaling component in younger birds compared to adults. Results from our guild-level analysis largely mirrored this, with the directionality of age effects remaining inconsistent across guilds, although adults had higher body condition for all three dietary guilds.

Implications for fitness

Our findings demonstrate the importance of short-term weather patterns to individual avian body condition, which can have broad ramifications across entire populations, including altered levels of

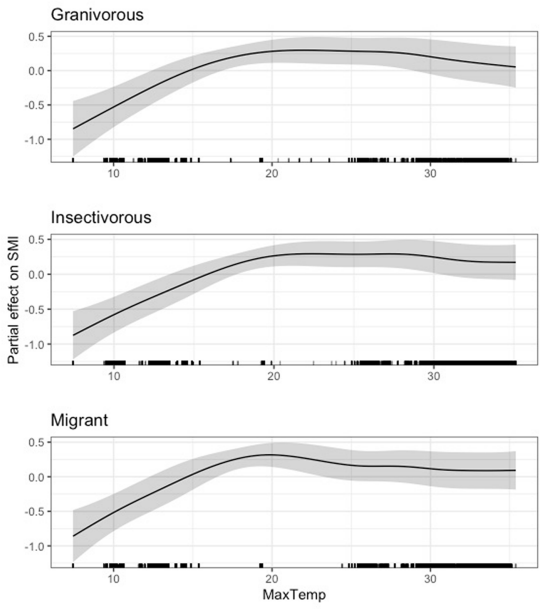

FIGURE 9
Partial effect of average daily maximum temperature (degrees Celsius) on scaled mass index for the granivorous guild (top), insectivorous guild (center), and Neotropical migrant guild (bottom).

individual fitness. Relationships between avian body condition, external variables such as habitat and climate, and fitness are not well established and frequently inconclusive at best (Kleist et al., 2017; Kouba et al., 2021) or suggest climate changes affect population growth rates predominantly independently of simultaneous body condition declines (McLean et al., 2020). However, body condition has been found to influence individual fitness in a large number of species, including a wide variety of birds (Stevenson and Woods, 2006) and other vertebrate taxa such as marine iguanas (Romero et al., 2001) and bison (Vervaecke et al., 2005). This is a result, in part, of reduced body condition negatively affecting the foraging success of an individual (Jakob et al., 1996) along with increasing susceptibility to disease and predators (Kouba et al., 2021). This uncertainty in the literature may reflect complexity in the interpretation of body condition, which can be influenced by fat storage for thermoregulation as well as energetic storage (Stevenson and Woods, 2006). Thus, high body condition may not always be adaptive, for example under high heat conditions where lower body mass results in more rapid cooling (McLean et al., 2020).

The results of our study indicate that a further increase in body condition past certain temperature and precipitation thresholds (i.e., average daily maximum temperature exceeding 20°C) may no longer be beneficial. Future research may help further elucidate if long-term changes to temperature and precipitation have negative effects on body condition, fitness, and population dynamics. In some cases, subsequent consequences such as reduced nestling survival may become apparent if individuals must spend more time foraging. Future studies could explore the relationship between fitness, body condition, and weather further by pairing adult body condition data with nesting success and annual survival, or by correlating current year population estimates with prior year body condition data. We also anticipate that these effects will differ across scale, species, and geographies, consistent with data from this study.

Conclusion

Overall, our results demonstrate that maximum temperature, minimum temperature, and precipitation frequently relate to avian body condition in a non-linear fashion and that threshold effects of weather are important for many species and guilds. We anticipate that threshold effects, such as those found for several species in this study (e.g., northern cardinal and indigo bunting), will become more relevant in the future as climate change increases extreme weather (Bregman et al., 2016) and pushes species closer to, or further from, these thresholds. Threshold effects are often witnessed at the scale of ecosystems (Burkett et al., 2005) and interactions between ecosystem, community, and species-specific thresholds may be expected and should be investigated in the future. Relationships between weather and body condition were also remarkably similar between most dietary, migratory, or habitat guilds. These trends often did not mirror those for individual species within guilds due to strong species-specific heterogeneity that was masked in a guild-level analysis. Thus, responses of birds to weather may be difficult to predict across species, at least regarding body condition. For migratory species, this may be partially due to spillover effects from conditions on the wintering grounds (Rockwell et al., 2012; Akresh et al., 2019a). For example, migratory species may arrive on their breeding grounds in variable energetic states because of conditions on their wintering grounds or migratory route, that then influence body condition during the breeding season. Finally, our results also suggest that temperature may have stronger effects than precipitation on avian body condition, which is consistent with existing literature indicating stronger effects of temperature than precipitation on avian populations (Pearce-Higgins et al., 2015).

Future directions

The strong species-specific heterogeneity that we observed in this study suggests that caution should be used when evaluating the effects of short-term weather on birds at the guild level, and that species-level approaches may be more informative albeit less consistent. This would be particularly important when dealing with imperiled or engendered species, or species of conservation concern, for which more exact knowledge of their responses are warranted. Dietary and habitat preferences of some species may change across seasons (Billerman et al., 2022), thus future studies should explore alternative guild assignments that may capture more nuance in species response. Future studies at different spatial or temporal scales may also further elucidate the drivers behind species-specific heterogeneity, and scales at which guild-level analyses are appropriate.

We foresee additional future research opportunities investigating the consequences of our observed relationships between weather dynamics and body condition for individual fitness (i.e., reproductive success and survival), and the implications of these relationships for short-term population dynamics and long-term evolutionary processes. Developing a better understanding of how birds respond to short-term weather at the level of the individual complements existing research at the level of the population/community and is an urgent area of research as climate change continues to shift short-term weather patterns (Jenouvrier, 2013; Langham et al., 2015).

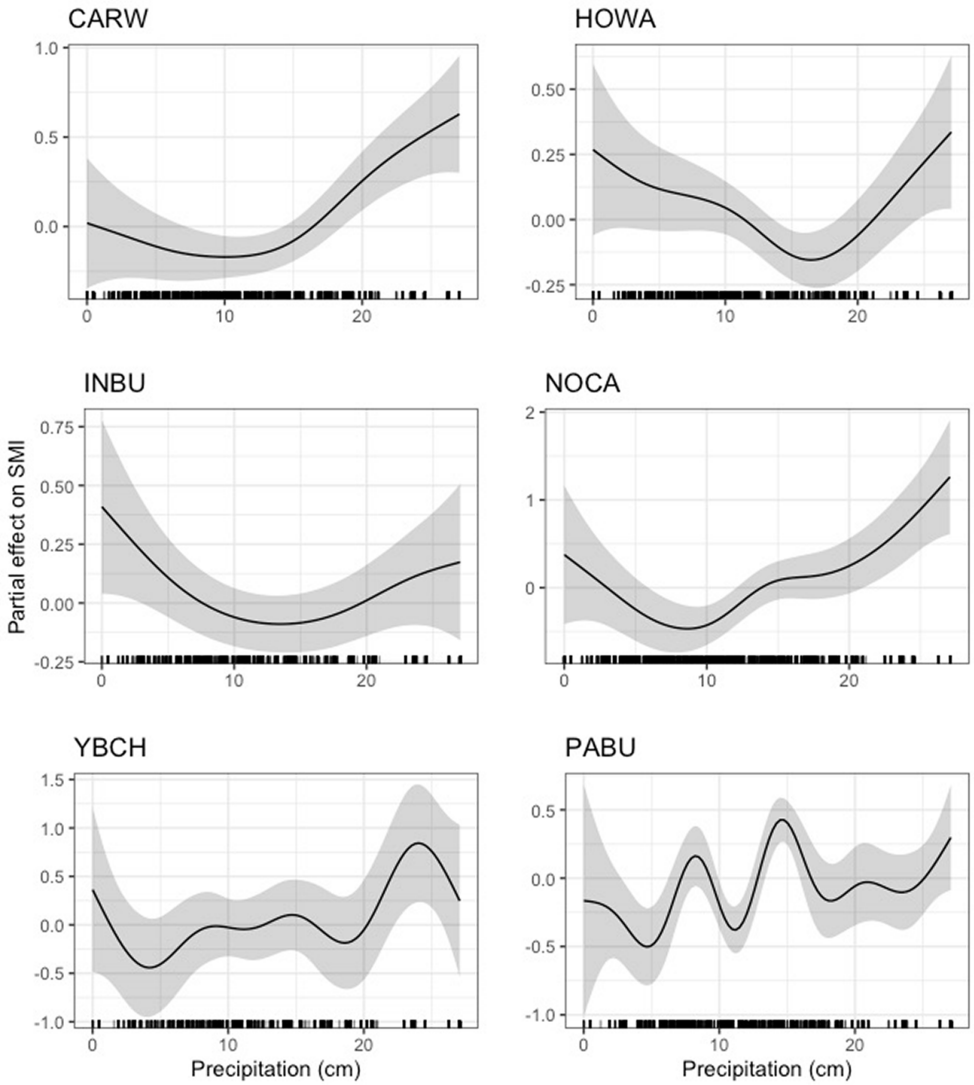

FIGURE 10
Partial effect of precipitation on scaled mass index for six species. Left: Carolina wren (CARW, top), indigo bunting (INBU, center), yellow-breasted chat (YBCH, bottom); right: hooded warbler (HOWA, top), northern cardinal (NOCA, center), painted bunting (PABU, bottom).

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: https://datadryad.org/stash/share/sYVnEPMQP-mTS5_-xBBDJDZDT3ocJKik8h53G3HuULw.

Ethics statement

Ethical review and approval was not required because this research was conducted through an existing dataset. No new field work or handling of individual organisms was conducted for this study.

Author contributions

MM spearheaded the study design, data collection of this research, designed figures, and tables. JG and MM both contributed to the analysis of data and contributed equally to the editing process. JG contributed significantly to the discussion section and interpretation of results. All authors contributed to the article and approved the submitted version.

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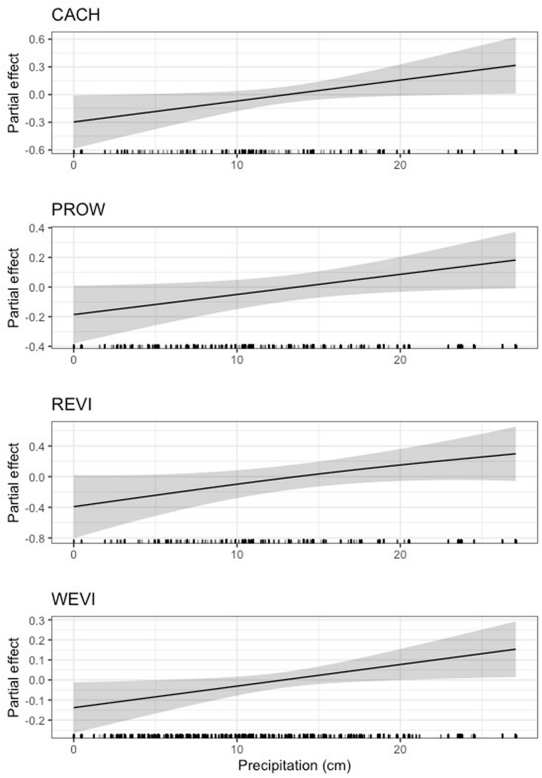

FIGURE 11
Partial effect of precipitation on scaled mass index for Carolina chickadee (CACH, top), prothonotary warbler (PROW, second from top), red-eyed vireo (REVI, third from top), and white-eyed vireo (WEVI, bottom).

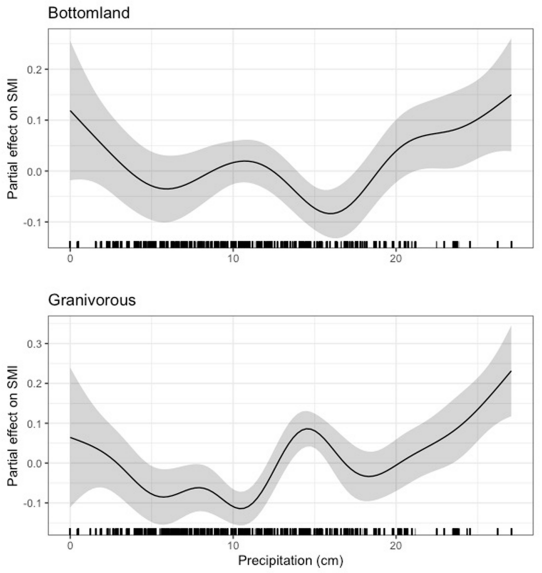

FIGURE 12
Partial effect of precipitation on scaled mass index for the bottomland guild (top) and the granivorous guild (bottom).

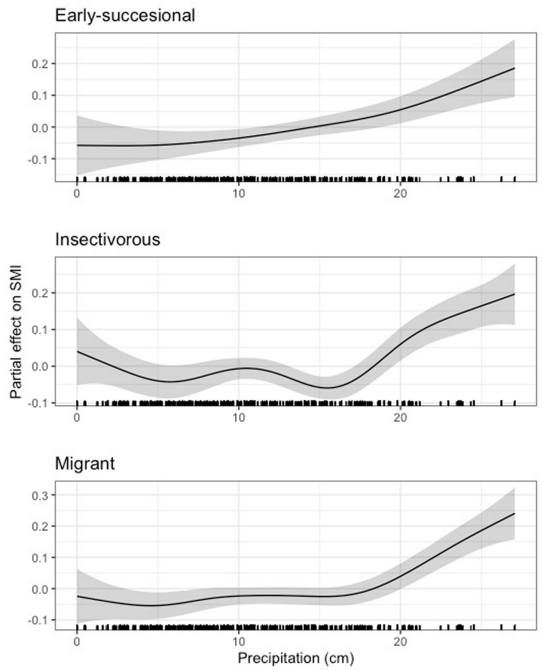

FIGURE 13
Partial effect of precipitation on scaled mass index for the early successional guild (top), the insectivorous guild (center), and the Neotropical migrant guild (bottom).

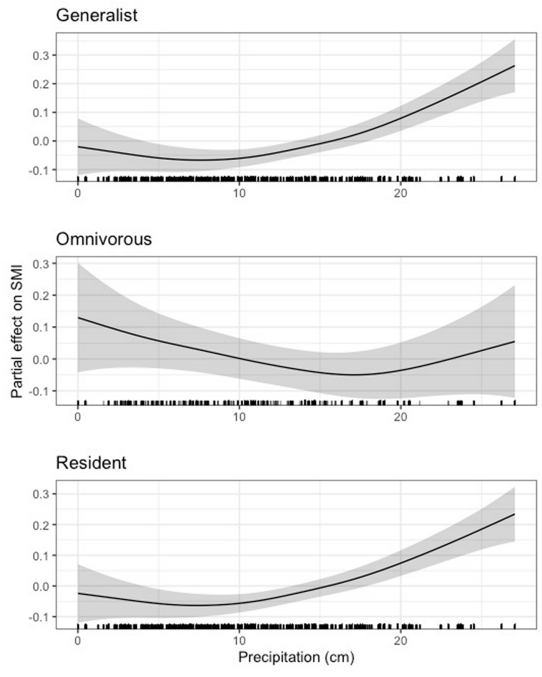

FIGURE 14
Partial effect of precipitation on scaled mass index for the habitat generalist guild (top), the omnivorous guild (center), and the resident guild (bottom).

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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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Supplementary material

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A simple biochemical plasma test as an indicator of maternal energy balance predicts offspring survival in bighorn sheep

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In species where offspring survival is highly variable relative to adult survival, such as bighorn sheep (*Ovis canadensis*), physiological indicators of maternal investment could clarify the functional mechanisms of life history trade-offs and serve as important predictors of population dynamics. From a management perspective, simple predictors of juvenile survival measured non-lethally from maternal samples could aid in identifying at-risk populations or individuals before significant mortality occurs. Blood biochemical parameters can offer low-cost insights into animal health and physiology, therefore we sought to develop a simple biochemical predictor of juvenile survival based on maternal blood samples. We measured biochemical indicators of energy balance in adult bighorn sheep at a single time point in January or February, and then monitored survival through August of the same year to assess how those measures related to survival of individual adults and their juvenile offspring. Juvenile survival was lower over the subsequent spring and summer when maternal adult serum beta-hydroxybutyric acid (β-HBA) concentration was high, indicating a negative energy balance in the mothers. However, serum β-HBA did not correlate with adult survival over the same period. Our findings suggest that even when maternal body condition is high, short-term caloric deficit may be sufficient trigger to decrease investment in offspring survival. This mechanism could protect adult females from investing heavily in juvenile survival when resources become too limited to support population growth. Our study suggests that β-HBA could be a powerful monitoring tool for bighorn sheep and other threatened ruminant populations under resource limitation.

KEYWORDS

bighorn sheep, energy balance, maternal effects, body condition, wildlife health monitoring, plasma biochemistry profile

Introduction

The relative influence of adult versus juvenile survival on population dynamics varies strongly across species depending on whether they are k-selected, i.e., having fewer young with greater parental investment, or r-selected, i.e., having more young with reduced parental investment (Mac Arthur and Wilson, 1967). For k-selected species such as large terrestrial

herbivores, which often exist near the carrying capacity of their environment, annual adult survival tends to be relatively high with little variation (Mac Arthur and Wilson, 1967). In contrast, juvenile survival tends to be variable, and therefore population growth is more sensitive to juvenile survival parameters (Gaillard et al., 2000). The trade-off of investing in adult versus juvenile survival has been documented across numerous species, including large terrestrial herbivores. However, the physiological mechanisms underlying these trade-offs remain unclear due to the challenges of obtaining long-term measures of survival and linking them to function (Ricklefs and Wikelski, 2002). Mechanistic understanding of life history choices could contribute to improved understanding of demographic trends in natural wildlife populations.

Reproductive success depends on the physiological propensity of an animal to breed, and subsequently to invest in the survival of its offspring (Wasser and Barash, 1983). In k-selected species that evolved near carrying capacity, we would expect reduced probability of breeding and investment in the survival of fewer offspring when resources become limited (Pianka, 1970). In capital breeders that rely on stored endogenous resources for reproduction and offspring rearing, fertility is heavily dependent on maternal energy balance (Boyd, 2000). This pattern is likely mediated by hormonal regulation (Garcia-Garcia, 2012; Clarke, 2014). Postpartum maternal expenditure is also dependent on resource availability. For example, in bighorn sheep, maternal expenditure decreases when competition for resources increases (Festa-Bianchet et al., 1997). Reduced fertility and maternal expenditure under resource limitation reflects a conservative reproductive strategy that favors maternal rather than offspring survival (Martin and Festa-Bianchet, 2010). While reproductive hormones mediate the relationship between energy balance and fertility, they do not explain the expected trade-off between maternal energy balance and offspring survival. In large mammals, lactation is the costliest part of raising offspring (Clutton-Brock et al., 1989), therefore reducing the quantity or quality of lactation as soon as resources become limited could significantly reduce energy expenditure and enhance maternal survival at the cost of juvenile survival. In k-selected species with high adult survival and variable juvenile survival, we would therefore expect reduced investment in lactation as soon as resources become limited and before significant loss of maternal body condition occurs. Biochemical indicators of current maternal energy balance are therefore likely to be good predictors of investment in offspring survival, because they reflect current resource availability and may be a proxy for lactation quality (Adewuyi et al., 2005).

In species where offspring survival is highly variable relative to adult survival, indicators of maternal investment could be important predictors of population dynamics. In populations of conservation concern, including a biochemistry profile of serum or plasma samples during routine monitoring could provide a low-cost and informative tool for managers to identify at-risk populations or individuals before juvenile mortality occurs. The utility of blood biochemical parameters for monitoring large ungulates has been demonstrated by Milner et al. (2003), who found that parameters reflecting energy balance and body condition were related to subsequent survival and successful calving in Svalbard reindeer. The relationships between blood biochemical parameters and offspring survival have not been evaluated in ungulates from temperate environments or that are subjected to predation, but it is reasonable to expect that energy balance would correlate to survival and reproduction in any resource-limited population.

Biochemical parameters that reflect energy balance in ruminants include serum concentrations of glucose, triglycerides, non-esterified fatty acids (NEFAs), and beta-hydroxybutyric acid (β-HBA). Blood glucose is an important source of fuel for glycolytic tissues, including nerves and muscles, and may decrease when energy stores become depleted (Chowdhury and Ørskov, 1994). However, blood glucose is also highly sensitive to stress and therefore may not accurately reflect energy balance in animals that are captured for sampling (Marco et al., 1997). Adipose tissue in mammals is composed of triglycerides which are broken down, in a process known as lipolysis, to form NEFAs during periods of negative energy balance (Herdt, 2000). NEFA concentrations in the blood could therefore indicate negative energy balance. β-HBA is a type of ketone made in response to negative energy balance in all mammals, but is most clinically apparent in lactating ruminants (Herdt, 2000). In addition to fueling tissues during energy restriction, β-HBA is a precursor to and regulator of milk fat in ruminants (Zhang et al., 2015). β-HBA is much less sensitive to acute stress than glucose, and is therefore unlikely to be affected by capture stress (Koeslag et al., 1980). Because β-HBA directly links maternal energy balance with milk quality in ruminants, this metabolite could accurately indicate when a lactating female is conserving energy versus investing in offspring survival. β-HBA levels in wild ruminant species could therefore represent an important physiological mechanism for life history trade-offs under resource limitation in wild ruminant species.

In this study, we assessed the utility of blood biochemical parameters (Table 1), including measurements of energy balance, organ health, and immune function for predicting adult and juvenile survival in bighorn sheep (*Ovis canadensis*) in southeastern Oregon and northern Nevada. Bighorn sheep are a large herbivore species historically distributed across the North American west, but now existing mainly in fragmented populations which are vulnerable to local extinction (Whittaker et al., 2004). In this ecosystem, bighorn sheep feed on grasses and shrubs, with diet selection varying by season and by reproductive status (Van Dyke et al., 1983). Adult survival is relatively stable between years, but juvenile survival varies significantly as is typical for k-selected species (Spaan, 2022). Persistence of local populations is therefore highly dependent on juvenile survival rates, and monitoring and predicting juvenile survival is critical for effective management. During our study period, annual pregnancy rates in adult females exceeded 90 and 78% were seen with juveniles (Spaan et al., 2021). Juvenile survival is influenced by many factors including the presence of the respiratory bacterial pathogen *Mycoplasma ovipneumoniae* and forage quality (Spaan et al., 2021), but the role of maternal health has not been investigated in this region. Maternal body condition, a long-term measure of nutritional status, is known to influence juvenile survival in other bighorn sheep populations (Festa-Bianchet et al., 2019), but the effect of short-term maternal energy balance has not been explored. We hypothesized that parameters reflecting maternal energy balance would predict offspring survival from late gestation through the first 4 months of life, a period when juvenile mortality tends to be particularly high (Spaan et al., 2021). We also hypothesized that these parameters would predict adult survival during the same time period, but to a lesser extent because adult survival rates tend to be relatively stable. We did not expect measures of organ function to predict survival because organ

TABLE 1 All biochemical and immune parameters that were investigated are listed.

Parameter	Associated organ/ system	Clinical significance
Total WBC	Immune system	Overall status of cellular immunity (i.e., non-humoral).
Total neutrophils	Immune system	Bacterial defense.
Total lymphocytes	Immune system	Cell mediated immunity.
Total monocytes	Immune system	Differentiate into macrophages once in the tissues to provide resident microbial defense in various tissues.
Total eosinophils	Immune system	Parasitic defense.
Total basophils	Immune system	Parasitic defense.
Blood urea nitrogen (BUN)	Liver/Kidney/GI (Ruminants)	The urea is produced by the liver in mammalian species. It is excreted by the kidney and in the saliva in ruminants. Urea is used by the rumen microbes for protein production. The main clinical use is to measure glomerular filtration rate.
Creatinine	Kidney/Muscle	Measurements of serum creatinine are directly related to glomerular filtration rate because creatinine is freely filtered by the kidneys. Significant muscle atrophy/damage can increase creatinine levels.
Glucose	Liver/Endocrine system/ Energy balance	Created or stored by the liver in response to various hormones (insulin, glucagon, catecholamines, growth hormone, and corticosteroids). Hyperglycemia associated with various endocrinopathies, iatrogenic overdose of drugs, and various other diseases. Hypoglycemia associated with various endocrinopathies, sepsis, and bovine ketosis.
Cholesterol	Liver/Biliary system/ Metabolic health	Made in the liver and excreted through the bile. Clinically associated with altered metabolism in various disease states including cholestasis.
Triglycerides	Liver/Metabolic health	Synthesized from NEFAs. Ruminants store triglycerides in their livers and use NEFAs to make ketones. Clinically associated with hepatic lipidosis and ketosis in ruminants.
Total protein	Various systems	Numerous physiological functions. Clinically significant ones include oncotic pressure maintenance, transportation of electrolytes and metabolites, inflammation, and hemostasis.
Albumin	Liver	Made in the liver. One of the main constituents of blood protein and main factor in plasma osmotic pressure.
Globulins	Liver/Immune system	Made in the liver. All non-albumin blood proteins. Made of three distinct subgroups (α, β, and γ). Gamma globulins are associated with inflammatory conditions.
Total bilirubin	Red blood cells/Liver/Biliary system	80% comes from breakdown of hemoglobin from red blood cells. Increased in diseases that cause significant hemolysis and diseases that are associated with cholestasis.
Creatine kinase (CK)	Muscle	Leakage enzyme from myocytes. Significant muscle damage leads to increases in CK.
Gamma-glutamyl transferase (GGT)	Liver/Biliary system	Membrane bound protein in various tissues. Main source of serum GGT is biliary epithelium. Increased in hepatobiliary disease, especially ones associated with cholestasis. In large animals, it is specific for biliary hyperplasia and structural cholestasis.
Aspartate aminotransferase (AST) or serum glutamic-oxaloacetic transaminase (SGOT)	Liver/Muscle	Non organ specific intracellular enzyme. Found in highest concentrations in muscle cells and hepatocytes. Increases are due to muscle and/or liver damage.
Sodium (Na)	Various vital functions	Sodium is involved in water balance, acid/base balance, and renal tubular absorption balance. Many physiologic and pathologic states can vary sodium concentration.
Potassium (K)	Various vital functions	Potassium is the primary intracellular cation and is primarily responsible for maintaining the membrane potential of cells.
Chloride (Cl)	Various vital functions	Chloride is the primary extracellular anion and provides electroneutrality for sodium. The main functions of chloride are acid/base balance and osmolality.
Phosphorus (P)	Bone	Phosphorus is always found in the body as phosphate. The main source of phosphate in the body is in the bone. Changes in phosphate are seen in various diseases including kidney disease, endocrinopathies, gastrointestinal disease, and nutritional issues. Ruminants excrete large amounts of phosphate in their saliva which acts to buffer the rumen pH and can be increased in dehydrated ruminants.
Magnesium (Mg)	Various vital biochemical reactions	Magnesium is the second most abundant intracellular cation. The main function of magnesium is its role in various enzymatic reactions including the making of ATP. Magnesium concentration is affected by various diseases which distort its absorption/excretion balance.
Total Carbon Dioxide (TCO_2)	Acid/Base status	TCO_2 is an indicator of blood pH. It is defined as the dissolved CO_2 + the bicarbonate concentration in the blood. It is lowered in metabolic acidosis and increased in metabolic alkalosis.

(Continued)

TABLE 1 (Continued)

Parameter	Associated organ/ system	Clinical significance
Sorbitol dehydrogenase (SDH)	Liver	SDH converts fructose to sorbitol and is a very specific indicator of hepatocellular damage.
Beta Hydroxybutyric acid (β-HBA)	Energy balance	Ketone produced from the metabolism of non-esterified fatty acids (NEFAs). It is used as a sensitive indicator for negative energy balance in all species but especially in ruminants.
Non-esterified fatty acids (NEFAs)	Energy balance	The main source of NEFAs is the breakdown of fat making it one of the most sensitive biochemical markers of negative energy balance.

Summaries of the clinical significance of each parameter were obtained from Cornell University's College of Veterinary Medicine's eClinPath website (https://eclinpath.com/).

failure is rare in wildlife except in instances of severe malnutrition or disease. Immune function is highly dependent on energy balance (Van Noordwijk and de Jong, 1986), therefore we did not expect immune-related parameters to improve upon survival predictions derived from indicators of energy balance.

To evaluate our hypotheses, we collected a blood sample from each individual in the study population in January or February of each study year and then monitored adult and juvenile survival through August. Our study design was made possible by an intensive monitoring program carried out by the Oregon Department of Fish and Wildlife (ODFW) and Nevada Department of Wildlife (NDOW), but this type of monitoring is not feasible in all bighorn sheep populations. However, periodic capture and blood sampling of individuals is a common management practice even in populations that are not intensively monitored. A simple biochemical indicator of adult and juvenile survival probability could therefore provide a useful tool for large-scale monitoring of bighorn sheep in populations where intensive, long-term monitoring is not possible.

Methods

Study site

Our study encompassed seven bighorn sheep populations, all located in southeastern Oregon and northern Nevada, United States (Figure 1). The populations included the Blue Mountain (BSP), Trout Creek (TCP), Bowden Hills (BHP), Rattlesnakes (RSP), Ten Mile (TMP), Santa Rosa Population (SRP), and the Upper Owyhee (UOP) populations in Oregon and Nevada. Elevation across the study area ranged from approximately 1,050 m in the Owyhee Canyon to 2,957 m in the Santa Rosa Mountains. Mean precipitation for the study area was approximately 22.5–35.0 cm *per annum* (Omernik and Griffith, 2014). The study area terrain types included elevated plateaus, sheer-walled canyons with intermittent lakes and ephemeral streams, and mountains of low to mid-elevation with primarily steep slopes and ephemeral or perennial streams (Omernik and Griffith, 2014). For more detailed information on geology, vegetation, wildlife, and land use practices, please refer to Spaan (2022).

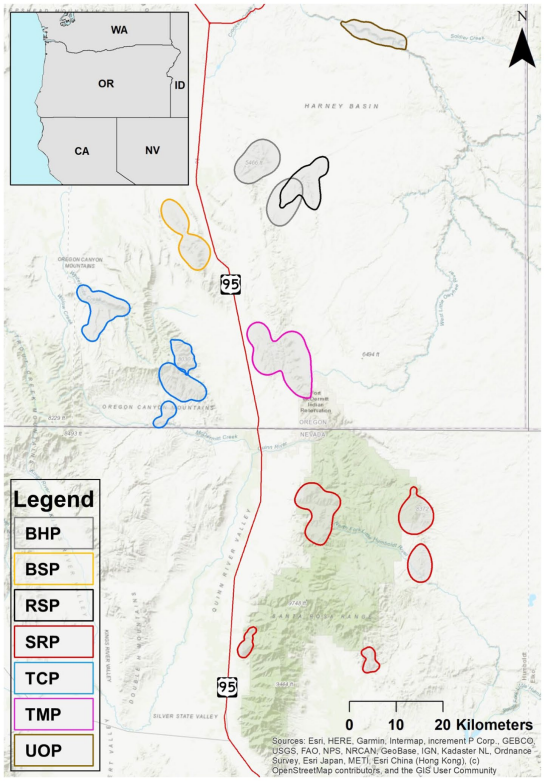

FIGURE 1
Bighorn sheep in this study were from 7 populations in southeastern Oregon and Northern Nevada. Polygons shown here for each population indicate female bighorn sheep 95% summer utilization distributions from Spaan (2022). Abbreviations are: Blue Mountain (BSP), Trout Creek (TCP), Bowden Hills (BHP), Rattlesnakes (RSP), Ten Mile (TMP), Santa Rosa Population (SRP), and the Upper Owyhee (UOP) populations.

Capture of bighorn sheep

All capture, handling, and disease testing were conducted by Oregon Department of Fisheries and Wildlife (ODFW) and Nevada Department of Wildlife (NDOW). Capture methodology followed the recommendations of Foster (2004) and the American Society of Mammalogists (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists, 2016). ODFW and NDOW captured, collared, and sampled adult bighorn sheep across the 7 populations between January 2016 and February 2018. Captures were conducted using a net gun fired from a helicopter, with individual bighorn sheep blindfolded and hobbled once captured (Krausman et al., 1985). Bighorn sheep were brought to a centralized area at the base of their range to be fitted with a telemetry collar and to collect

biological samples, except where capture location was too far from basecamp to transport them quickly, in which case they were field processed, at the capture location.

At capture, each adult female was fitted with a Vertex Survey Globalstar collar (Vectronic Aerospace, Berlin, Germany) and each adult male was fitted with one of two collars ($n = 41$ Vertex Survey Globalstar collars; $n = 5$ with Telonics Globalstar collars). The age of each adult was estimated from horn growth rings (Geist, 1966; Hoefs and Konig, 1984). Blood was obtained via jugular venipuncture into a vacutainer tube with no additive and placed on ice for transport back to the mobile laboratory within 12 h.

Laboratory analyses from capture samples

At the mobile laboratory the no-additive tubes containing whole blood were spun at 5000xg for 10 min to separate the serum. The serum was pipetted into sterile cryotubes and frozen in liquid nitrogen for transport back to Oregon State University. The large animal metabolic chemistry panels were run at the Veterinary Diagnostic Laboratory of the Carlson College of Veterinary Medicine at Oregon State University[1] on an automated analyzer from the frozen serum in one batch each year to detect all analytes shown in Table 1.

Population-level *M. ovipneumoniae* presence was determined as described in Spaan et al. (2021). Briefly, testing of adult sheep was performed at Washington Animal Disease Diagnostic Laboratory (WADDL) using PCR (Manlove et al., 2019) and cELISA (Ziegler et al., 2014). Testing of juvenile sheep was done opportunistically, where dead juveniles were located in a population and samples (including either the entire corpse, heart, liver, lungs, the head, nasal and ear swabs, and/or tissue samples depending on the state of decomposition) were submitted to WADDL for gross-and histo-pathology on lung tissue sample and PCR tests on swabs. Similarly to Spaan et al. (2021), for survival analyses, each juvenile bighorn sheep was encoded as *M. ovipneumoniae*-exposed if it came from a population with dead *M. ovipneumoniae* juveniles in the same year.

Monitoring of bighorn sheep

The GPS collars fitted to the adults provide a GPS location every 13 h and were set to report a mortality if stationary for 12 h, allowing us to accurately determine mortality date for each adult. Each collar also transmitted its own unique VHF frequency, with the occasional duplicate placed on individuals in different populations between which dispersal was deemed unlikely. The collars were also fitted with colored tags with unique numbers, allowing for identification of individuals observed in the field.

From 2016 to 2018, as described in Spaan et al. (2021), we conducted semi-monthly observations of all collared adult females between April 1 and August 31 to assess the survival of their juvenile. Juvenile identification was determined via observation of physical contact between adult females and juveniles, such as nursing or bedding down together. Juveniles are weaned at approximately 4 months of age (Festa-Bianchet, 1988); thus, our observation period was intended to cover birth through weaning. We located adult females for observation using an R-1000 telemetry receiver fitted with an RA-23 K VHF directional antenna (Telonics, Inc., Mesa, AZ). We conducted observations with Kowa TSN-601 spotting scopes fitted with a 20–60x magnification mounted on tripods. Once an adult female was confirmed to not or no longer have a juvenile, i.e., two consecutive observations where the adult female was observed without a juvenile, we stopped tracking that individual adult female (e.g., Cassirer and Sinclair, 2007).

Statistical analyses

We conducted the following analyses separately for adult bighorn sheep and juveniles. We estimated survival functions of adults and juveniles using Kaplan–Meier survival estimates (Kaplan and Meier, 1958) using the R package survival (Therneau, 2021). For an initial selection of covariates for model selection, we used extreme gradient boosting (Chen and Guestrin, 2016) in the package xgboost (Chen et al., 2021) to estimate the relative importance of multiple physiological parameters (Supplementary Appendix I) in predicting binary outcomes of known deaths vs. survival or unknown fates. We chose covariates with a relative importance greater than 0.05 to use in the model selection process for Cox proportional hazards models (Cox, 1972) using right-censored data to estimate correlates of selected covariates with survival. We calculated the pairwise Pearson's correlation coefficient for all selected covariates, and using a correlation threshold of 0.5, we chose one of two correlated variables for the next model selection step. Using an exhaustive model selection process, we compared the Akaike's information criteria (AIC) score for each model that included sex, population, and age for adult survival, and population and adult female's age for juvenile survival.

With the most parsimonious models for adult and juvenile survival, we used the Cox proportional hazards (hereafter Coxph) model to investigate the association between survival time and predictor covariates identified in the previous analysis. Only complete cases (i.e., no missing data) were used in the Coxph models. We used survival data for eight observation periods, each lasting approximately 2 weeks. We used an alpha value of 0.05 for thresholds of model and parameter significance, and after Bonferroni correction for multiple comparisons using the same data (two tests), we used an alpha value of 0.025. For visualization of variation in hazard ratio in relation to individual continuous covariates, we used the R packages rankhazard (Karvanen and Koski, 2016) and smoothHR (Araújo and Meira-Machado, 2022) to examine relationships between individual covariates and survival. All analyses were performed in R version 3.6.3 (R Core Team, 2020).

Results

We observed the survival outcomes of 129 adult sheep (82 female, 47 male) and 61 juveniles across seven populations during the study period (Figure 2). Adult estimates of survival over the study period were high (0.92, Figure 3) while juvenile estimates of survival were relatively low (0.44, Figure 3).

1 https://vetmed.oregonstate.edu/ovdl/test-catalog

10.3389/fevo.2023.1106635

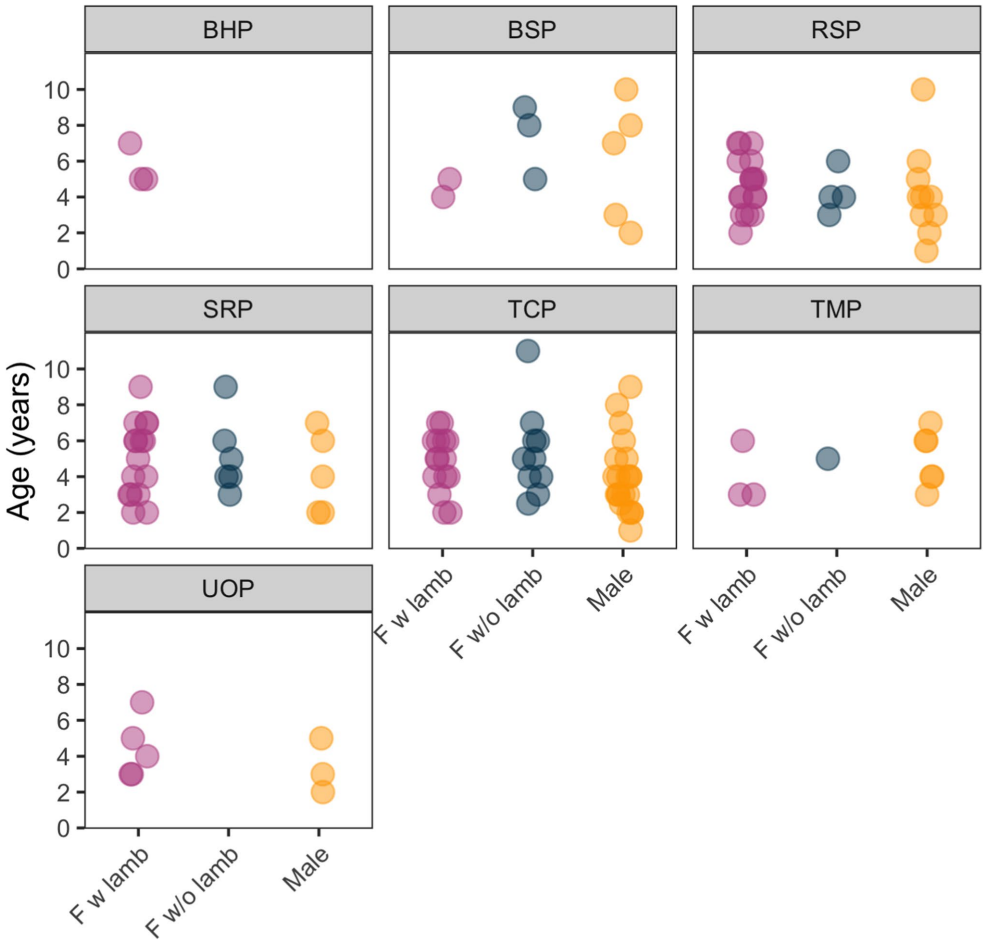

FIGURE 2
Sample sizes and demographic distribution of Bighorn Sheep (*Ovis canadensis*) in seven populations in Nevada and Oregon, United States. Sample sizes for juveniles are reflected in "F with lamb" categories; i.e., each pink point represents two individuals used in this study (a female and its juvenile), blue points represent one female without a juvenile, and yellow points represent one male.

Correlates of adult survival

Covariates considered are listed in Table 1. The covariates that were important in predicting survival in the extreme gradient boosting analysis (Supplementary Appendix I) were age, P (phosphate), total CO_2, creatinine, and β-HBA, cholesterol and Cl (chloride). P and TCO_2 were correlated with each other (Supplementary Appendix VII) so we did not include both in the Coxph model. We chose to use P, because the distribution of TCO_2 data for bighorn sheep with different survival outcomes was highly variable (Supplementary Appendix II). However, we reported results from models including TCO_2 to inform future studies (Supplementary Appendix V). Cholesterol and the total number of monocytes had >0.05 relative importance in predicting binary sheep survival (Supplementary Appendix I), but inclusion of these covariates substantially lowered sample size of full cases ($n = 125$ to 95), therefore we left them out of the Coxph model selection step. The most parsimonious Coxph model included P, creatinine, and β-HBA (Supplementary Appendix III). We observed a significant and positive correlation between P and bighorn sheep mortality (Table 2; Figure 4). Age was also significantly and positively correlated with bighorn sheep mortality (Table 2). The effect size of age on adult

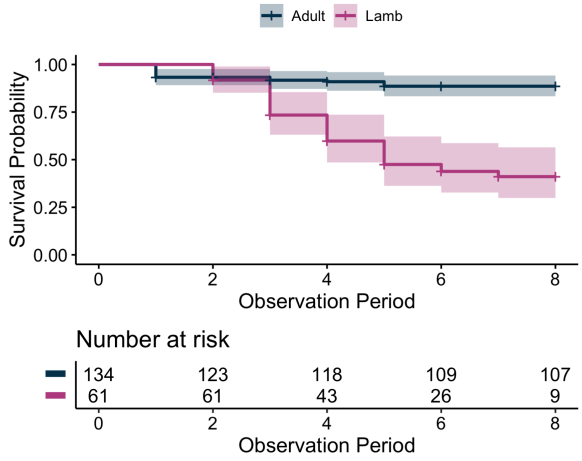

FIGURE 3
Kaplan–Meier survival estimates and sample sizes for adult bighorn sheep and juveniles across 8 observation periods. Each observation period lasted approximately two weeks.

TABLE 2 Results of Cox proportional hazard models for adult bighorn sheep (*Ovis canadensis*).

Covariate	Coefficient	*z*	Pr(>|z|)
Age	**0.485**	**2.618**	****0.009**
PopBSP	20.065	0.000	1.000
PopRSP	0.750	0.000	1.000
PopSRP	21.064	0.000	1.000
PopTCP	19.242	0.000	1.000
PopTMP	21.733	0.000	1.000
PopUOP	−0.349	0.000	1.000
Sex_male	−1.301	−1.007	0.314
P (phosphate)	**0.845**	**2.214**	****0.027**
Creatinine	1.139	0.829	0.407
β-HBA	−1.021	−1.178	0.239

Covariates significantly correlated with survival are bolded. "Pop" represents categorical variables for different populations. Overall Cox proportional hazards model significant (Likelihood ratio test = 29.66, DF = 11, $p = 0.002$). Four of the observations were removed due to missing data, resulting in $n = 125$ with 10 deaths. Coefficients can be exponentiated to produce the hazard ratio (i.e., effect size) of covariates.

mortality was 1.62 (95% CI 1.12–2.33), indicating that a one-year increase in age was associated with a 1.62 increase in the chances of mortality. The effect size of P on adult mortality was 2.29 (95% CI 1.05–4.98), indicating that a 1 mg/dL increase in P was associated with a 2.29 increase in the chances of mortality.

Correlates of juvenile survival

Covariates considered are listed in Table 1. The covariates that were important in predicting juvenile survival in the extreme gradient boosting analysis (Supplementary Appendix 1) were the presence/absence of *M. ovipneumoniae*, adult female β-HBA, adult female K (potassium), total number of adult female monocytes and total number of adult female neutrophils. None of these covariates were correlated with each other (Supplementary Appendix VII). The total number of adult female monocytes and neutrophils had >0.05 relative importance in predicting binary juvenile survival (Supplementary Appendix I), but inclusion of these covariates substantially lowered sample size of full cases ($N = 59$ to 46) for juveniles, therefore we left them out of the Coxph model selection step. The most parsimonious Coxph model included all three covariates (*M. ovipneumoniae*, adult female's β-HBA and K, Supplementary Appendix IV). We observed a significant and positive correlation with adult females' β-HBA and juvenile mortality (Table 3; Figure 5). When the β-HBA outlier was removed, the overall result remained unchanged (Supplementary Appendix VI). With the full dataset, the effect size of β-HBA was 1.83 (95% CI 1.02–3.28), indicating that a 1 mg/dL increase in maternal β-HBA was associated with a 1.83 increase in the chances of juvenile mortality.

Discussion

Maternal β-HBA, an indicator of negative energy balance in ruminants, was a significant predictor of bighorn sheep juvenile mortality during the 16 weeks after sampling in our study. Because β-HBA supplies energy to tissues during periods of caloric deficit and is a regulator of milk fat production (Zhang et al., 2015), this metabolite may be an important biochemical mediator of the trade-off between adult versus offspring survival in k-selected ruminant species. Our findings align with previous work in Svalbard reindeer, where maternal β-HBA concentration was also associated with reduced juvenile survival (Milner et al., 2003). Together, results from these studies suggest that maternal β-HBA could be a consistent indicator of stage-specific survival probability in capital breeders. In species where population dynamics are largely determined by juvenile survival rates, the ability to predict offspring survival rates from a simple biochemical indicator could contribute to large-scale monitoring efforts and facilitate targeted conservation actions.

In contrast to juvenile survival, serum concentration of β-HBA was not a significant predictor of adult survival, possibly because it is an indicator of current energy balance and does not directly correspond to total energy stores. Body mass and fat percentage, which are more direct measures of stored energy, have previously been found to correlate with overwinter survival of bighorn adult females (Festa-Bianchet et al.,

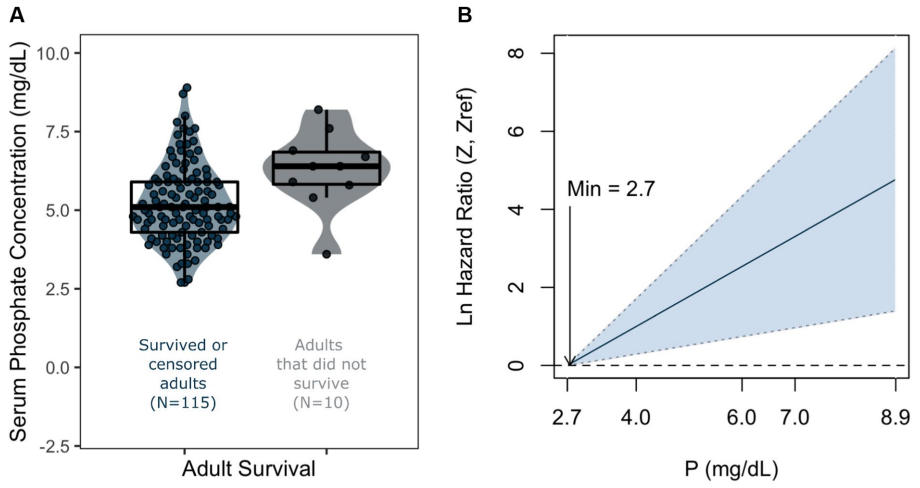

FIGURE 4
Violin plots overlaid with box plots of phosphate (P) in adult bighorn sheep, grouped by survival outcome **(A)**, and corresponding smoothed hazard functions of the same variables with 95% confidence intervals for adults **(B)**. Minimum values refer to values of x when the natural log of hazard ratio is 0. Violin plots indicate probability density of the raw phosphate data points, and boxplots indicate median and interquartile range of the same data points.

TABLE 3 Results of Cox proportional hazard models for juvenile bighorn sheep (*Ovis canadensis*, Likelihood ratio test=26.66, DF=9, p=0.002).

Covariate	Coefficient	z	Pr(>|z|)
adult female B-HBA	**0.455**	**2.400**	****0.016**
Juvenile *Mycoplasma*	−0.329	−0.320	0.749
adult female K	0.060	0.212	0.832
adult female age	−0.039	−0.372	0.710
PopBSP	1.704	1.302	0.193
PopRSP	0.996	1.308	0.191
PopSRP	−1.073	−1.304	0.192
PopTCP	−1.397	−1.499	0.134
PopTMP	0.482	0.448	0.654
PopUOP	NA	NA	NA

The covariate significantly correlated with survival is bolded. "Pop" represents categorical variables for different populations. Two of the observations were removed due to missing data, resulting in n = 59 with 33 deaths. Coefficients can be exponentiated to produce the hazard ratio (i.e., effect size) of covariates.

1997; Denryter et al., 2022). However, we only monitored adult survival from January or February through August, so it is unknown how the short-term measures of energy balance in our study might correlate with overwinter survival. Among the biochemical parameters that we measured, serum phosphate concentration was the only significant predictor of adult survival. Adult ruminants excrete most of their phosphorus as phosphate in saliva to support rumen microorganisms and to buffer rumen pH (Grünberg, 2014), therefore increased serum phosphate could indicate decreased saliva production due to dehydration. Dehydration could reflect a number of factors such as capture stress, underlying disease, or unknown environmental stressors that decrease probability of survival in adults.

Survival probability for juveniles was only 44% over the 16 week monitoring period of our study, compared with 92% survival in adults. This highlights the extreme vulnerability of bighorn sheep juveniles and the important role of juvenile mortality rates in driving population dynamics. In addition to maternal energy balance, juvenile mortality is known to be related to *M. ovipneumoniae* infection in our study system. Using data from the same populations, Spaan et al. (2021) found that the populations without *M. ovipneumoniae* have juvenile survival rates

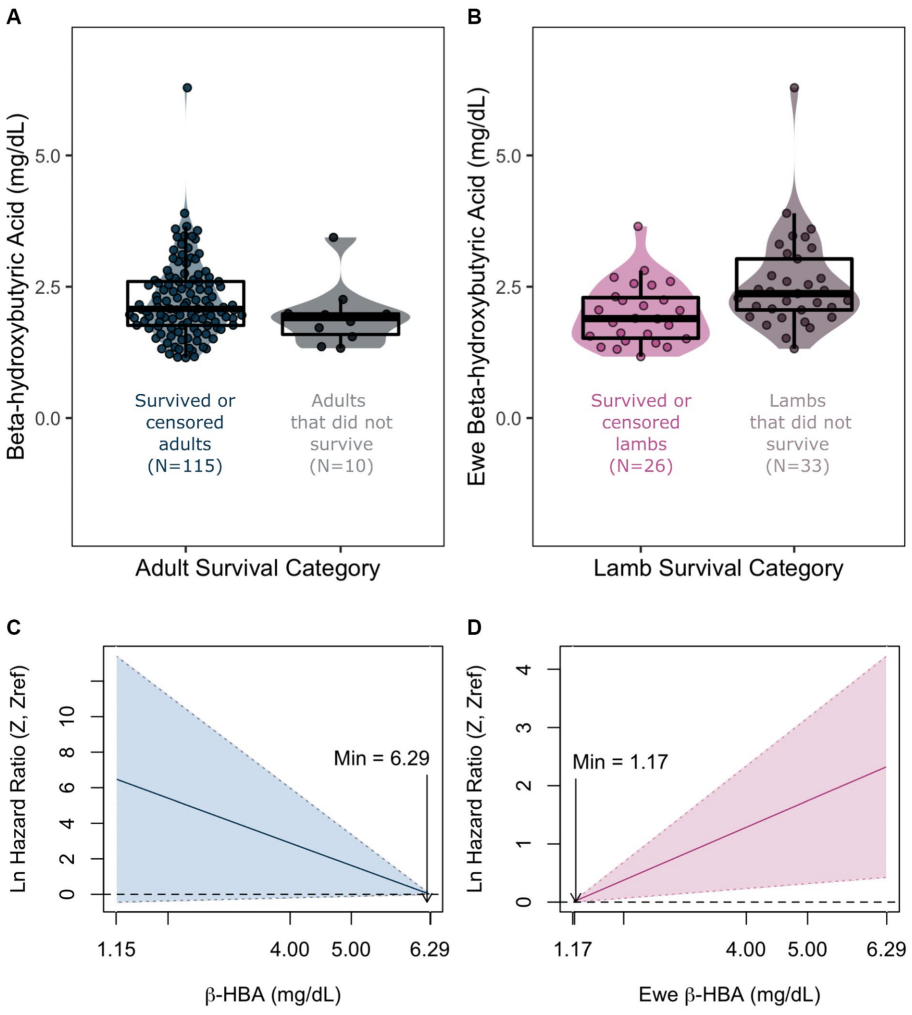

FIGURE 5
Violin plots overlaid with box plots of beta-hydroxybutyric acid (β-HBA) in adult bighorn sheep **(A)** and maternal β-HBA in juveniles **(B)**, grouped by survival outcome. Corresponding smoothed hazard functions of the same variables with 95% confidence intervals for adults **(C)** and juveniles **(D)**. Minimum values refer to values of x when the natural log of hazard ratio is 0. Violin plots indicate probability density of the raw β-HBA data points, and boxplots indicate median and interquartile range of the same data points.

that are 20 times higher than populations with *M. ovipneumoniae*. Similarly, our analysis found that *M. ovipneumoniae* presence in the population was important in predicting binary outcomes of juvenile survival, although not statistically significant in Cox survival models once we controlled for population (due to reduced sample sizes and population structure compared to Spaan et al., 2021). However, it is important to consider the effects of maternal energy balance in the context of disease, predation, and other environmental stressors on juvenile survival.

Our study found that maternal β-HBA, a simple biochemical indicator of negative energy balance, significantly predicted juvenile survival from late pregnancy through the first 4 months post-birth, but did not predict adult survival during the same period. This finding may represent a snapshot of the physiological processes involved in life history trade-offs of a k-selected herbivore species. Even when maternal body condition is high, short-term caloric deficit may be a sufficient trigger to decrease investment in offspring survival. This mechanism could protect adult females from investing heavily in juvenile survival when resources become too limited to support population growth. Our study suggests that β-HBA could be a powerful monitoring tool for bighorn sheep and other threatened capital breeding populations under resource limitation. However, maternal energy balance is only one factor that affects juvenile survival, and it must be interpreted in the context of environmental and disease factors that vary over time and among populations. For example, although we did not detect an interaction effect of *M. ovipneumoniae* and β-HBA on lamb survival in our study, such a relationship could manifest in other ecosystems or on different time scales. Additionally, the drivers of maternal energy balance in bighorn sheep are not fully understood. Previous research found that forage availability did not strongly influence juvenile survival, suggesting that competition or other behavioral traits might underlie the differences in maternal energy balance we observed in our study. Future research should seek to determine the relative importance of maternal energy balance, disease, and other variables for predicting juvenile survival, as well as the underlying causes of variation in maternal energy balance.

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 at: https://github.com/devansong/BHS.

Ethics statement

The animal study was reviewed and approved by Oregon State University Institutional Animal Care and Use Committee.

Author contributions

CL helped with data analysis design, interpreted finding, and led the writing. MG helped with early data analysis and participated in editing of drafts. AD-S performed the data analysis. JB, CC, and RS helped with data collection and interpretation. CE and BB advised students on the project, helped with data collection, and conceived of the project. 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

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Spatial and individual factors mediate the tissue burden of polycyclic aromatic hydrocarbons in adult and chick brown pelicans in the northern Gulf of Mexico

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The northern Gulf of Mexico supports a substantial level of oil and gas extraction in marine waters and experiences acute and chronic exposure to marine pollution events. The region also supports a diverse array of breeding and migratory seabirds that are exposed to these pollutants during foraging and other activities. Among the pollutants of highest concern within the region are polycyclic aromatic hydrocarbons (PAHs) which tend to be toxic, carcinogenic, mutagenic, or teratogenic. We assessed PAH loads in blood from adult brown pelicans and from feathers of adults and chicks of brown pelicans in relation to individual (e.g., body condition, sex) and spatial (e.g., breeding location within the Gulf, home range size, migration distance) factors. Of the 24 PAHs assessed, 17 occurred at least once among all samples. There were no PAHs found in chicks that were not also found in adults. Alkylated PAHs occurred more commonly and were measured at higher summed concentrations compared to parent PAHs in all samples, indicating that exposure to oil and/or byproducts of oil may have been a substantial source of PAH contamination for brown pelicans during this study. Within adults, PAHs were more likely to occur, and to increase in concentration, in blood samples of females compared to males, although no difference was found in feather samples. We also found that occurrence of and concentration of PAHs increased in adults that migrated longer distances. In adults and chicks, the background levels of oil and gas development within the region of the colony was not a consistent predictor of the presence of or concentration of PAHs. We also found correlations of PAHs with hematological and biochemical biomarkers that suggested compromised health. Our results indicate that both short- and long-term exposure (i.e., blood and feathers, respectively) are occurring for this species and that even nest-bound chicks can accumulate high levels of PAHs. Long-term tracking of PAHs, as well as an assessment of sublethal effects of PAHs

on pelicans, could enhance our understanding of the persistence and effects of this contaminant in the northern Gulf as could increasing the breadth of species studied.

KEYWORDS

body condition, brown pelicans, Gulf of Mexico (GOM), home range, migration, petrogenic, polycyclic aromatic hydrocarbons (PAHs)

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are among the pollutants of highest concern for wildlife and ecosystem health (Eisler, 1987). Though often associated with catastrophic events such as oil spills, PAHs are also ubiquitous background contaminants in both aquatic and terrestrial environments (Marvin et al., 2021). In the United States, 16 PAHs are listed as priority environmental pollutants because of their persistence through time, and their propensity to be carcinogenic to humans (Menzie et al., 1992). In wildlife, even relatively low exposure to PAHs can negatively affect growth and metabolism, blood biochemistry, liver health, and immunity (Briggs et al., 1996; King et al., 2021). Parent PAHs, which typically have higher molecular weights usually originate from pyrogenic sources, tend to have higher bioavailability in the marine environment, and are acutely toxic, whereas alkylated PAHs with lower molecular weights tend to be carcinogenic, mutagenic, or teratogenic (Eisler, 1987; Albers, 2006). Even trace exposure to PAH residue can lead to elevated PAH levels, as can exposure to chronic spills and produced waters (King et al., 2021). PAHs tend not to biomagnify or bioaccumulate, however, because most vertebrates can metabolize PAHs within weeks of ingestion (Albers, 2006; Hylland, 2006; Nfon et al., 2008). Nonetheless, both parent (PAR) and alkylated (ALK) PAHs can still accumulate in body tissues at low concentrations when the rate of uptake exceeds elimination, with potentially detrimental effects (Albers, 2003; Laffon et al., 2006; Champoux et al., 2020). Documenting PAHs in wildlife populations therefore requires intensive sampling to detect exposure pathways and trends while accounting for individual, spatial, and temporal variation (Nicholson et al., 2023).

In mobile wildlife, assessing PAH burdens requires sampling across a wide spatial range. This is particularly true in marine systems, in which habitat features are highly dynamic and contaminants can be transported long distances from their sources. The two primary routes of PAH contamination for wildlife are through direct physical interaction (i.e., contact) or foraging on a contaminated item (i.e., ingestion). Wildlife can be exposed to PAHs during daily activities and behaviors such as foraging, movement within and between habitat patches, and bouts of inactivity or resting (e.g., as they come into contact with a contaminated surface). Across the annual cycle, individuals from migratory populations can be exposed to contaminants across a wide spatial range that might also include different ecosystems, food sources, and regulatory environments (i.e., different political jurisdictions with different regulations pertaining to pollutants) (Seegar et al., 2015; Paruk et al., 2016; Champoux et al., 2020). For example, when individuals within a population vary in their migratory strategies or destination (i.e., partial migration), exposure to contaminants may be more difficult to assess or predict for individuals within a population because of the difficulty in knowing which individuals migrate and the locations to which they migrate (Lamb et al., 2017b; Wilkinson and Jodice, 2023). Outside of the migration period, exposure also can be difficult to assess if individuals have extensive home ranges and if the size of the home range is not consistent among populations (Lamb et al., 2017b, 2020). Evaluating the effects of environmental contaminants that are temporally or spatially heterogeneous on mobile wildlife therefore requires detailed data on movement patterns, behavior, and foraging activities of the population in question, as well as background information on the potential sources of contamination. Hence, sampling that is spatially and temporally both intensive and extensive is often required.

The northern Gulf of Mexico is a heavily industrialized sea particularly with respect to oil and gas extraction. The region has been under development for marine energy extraction since the early 1940s, and currently there are ~ 3,200 active rigs[1] along with ~42k km of pipelines and numerous shipping ports throughout the region. Oil and gas activity is heterogeneous across the northern Gulf; most offshore platforms and pipelines are concentrated in the central and western Gulf along the coasts of Louisiana, Alabama, Mississippi, and Texas, while the eastern Gulf along the Florida coast remains undeveloped (**Figure 1**). Due to the substantial levels of oil and gas activity in the region, the northern Gulf also experiences both acute and chronic levels of oil pollution. While spills that are large in quantity and are spatially and temporally extensive tend to be the focus of most research, monitoring, and mitigation efforts, the many small spills that occur in the region also impact the estuarine, coastal, and marine habitats of the Gulf. For example, from 1972- to 2017, 149 spills occurred in the region with only eight of these classified as large (>1,000 barrels) spills (Absg Consulting Inc, 2018). The release of produced waters, which contain byproducts of oil extraction, are also a source of petrochemical pollution (Beyer et al., 2020). Between 2000 and 2015, ~558 million barrels of produced oil were released annually into the northern Gulf (Bureau of Ocean Energy Management [BOEM], 2016). Furthermore, ~1,600 natural oil seeps occur throughout the region, tending to be most common in the central area of the northern Gulf (O'Reilly et al., 2022). Given this extent of oil exposure, the impact of oiling and its resulting contamination, including PAHs has become a

1 https://www.ncei.noaa.gov/maps/gulf-data-atlas/atlas.htm

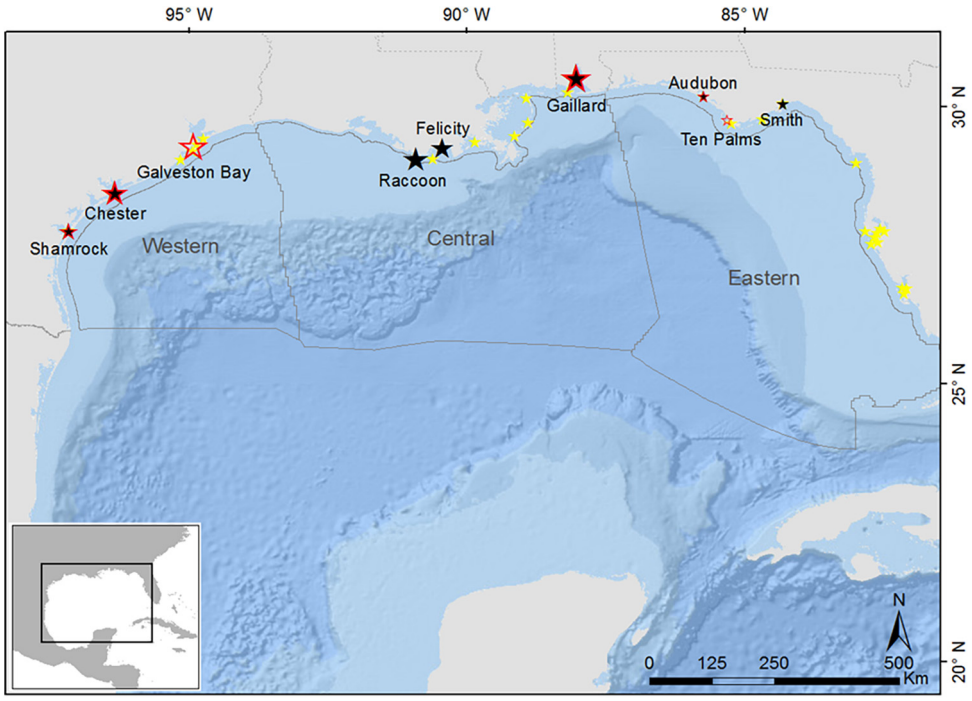

FIGURE 1
Location of Brown Pelican colonies in the northern Gulf of Mexico. Adults were sampled at colonies marked by a black symbol and chicks were sampled at colonies marked by a red-outlined symbol. Yellow symbols represent colonies not sampled. Size of symbol is relative to colony size (75–5,000 nesting pairs). The three planning areas of the Bureau of Ocean Energy Management (BOEM) are designated. Oil and gas activity is highest in the central, intermediate in the West, and least in the East (Base layer: ESRI, DeLorme, GEBCO, NOAA, NGDC, and other contributors).

primary focus of research and management activities in the region (Fallon et al., 2018; Paruk et al., 2021; Polidoro et al., 2021; Michael et al., 2022; Woodyard et al., 2022).

The brown pelican (*Pelecanus occidentalis*) is a high-profile, nearshore seabird that breeds within the northern Gulf of Mexico and throughout the southeastern USA. Due to their documented sensitivity to contaminants and other environmental stressors (both natural and anthropogenic), brown pelicans are recognized as a high priority species for monitoring and research in the Gulf of Mexico (Jodice et al., 2019). For example, the species was reduced to near-extinction by exposure to dichlorodiphenyltrichloroethane (DDT) and other toxic contaminants during the mid-twentieth century (McNease et al., 1992) and continues to experience detrimental effects at the individual and population levels from oil exposure and other environmental stressors (U.S. Fish and Wildlife Service, 2011; Walter et al., 2013; Deepwater Horizon Natural Resource Damage Assessment Trustees, 2016; Fallon et al., 2018; Jodice et al., 2019). The species, therefore, has been and continues to be one of focus for assessing stressors in this geographic area (Jodice et al., 2019). As part of a larger effort to explore various aspects of the ecology and conservation of the species in the northern Gulf (Lamb et al., 2020), we set out to establish baseline levels of health and contaminant exposure for the species. Our prior research (Jodice et al., 2022) indicates that baseline indices of health and condition of pelicans do not consistently match the levels of oil and gas development in the breeding region, suggesting other factors played a role in the physiology of these individuals. PAH exposure could play an important role in mediating physiological parameters. Little is currently known, however, about underlying PAH levels in brown pelicans, nor how individual variability in movement may impact exposure. For example, home range sizes vary substantially across the northern Gulf during the breeding season and, because the species exhibits partial migration, the spatial extent of movements during the non-breeding season can vary greatly as well (Lamb et al., 2017b, 2020).

In this paper, we expand on prior efforts to document baseline health and condition of brown pelicans in the northern Gulf of Mexico by examining the levels of PAH contamination in breeding adult pelicans and chicks across a gradient of oil and gas development within the northern Gulf. Because the amount of contamination and the repercussions of contact with a contaminant can differ with the route of contamination, we measured the levels of PAHs in the blood of adults and in the feathers of adults and chicks. Blood samples provide a recent assessment of exposure and represent substances currently circulating within an individual (Paruk et al., 2014, 2016). PAHs in blood likely derive from consumption of contaminated prey or ingestion of preen oil, while bioaccumulation through food webs is thought to be low (Paruk et al., 2014, 2016; Acampora et al., 2018). PAHs detected from adult feathers, however, may provide a different spatial, temporal, or source signature compared to PAHs detected in blood or other fluids (e.g., preen oil) that turnover relatively quickly (Jaspers et al., 2004; Acampora et al., 2018). For example, sources of PAHs in feathers may include blood supply (during feather growth), contact with the external environment, or contact with contaminated preen oil (Jaspers et al., 2004, Acampora et al., 2018). Feather samples provide an assessment of local and/or transboundary contamination in migratory species with a longer

temporal signature compared to blood (Acampora et al., 2018). Multiple tissues also respond to different exposure pathways and offer a wider temporal frame of reference, with blood representing more recent exposure (e.g., contamination during the breeding season proximal to breeding colonies) and feathers representing longer term exposure (e.g., migratory or post-breeding locations).

We assessed PAH levels in relation to spatial and individual factors including colony location (adults and chicks), individual attributes (adults and chicks), home range size (adults), migration distance (adults), and select blood biochemistry parameters indicative of health and hepatic damage (adults and chicks). We included both PAR and ALK PAHs in our analyses because petroleum studies have traditionally focused on parent PAHs, and exposure can be underestimated if both PAR and ALK are not assessed (King et al., 2021). We report on the sum of PAHs and not the detection levels of each individual PAH in each individual because our focus was on the total tissue burden among individuals and not necessarily the exact exposure for each individual PAH. By taking this approach, we sought to better understand the PAH tissue burden in brown pelicans throughout the region, which could subsequently enhance our ability to improve targeted mitigation efforts by linking exposure pathways across nesting, foraging, and non-breeding habitats. Ultimately, these data may improve our ability to predict which portions of the Gulf-wide metapopulation are likely to be affected by future contamination events.

TABLE 1 Study sites sampled for polycyclic aromatic hydrocarbons (PAHs) in adult and chick brown pelicans from breeding colonies in the northern Gulf of Mexico, 2013–2015.

Region/Colony location	Adult blood	Adult feathers	Chick feathers
Eastern planning area			
Audubon Island	■	■	■
Smith Island	■	■	
Ten Palms Island			■
Central planning area			
Gaillard Island	■	■	■
Felicity Island	■	■	
Racoon Island	■	■	
Western planning area			
Chester Island	■	■	
Shamrock Island	■	■	
Galveston Bay Colonies			■

Green fill within a cell indicated samples of that type were collected from that site during the study period. See Figure 1 for boundaries of planning areas and colony locations. Descriptions of the planning areas appear in methods.

Materials and methods

Ethics statement

This study is one component of a broader research program that was focused on the ecology of Brown Pelicans in the northern Gulf of Mexico (Lamb et al., 2020; Jodice et al., 2022). Research was authorized by permits from the Clemson University Animal Care and Use Committee (2013-026), U.S. Geological Survey Bird Banding Lab (#22408), Texas Parks and Wildlife (SPR-0113-005), Audubon Texas, The Nature Conservancy in Texas, Louisiana Department of Wildlife and Fisheries (LNHP-13-058 and LNHP-14-045), and the Florida Fish and Wildlife Conservation Commission (LSSC-13-00005).

Study area

Study sites extended from the Florida panhandle to the Texas coast and represent each of the three planning areas designated by the Bureau of Ocean Energy Management (BOEM; the federal agency responsible for managing oil and gas activities in U.S. waters). These are defined as the Eastern Planning Area (EPA), Central Planning Area (CPA), and Western Planning Area (WPA). The CPA contains the highest level of oil and gas infrastructure (platforms and pipelines) among the three, while the WPA is moderately developed and the EPA is undeveloped. We collected samples from nine sites throughout the study area (Figure 1), although we did not sample both age classes or both tissue types from all sites (Table 1). We sampled adults from seven colonies; Audubon and Smith islands, Florida (EPA); Gaillard Island, Alabama (CPA); Felicity and Raccoon islands, Louisiana (CPA); and Chester and Shamrock islands, Texas (WPA) (Figure 1). We sampled chicks from seven colonies: Audubon and Ten Palms islands, Florida (EPA); Gaillard Island, Alabama (CPA); Marker 52 and North Deer (regrouped as Galveston Bay colonies), Chester, and Shamrock islands, Texas (WPA). Data were analyzed at the spatial scale of the planning areas (i.e., not at the colony level) due to limited samples from some colonies. All study colonies and planning areas were, however, within a single marine ecoregion (Northern Gulf of Mexico within the Temperate North Atlantic Realm: Spalding et al., 2007).

Sampling

Adult brown pelicans were sampled from active nests during the breeding seasons of 2013–2015. Adults were captured on nests using leg nooses during the late incubation and early chick-rearing stages. If eggs were present in the nest, they were replaced with porcelain eggs during capture to prevent damage. If chicks were present, they were moved to the nest edge to avoid injury. Median handling time was 17.5 min (range = 11–35 min) from capture to release. After release, we observed individuals for several minutes to ensure that they displayed normal flight, swimming, and balance capabilities. Observation methods and results are described in detail in Lamb et al. (2020). We collected 3–4 scapular feathers from 92 breeding adults. We collected blood samples within 2 min of capture from the tarsometatarsal vein. After sterilizing the collection site, we collected a 5 mL blood sample using a 23-gauge needle and VacuTainer tube (Becton Dickinson, Franklin Lakes, NJ, United States) with lithium heparin anticoagulant. We stored samples over cold packs until returning from the field (~5–10 h), after which we centrifuged samples, separated red blood cells from

plasma, and froze samples for storage until lab analyses could be completed. For PAH analysis, we included all adult feather samples (N = 92) as well as red blood cell samples from up to five randomly selected individuals per colony (N = 33).

Pelican chicks were captured by hand at or near nest sites. We collected ~4 scapular feathers from 606 brown pelican chicks during the breeding season of 2014–2015 of which we randomly selected five individuals per colony (N = 35) for PAH analysis. Since feathers in young chicks represent approximately the same exposure period as blood samples (i.e., the weeks preceding capture), we did not collect blood from chicks for PAH analysis.

For adults and chicks, we measured body mass (±50 g), culmen length (±1 mm), tarsus length (±1 mm), and wing length (±5 mm) and created a body condition index (BCI) specifically for the individuals used in this study. The BCI provides an index for the mass of the bird in relation to its size and is calculated as the residual of the linear relationship between mass and culmen length (Lamb et al., 2017a). In brown pelicans, sex cannot be easily determined *in situ*. Therefore, the distribution of samples between sexes is opportunistic. Sex of adults was determined from collected blood samples through PCR amplification of sex-linked molecular markers (Itoh et al., 2001). We did not sex chicks.

Sample processing

Analyses of polycyclic hydrocarbons were conducted at the University of Connecticut Center for Environmental Sciences and Engineering (Storrs, Connecticut). In the lab, we weighed 0.2 g of blood sample into a 1.5 mL plastic centrifuge tube. Samples were spiked with quality control standard solutions and vortexed for 1 min at 2,500 rounds per minute. Methanol or acetonitrile (500 μL) were added to each tube along with $MgSO_4$. Samples were then vortexed for 5 min at 2,500 rounds per minute, then centrifuged for 10 min at 14,000 rounds per minute. Next, 190 μL of the supernatant were transferred to a 300 μL liquid-chromatography vial. These samples were then spiked with an internal standard and vortexed.

Following extraction, the samples were analyzed for alkylated PAHs using an Agilent 6890 gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, United States) equipped with a Restek Rxi-5Sil MS column (Restek, Bellefonte, PA, USA; 30 m) using splitless injection coupled to a Waters QuattroMicro triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). Parent PAHs were quantified using a Waters Acquity ultra-performance liquid chromatograph (UPLC; Waters Corporation, Milford, MA, USA) with fluorescence and photo diode array detection, which was equipped with an Acquity UPLC BEH C18 column (Waters Corporation, Milford, MA, USA; 1.7 μm, 2.1 × 100 mm). All peaks were quantified against the internal standard, and the extraction efficiency was evaluated using a surrogate standard of naphthalene-d8. Standard quality assurance procedures were employed, including analysis of duplicate samples, method blanks, post-digestion spiked samples, and laboratory control samples.

Feathers were washed three times in acetone, three times in high performance liquid chromatography water, and one additional time in acetone before allowing them to dry overnight (ca. 10 h). Feathers were weighed (±0.2 g) on folded weighing paper and transferred directly into the accelerated solvent extraction (ASE) cell using forceps when needed. Hydromatrix powder was added to pack the 11 mL ASE cells. Using gelatin as the matrix for the blank and laboratory control samples, a 0.2 g sample was weighed out and transferred to ASE cells. Samples were then spiked with quality control standards. ASE extracts were subsequently run and collected utilizing acetonitrile solvent, and the solution was transferred into the pre-marked conical evaporation vials and evaporated to just below 0.5 mL under a gentle nitrogen stream (set flowrate on N-Evap unit to 180 mL/min). Samples were spiked again with internal standard. The volume was then brought up to 500 μL with acetonitrile and vortexed for a few seconds to mix. Filtered samples were injected into liquid-chromatography vials using 1 mL plastic syringes and 4 mm, 0.2 μm syringe filter. The detection limit for all PAHs was 5 ng g^{-1} (i.e., parts per billion) and values for PAHs were reported as wet weight for blood and dry weight for feathers.

Statistical analyses

We assessed the concentration of PAHs in adult blood, adult feathers, and chick feathers via three dependent variables: the sum of all PAHs detected (sumPAH), the sum of Parent PAHs detected (sumPAR), and the sum of Alkylated PAHs detected (sumALK). We assessed the relationship for each with a suite of independent variables, including: BCI (adults and chicks; continuous), planning area (adults and chicks; WPA, CPA, or EPA with EPA set as the reference level), sex (adults; male of female), migration class (adults; categorical, see below) and home range size (adults; continuous). Methods for deployment of satellite tags, calculation of home range size, and calculation of migration distance are detailed in Lamb et al. (2017a,b, 2020). Home range was reported as the area of the 95% kernel density contour for any individual from which a sample was collected that was also equipped with a satellite transmitter (n = 64). We selected the 95% use area for PAH analysis rather than a core use area (e.g., 25 or 50%) because we assumed that exposure to PAHs could occur anywhere within an individual's habitat regardless of intensity of use. Migration distance was calculated as the distance between the center of breeding home range and the center of winter home range as determined from locations of satellite tagging (Lamb et al., 2017b, 2020), and classified as short (i.e., resident: < 200 km), medium (200–800 km), and long (>800 km). The migration class defined as long-distance was set as the reference level.

Because the concentration of PAHs was below detectable limits for many samples from both age classes and sample types (i.e., a high proportion of 0's in the data set, see Results), we analyzed our data using a hurdle model approach. A hierarchical hurdle model approach allows for the consideration of two ecological processes, one that may affect the occurrence of an exposure to a PAH contaminant (i.e., presence/absence) and another that may affect the dose or concentration of the PAH contaminant (i.e., abundance) (Zuur et al., 2009). For step one of the hurdle approach we used a binomial logistic regression with a log link function, using the presence (value > 0) or absence (value = 0, or undetectable) of each of the three PAH variables (sumPAH, sumPAR, sumALK)

as the response variable. For step two of the hurdle approach we used a generalized linear model (GLM) to assess the change in the concentration of PAHs only in tissue samples where PAHs exceeded detectable limits. We used a GLM with a gamma distribution and a log link function, with the sum of the concentration of each of the three PAH variables as the response variable.

We used the same set of candidate models in each of the two steps of the hurdle approach and compared the strength of the candidate models using Akaike's Information Criteria (AIC; Table 2). We built 12 models for analysis of adult blood samples, 16 models for adult feather samples, and three models for chick feather samples. Variables that were correlated were not included within the same model but each independent variable appeared in at least one model. We reported coefficient estimates from the top performing models if no other model was within two points of the model with the lowest AIC value, or averaged coefficient estimates if multiple models were supported (delta AIC $\leq$ 2.0 relative to the lowest AIC value, n = 2 cases). We provided odds ratios and confidence intervals on odds ratios for coefficient estimates ($\pm$SE) from binomial logistic models of PAH detection (the odds of a PAH being detected for a change in categorical levels, or for a one unit increase in a continuous variable) and from gamma models of PAH concentration where present (the odds of the concentration of a PAH increasing by 1 ng g^{-1} for a change in categorical levels, or for a one unit increase in a continuous variable).

Because exposure to PAHs can lead to sublethal effects, we also assessed the correlation between levels of exposure to all PAHs (sumPAH) and attributes that may reflect individual condition or health. We collected blood samples to assess hematological and physiological parameters from many of the same birds from which PAH was measured as part of a component study among these same pelican colonies (Jodice et al., 2022). We assessed the correlation of PAH exposure with total protein (g dL^{-1}), corticosterone (mg dL^{-1}), packed cell volume (PCV; %), heterophil count (10–3 ml^{-1}), lymphocyte count (10^{-3} ml^{-1}), and the ratio of heterophils to lymphocytes (H:L). We also assessed correlations with AST and CPK, two enzymes indicative of hepatic damage (Harr, 2002; Alonso-Alvarez et al., 2007). Laboratory methods for the measurement of these attributes are described fully in Jodice et al. (2022). Because the distribution of the sumPAH data was non-normal and not able to be transformed to a normal distribution, we used Kendall's tau to perform a non-parametric correlation. We correlated physiological markers with sumPAH in adult blood, sumPAH in adult feathers, and sumPAH in chick feathers.

Results

PAH profiles in adult blood

We analyzed blood for PAHs from 33 adult pelicans (Table 3). The most frequently occurring PAHs were of intermediate molecular weight (Figure 2). We failed to detect two alkylated compounds and eight parent compounds out of the full test set. The two most frequently detected PAHs were 1,3-dimethylnaphthalene and 2,3,5-trimethylnaphthalene. Of the 24 PAHs assessed, 14 were detected in at least one individual (Table 3). Alkylated PAHs were detected in 48% of individuals and parent PAHs were detected in 30% of individuals. Three alkylated compounds were each detected in >10% of individuals; 2,6-dimethyl naphthalene (n = 4), 1,3-dimethylnaphthalene (n = 5), and 2,3,5-trimethylnaphthalene (n = 6). Of 33 birds sampled, 21 (63.6%) had at least 1 PAH detected (1 PAH: n = 10; 2 PAHs: n = 10; four PAHs: n = 1). Parent

TABLE 2 Models used in an information theoretic approach to assess relationships among polycyclic aromatic hydrocarbons (PAHs) and independent variables for brown pelican adults and chicks sampled from breeding colonies in the northern Gulf of Mexico, 2013–2015.

	Variables included	Adult blood	Adult feathers	Chick feathers	Comments
Model 1	Sex	Yes	Yes	No	Sex not available for chicks
Model 2	BCI	Yes	Yes	Yes	–
Model 3	Planning area	Yes	Yes	Yes	–
Model 4	Home range size	Yes	Yes	No	Home range irrelevant for chicks
Model 5	Sex + BCI	Yes	Yes	No	Sex not available for chicks
Model 6	Sex + planning area	Yes	Yes	No	Two terms are related so not modeled together
Model 7	Sex + home range size	Yes	Yes	No	Home range irrelevant for chicks
Model 8	BCI + planning area	Yes	Yes	No	Two terms are related so not modeled together
Model 9	BCI + home range size	Yes	Yes	No	Home range irrelevant for chicks
Model 10	Planning area + home range size	Yes	Yes	No	Home range irrelevant for chicks
Model 11	Sex + BCI + planning area + home range size	Yes	Yes	No	See above
Model 12	Migration class	No	Yes	No	Temporal mismatch w/adult blood; not relevant for chicks
Model 13	Migration class + BCI	No	Yes	No	Temporal mismatch w/adult blood; not relevant for chicks
Model 14	migration class + sex	No	Yes	No	Temporal mismatch w/adult blood; not relevant for chicks
Model 15	Migration class + planning area	No	Yes	No	Temporal mismatch w/adult blood; not relevant for chicks
Model 16	Null model	Yes	Yes	Yes	Temporal mismatch w/adult blood; not relevant for chicks

Models were compared using Akaike's Information Criteria. BCI, body condition index (see methods for definition).

TABLE 3 Frequency of detection, range and sum of concentration (ng/g wet weight), and percent contribution to total burden of individual polycyclic aromatic hydrocarbons (PAHs) from blood of adult brown pelicans (n = 33) breeding in the northern Gulf of Mexico, 2013–2014.

	Number birds > detection limit, <detection limit	Range (ng/g wet weight)	Sum (Σ) of PAHs (ng/g wet weight) and (% contribution to total PAH burden from each compound)
Alkylated compounds			
2-methyl naphthalene	3, 30	80.7–86.3	252.9 (8.2%)
2,6-dimethyl naphthalene	4, 29	79.3–98.1	354.7 (11.5%)
1,3-dimethylnaphthalene	5, 28	82.8–128.1	545.2 (17.7%)
1,5-dimethylnaphthalene	0, 33	–	–
2,3,5-trimethylnaphthalene	6, 27	81.4–127.6	560.3 (18.2%)
1-methylfluorene	0, 33	–	–
3-methylphenanthrene	1, 32	78.0	78.0 (2.5%)
9-methylphenanthrene	2, 30	58.5–61.9	120.4 (3.9%)
Parent compounds			
Naphthalene	2, 31	83.9–102.2	186.1 (6.1%)
Acenaphthylene	0, 33	–	–
Acenaphthene	2, 31	102.2–108.5	210.7 (6.8%)
Fluorene	2, 31	161.1–165.4	326.6 (10.6%)
Phenanthrene	1, 32	48.9	48.9 (1.6%)
Anthracene	3, 29	73.9–84.3	238.7 (7.8%)
Fluoranthene	1, 32	42.4	42.4 (1.4%)
Pyrene	1, 32	50.0	50.0 (1.6%)
Benzo(a)anthracene	1, 32	57.8	57.8 (1.9%)
Chrysen	0, 33	–	–
Benzo(b)fluoranthene	0, 33	–	–
Enzo(k)fluoranthene	0, 33	–	–
Benzo(a)pyrene	0, 33	–	–
Indeno(1,2,3-cd)pyrene	0, 33	–	–
Dibenz(a,h)anthracene	0, 33	–	–
Benzo(g,h,i)perylene	0, 33	–	–

compounds were detected in 10 of 33 birds, alkylated compounds were detected in 16 of 33 birds, and both parent and alkylated PAHs were detected in 5 of 33 birds. PAHs were detected in 75% of females sampled and in 57% of males sampled.

Among adult pelicans with PAH concentrations above detectable limits, the sum of all PAHs ranged from 42.44 to 463.75 ng g^{-1}, the sum of alkylated PAHs ranged from 58.46 to 220.51 ng g^{-1}, and the sum of parent PAHs ranged from 42.44 to 245.47 ng g^{-1} in blood samples (Figure 2). There was no significant difference in the concentration of parent compared to alkylated PAHs in blood samples from adults using either the full data set or using only data from birds above detectable limits (Wilcoxon rank sum test $P > 0.13$ for each). When all individuals were considered, there was no correlation between summed concentrations of alkylated and parent PAHs (Kendall tau r = −0.05, Pearson r = −0.09). Similarly, when only individuals with detectable limits of PAHs were considered, there was no correlation between summed concentrations of alkylated and parent PAHs (Pearson r = 0.25). The individual with the highest concentration of summed parent PAHs (245.47 ng g^{-1}) did, however, present with the second highest level of alkylated PAHs (218.28 ng g^{-1}; female, Audubon Island, Florida). The PAH with the highest concentration detected was fluorene (165.4 and 161.1 ng g^{-1}; both birds from Audubon Island, Florida). The two most frequently detected PAHs (1,3-dimethylnaphthalene and 2,3,5-trimethylnaphthalene) also had the widest range in measured concentrations (∼80∼28 ng g^{-1}; Table 3).

Five models for the presence of sumPAH in pelican blood received support, and these included each of the main variables we assessed except planning area (Supplementary Table 1). There was little separation among these five models, and the most supported model only carried an AICc weight = 0.21. The only variable that appeared to have a measurable relationship with sumPAH was sex (model averaged coefficient estimate = −1.12 ± 0.95). Females were 3.1 times more likely to be detected with a PAH compared to males although there was substantial variability around the estimate (90% CI for odds ratio = 0.7, 16.7). The concentration of sumPAH in pelican blood was best described by a single model that included sex and home range size (Supplementary Table 1). There was substantial separation among the first and second

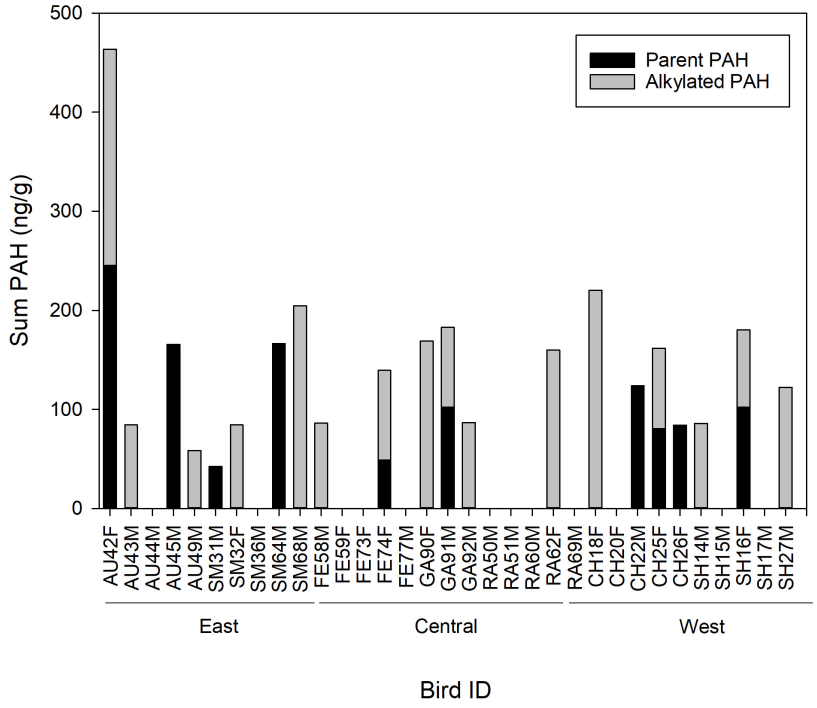

FIGURE 2
Concentration (ng g^{-1}; wet weight) of parent and alkylated polycyclic aromatic hydrocarbons (PAHs), and the sum of parent plus alkylated PAHs, in blood samples of adult brown pelicans breeding in the northern Gulf of Mexico, 2013–2015. Bird ID, two letter abbreviation for the colony of origin of the sample (AU, Audubon Island; SM, Smith Island; FE, Felicity Island; GA, Gaillard Island; RA. Raccoon Island; CH, Chester Island; SH, Shamrock Island; see Figure 1), a unique identification number, and F, Female or M, Male.

ranked model, and the most supported model carried 90% of the AICc weight. There was a strong relationship between sumPAH concentrations and sex (coefficient estimate = −0.73 ± 0.23). The odds of the sumPAH concentration increasing by 1 ng g^{-1} increased by 2.1 times for females compared to males (90% CI for odds ratio = 1.4, 3.0). There was a weak negative relationship between sumPAH concentrations and home range size which was strongly leveraged by a single individual and therefore discounted (coefficient estimate = −0.00022 ± 0.000099).

Three models for the presence of sumPAR in pelican blood received support, and these included the variables home range, sex, and BCI (Supplementary Table 2). There was little separation among these three models, with the most supported model only carrying an AICc weight = 0.28. Sex was weakly associated with sumPAR (coefficient estimate = −0.94 ± 0.89). Females were approximately 2.5 times more likely to be detected with a PAR PAH compared to males, although there was substantial variability around the estimate (90% CI for odds ratio = 0.6, 11.1). Coefficient estimates for home range size and BCI had standard errors that overlapped zero indicating little evidence of a statistical relationship. Four models for the concentration of sumPAR in pelican blood received support, and these included each of the main variables we assessed (Supplementary Table 1). There was little separation among these four models, and the most supported model carried an AICc weight = 0.38. The only variable with a measurable, yet weak, effect on sumPAR was planning area. Coefficient estimates indicated that the concentrations of sumPAR were lower in the CPA compared to the EPA (coefficient estimate = −0.70 ± 0.41; Figure 3). The odds of the sumPAR concentration increasing by 1 ng g^{-1} increased by 2.0 times for birds in the EPA compared to the CPA, although there was substantial variability around the estimate (90% CI for odds ratio = 1.0, 4.0).

Two models for the presence of sumALK in pelican blood received support, and these included the variables sex and home range size (Supplementary Table 3). There was little separation among these two models, and the most supported model was 1.5x as likely to be the best model compared to the second-ranked model. Sex had a measurable relationship with sumALK (coefficient estimate = −1.45 ± 0.91) while the coefficient estimate for home range had standard errors that overlapped zero indicating no relationship. Females were 4.3 times more likely to be detected with an ALK PAH compared to males, although there was substantial variability around the estimate (90% CI for odds ratio = 1.1, 25.0). Only the full model for the concentration of sumALK in pelican blood, which included each main variable and carried an AICc weight = 0.86, received support (Supplementary Table 3). Coefficient estimates indicated that the concentrations of ALK PAHs were likely to decrease in the CPA compared to the EPA (coefficient estimate = −0.36 ± 0.24; Figure 3), decrease in males compared to females (coefficient estimate = −0.67 ± 0.21), decrease with an increase in BCI (coefficient estimate = −0.0011 ± 0.0004), and decrease with an increase in home range size (coefficient estimate = −0.00019 ± 0.00011). The odds of the sumALK concentration increasing by 1 ng g^{-1} increased by 1.4 times for birds in the EPA compared to the CPA (90% CI for odds ratio = 1.0, 2.2), and increased by 1.9 times for females compared to males (90% CI for odds ratio = 1.4, 2.8). The odds of a change in sumALK

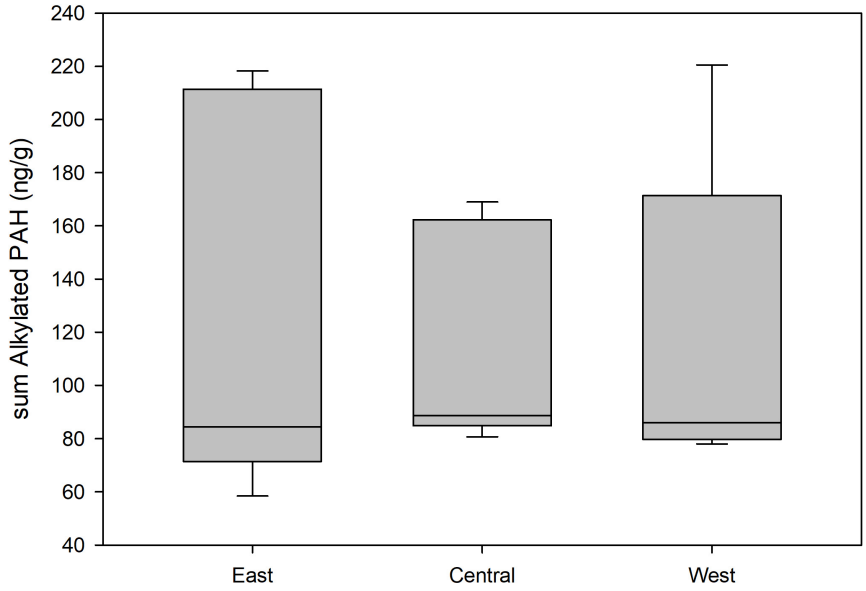

FIGURE 3
Concentrations (ng g^{-1}; wet weight) of alkylated polycyclic aromatic hydrocarbons (PAHs) by planning area from blood samples of adult brown pelicans breeding in the northern Gulf of Mexico, 2013–2015. Only samples above detectable limits are included.

for BCI and home range did not differ from 1.0 even when each independent variable was scaled up one order of magnitude.

We found a positive correlation between sumPAH in adult blood with total protein (tau = 032, p = 0.02) and with lymphocyte count (tau = 0.25, p = 0.08). All other correlations with hematological variables were not significant.

PAH profiles in adult feathers

We analyzed feathers for PAHs from 92 adult pelicans. The occurrence and concentrations of each PAH are summarized in Table 4 and reported as dry weight (ng g^{-1}). The most frequently occurring PAHs were of intermediate molecular weight. The PAHs with the highest concentrations detected were 1,3-dimethylnaphthalene (256.2 and 221.8 ng g^{-1}; both birds from Felicity Island, Louisiana) and 2-methyl naphthalene (229.0 ng g^{-1}; Shamrock Island, Texas). All of the alkylated compounds were detected in ≥2 birds while nine parent compounds were not detected. 1,3-dimethylnaphthalene, the most frequently detected PAH, also had the widest range in measured concentration (~72–~256 ng g^{-1}; Table 4). Of the 24 PAHs assessed, 15 were detected in at least one individual. Alkylated PAHs were detected in 46% of individuals and parent PAHs were detected in 26% of individuals. Three alkylated compounds were detected in >10% of individuals [2,6-dimethyl naphthalene (n = 10), 1,3-dimethylnaphthalene (n = 11), and 2,3,5-trimethylnaphthalene (n = 10)]. Of 92 birds sampled, 56 (60.8%) had at least 1 PAH detected (1 PAH: n = 39; 2 PAHs: n = 13; 3 PAHs: n = 2; 4 and 6 PAHs: n = 1). Parent compounds were detected in 24 of 92 birds, alkylated compounds were detected in 42 of 92 birds, and both parent and alkylated PAHs were detected in 10 of 92 birds. PAHs were detected in 67% of females sampled and in 55% of males sampled.

Among adult pelicans with detectable limits of PAHs, the sum of all PAHs ranged from 60.20 to 623.80 ng g^{-1}, the sum of alkylated PAHs ranged from 71.50 to 479.00, and the sum of parent PAHs ranged from 32.40 to 166.70 in feather samples (Figure 4). The mean (±SE) of the sum of alkylated PAHs (78.08 ± 10.94) was greater than the sum of parent PAHs (27.21 ± 5.13) for the full data set (i.e., including samples with no detectable PAHs; W = 3135.5, p-value = 0.0004). The mean (± SE) of the sum of alkylated PAHs (171.03 ± 9.41) was greater than the sum of parent PAHs (104.30 ± 3.62) for the subset of samples above detectable limits (W = 235.5, p-value = 0.0003).When all individuals were considered, there was no correlation between summed concentrations of alkylated and parent PAHs (Kendall tau r = −0.06, Pearson r = −0.03). When only individuals with detectable limits of PAHs were considered, there was a moderate positive correlation between summed concentrations of alkylated and parent PAHs (Pearson r = 0.57).

One model for the presence of sumPAH in pelican feathers received support, and it included BCI and migration class (Supplementary Table 4). This two-variable model carried 63% of the AIC weight and was 3x as likely to be the best model compared to the second ranked model (which was a single variable model for migration class). Both BCI (coefficient estimate = −0.002 ± 0.001) and migration class (coefficient estimates: medium −1.58 ± 0.77, short −1.38 ± 0.76) appeared to have a measurable relationship with sumPAH. The odds of a bird having a PAH increased 1.002 times (90% CI for odds ratio = 1.0006, 1.004) for every unit decrease in BCI (i.e., 1.2 times for every 100 unit decrease in BCI). The odds of a bird having a PAH decreased 4.8 times for medium v. long distance migrants (90% CI for odds ratio = 1.4, 20.0) and decreased 4.0 times for short v. long distance migrants (90% CI for odds ratio = 1.2, 16.7). One model for the concentration of sumPAH in pelican feathers received support, and it included BCI

TABLE 4 Frequency of detection, range and sum of concentration (ng/g wet weight), and percent contribution to total burden of individual polycyclic aromatic hydrocarbons (PAHs) from feathers of adult brown pelicans (n = 92) breeding in the northern Gulf of Mexico, 2013–2014.

	Number birds > detection limit, <detection limit	Range (ng/g wet weight)	Sum (Σ) of PAHs (ng/g wet weight) and (% contribution to total PAH burden from each compound)
Alkylated compounds			
2-methyl naphthalene	6, 86	56.3–229.0	764.6 (7.9%)
2,6-dimethyl naphthalene	10, 82	71.5–208.9	126.7 (13.1%)
1,3-dimethylnaphthalene	11, 81	71.7–256.2	1694.6 (17.5%)
1,5-dimethylnaphthalene	6, 86	84.0–190.0	863.4 (8.9%)
2,3,5-trimethylnaphthalene	10, 82	50.3–183.1	1067.6 (11.0%)
1-methylfluorene	7, 85	49.7–146.3	656.8 (6.8%)
3-methylphenanthrene	2, 90	120.5–181.5	302.0 (3.1%)
9-methylphenanthrene	3, 89	124.4–261.8	566.5 (5.8%)
Parent compounds			
Naphthalene	4, 88	90.7–132.2	448.9 (4.6%)
Acenaphthylene	2, 90	101.3–137.2	238.5 (2.5%)
Acenaphthene	3, 89	89.3–166.7	377.3 (3.9%)
Fluorene	0, 92	–	–
Phenanthrene	4, 88	32.4–100.0	290.5 (3.0%)
Anthracene	2, 90	63.2–70.5	133.7 (1.4%)
Fluoranthene	6, 86	60.2–121.6	513.6 (5.3%)
Pyrene	5, 87	55.8–160.5	500.9 (5.2%)
Benzo(a)anthracene	0, 92	–	–
Chrysene	0, 92	–	–
Benzo(b)fluoranthene	0, 92	–	–
Benzo(k)fluoranthene	0, 92	–	–
Benzo(a)pyrene	0, 92	–	–
Indeno(1,2,3-cd)pyrene	0, 92	–	–
Dibenz(a,h)anthracene	0, 92	–	–
Benzo(g,h,i)perylene	0, 92	–	–

and migration class (Supplementary Table 4). Although the best-performing model carried 94% of the AIC weight, neither BCI nor migration class had a measurable effect on the concentration of sumPAH (i.e., SE > coefficient estimate).

Three models for the presence of sumPAR in pelican feathers received support (Supplementary Table 5). The top ranked model carried 31% of the AIC weight and was 1.2 times as likely to be the best model compared to the second ranked model. Home range size appeared to have a measurable relationship with sumPAR (coefficient estimate = 0.00081 ± 0.0003). The odds of a bird having a PAR increased 1.08 times for every 100 km^2 unit increase in home range. There was a marginal increase in the odds of a bird having a PAR PAH for females compared to males (2.4 times, 90% CI for odds ratio = 0.8, 7.1). The odds of a bird having a PAR PAH decreased 4.3 times for medium compared to long distance migrants (90% CI for odds ratio = 1.4, 20.0). The odds of a bird having a PAR PAH increased 4.4 times for birds in the CPA compared to the EPA (90% CI for odds ratio = 1.3, 15.9). One model for the concentration of sumPAR in pelican feathers received support, and it included migration class and BCI (Supplementary Table 5). This two-variable model carried 70% of the AIC weight and was 4.4x as likely to be the best model compared to the second ranked model which was >2 AIC points removed. Only migration class (coefficient estimates: medium −0.31 ± 0.21, short −0.39 ± 0.15) had a measurable relationship with sumPAR (Figure 5). The odds of the sumPAR concentration increasing by 1 ng g^{-1} increased by 1.4 times for birds in the long-distance compared to medium-distance migrant class (90% CI for odds ratio = 0.9, 2.0) while the odds of the sumPAR concentration increasing by 1 ng g^{-1} increased by 1.5 times for birds in the long-distance compared to short-distance migrant class (90% CI for odds ratio = 1.1, 1.9).

Two models for the presence of sumALK received support, and both included migration class (Supplementary Table 6). The first-ranked model carried 68% of the model weights. This top ranked model included migration class and BCI and was 2.6 times as likely to be the best model compared to the second ranked model which included only migration class. BCI was negatively related to the presence of sumALK (coefficient estimate = −0.0014 ± 0.0009). The odds of a bird having an ALK increased 1.001 times (90% CI

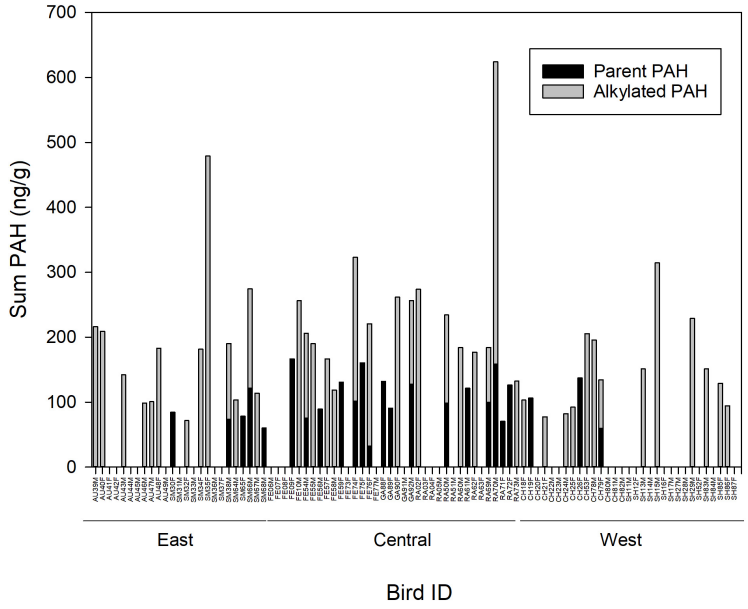

FIGURE 4
Concentration (ng g^{-1}; dry weight) of parent and alkylated polycyclic aromatic hydrocarbons (PAHs), and the sum of parent plus alkylated PAHs, in feather samples of adult brown pelicans breeding in the northern Gulf of Mexico, 2013–2015. Bird ID, two letter abbreviation for the colony of origin of the sample (AU, Audubon Island; SM, Smith Island; FE, Felicity Island; GA, Gaillard Island; RA, Raccoon Island; CH, Chester Island, SH, Shamrock Island; see **Figure 1**), a unique identification number, and F, Female or M, Male.

for odds ratio = 1.0002, 1.003) for every 1 unit decrease in BCI (i.e., 1.15 times for every 100 unit decrease in BCI). Migration class did not have a measurable effect on sumALK. The concentration of sumALK in pelican feathers was represented by one model that included variables for migration class and planning area (**Supplementary Table 6**). The best performing model carried 74% of the AIC weight and was 5.7 times as likely to be the best model compared to the second ranked model. Neither variable, however, had a measurable effect on the concentration of sumALK (i.e., SE > coefficient estimate).

Table 6 summarizes the model results. Among adults, sex and migration distance appeared as relevant variables for 50% of the dependent PAH variables. In all cases PAH levels were higher in females compared to males, and higher in longer distance migrants compared to short or medium distance migrants. BCI and planning area appeared as relevant variables for 25% of the dependent PAH variables. BCI was negatively related to PAHs in all cases while the relationship with planning area was inconsistent. Home range appeared as a relevant variable for 17% of the dependent PAH variables and had a positive relationship in one case but a negative relationship in the other case.

We found a positive correlation between sumPAH in adult feathers with PCV (tau = 0.20, $p = 0.03$). All other correlations were not significant ($P > 0.10$).

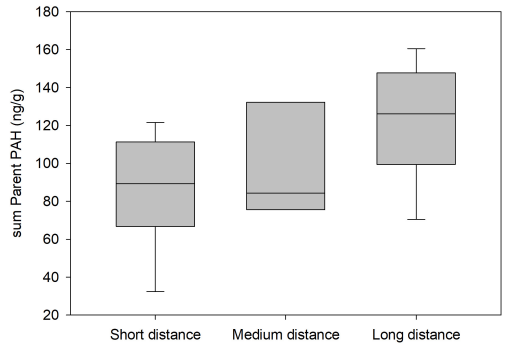

FIGURE 5
Concentrations (ng g^{-1} dry wet weight) of Parent polycyclic aromatic hydrocarbons (PAHs) by migration class from feather samples of adult brown pelicans breeding in the northern Gulf of Mexico, 2013–2015. Only samples above detectable limits are included.

PAH profiles in feathers of chicks

We analyzed feathers for PAHs from 35 pelican chicks. The occurrence and concentrations of each PAH are summarized in **Table 5** and reported as dry weight (ng g^{-1}). Of the 24 PAHs assessed, 11 were detected in at least one individual (**Table 5**). Alkylated PAHs were detected in 34% of individuals and each alkylated compound was detected within our sample of individuals. Parent PAHs were detected in 9% of individuals and we failed to detect 13 of the parent compounds within our sample. Two alkylated compounds were each detected in >10% of individuals; 2,6-dimethyl naphthalene ($n = 4$) and 2,3,5-trimethylnaphthalene ($n = 4$). Of 35 birds sampled, 13 (37.1%) had at least 1 PAH detected (1 PAH $n = 8$; 2 PAHs $n = 5$). Parent compounds were detected in 3 of 35 birds, alkylated compounds were detected in 12 of 35 birds, and both parent and were alkylated PAHs detected in 2 of 35 birds.

Among birds with detectable levels of PAHs, the sum of all PAHs ranged from 100.5 to 309.6, the sum of alkylated PAHs ranged

TABLE 5 Frequency of detection, range and sum of concentration (ng/g wet weight), and percent contribution to total burden polycyclic of individual aromatic hydrocarbons (PAHs) from feathers of chick brown pelicans (n = 35) breeding in the northern Gulf of Mexico, 2014–2015.

	Number birds > detection limit, <detection limit	Range (ng/g wet weight)	Sum (Σ) of PAHs (ng/g wet weight) and (% contribution to total PAH burden from each compound)
Alkylated compounds			
2-methyl naphthalene	1, 34	69.0	69.0 (3.2%)
2,6-dimethyl naphthalene	4, 31	50.3–250.0	642.8 (30.2%)
1,3-dimethylnaphthalene	2, 33	62.7–116.1	178.8 (8.4%)
1,5-dimethylnaphthalene	1, 34	140.7	140.7 (6.6%)
2,3,5-trimethylnaphthalene	4, 31	69.0–124.7	367.6 (17.3%)
1-methylfluorene	1, 34	104.3	104.3 (4.9%)
3-methylphenanthrene	1, 34	81.3	81.3 (3.8%)
9-methylphenanthrene	1, 34	100.5	100.5 (4.7%)
Parent compounds			
Naphthalene	1, 34	186.9	186.9 (8.8%)
Acenaphthylene	0, 35	–	–
Acenaphthene	0, 35	–	–
Fluorene	0, 35	–	–
Phenanthrene	1, 34	57.9	57.9 (2.7%)
Anthracene	0, 35	–	–
Fluoranthene	0, 35	–	–
Pyrene	1, 34	193.5	193.5 (9.1%)
Benzo(a)anthracene	0, 35	–	–
Chrysene	0, 35	–	–
Benzo(b)fluoranthene	0, 35	–	–
Benzo(k)fluoranthene	0, 35	–	–
Benzo(a)pyrene	0, 35	–	–
Indeno(1,2,3-cd)pyrene	0, 35	–	–
Dibenz(a,h)anthracene	0, 35	–	–
Benzo(g,h,i)perylene	0, 35	–	–

from 50.3 to 265.3, and the sum of parent PAHs ranged from 57.9 to 193.5 (Figure 6). The sum of alkylated PAHs (48.14 ± 77.03) was significantly higher compared to the sum of parent PAHs (12.52 ± 45.44) when using the full data set (W = 458, P = 0.01) but did not differ when only birds above detectable limits were examined (W = 21.0, P = 0.7). When all individuals are considered, there was no correlation between summed concentrations of alkylated and parent PAHs (Kendall tau r = 0.13, Pearson r = 0.05). Sample sizes were insufficient (n = 3 birds) to assess the correlation between alkylated and parent PAHs when only individuals with detectable limits of PAHs were considered.

Two models for the presence of sumPAH in chick feathers received support. The top ranked model included planning area and the null model also was supported (Supplementary Table 7). The planning area model carried 54% of the AIC weight and was 1.9 times as likely to be the best model compared to the second ranked model. Birds in the CPA were six times as likely to have a PAH compared to birds in the EPA (coefficient estimates = 1.79 ± 1.29) although the variability on the estimate was wide (90% CI for odds ratio = 0.9, 75.9). All three models received support for the concentration of sumPAH in chick feathers (Supplementary Table 7). The model including planning area carried 41% of the AIC weight and was 1.1 times as likely to be the best model compared to the null model which ranked second. Planning area (coefficient estimates: central = −0.59 ± 0.29, western = −0.36 ± 0.27) had a measurable relationship with sumPAH. Birds in the EPA were 1.8 times (90% CI for odds ratio = 1.1, 2.9) more likely to have elevated sumPAH concentrations with respect to birds in the CPA and birds in the EPA were 1.4 times (90% CI for odds ratio = 0.9, 2.3) more likely to have elevated sumPAH concentrations with respect to birds in the WPA.

Only three birds had detectable levels of sumPAR. Although the model including BCI received support for the presence of sumPAR, it ranked second to the null model which was 2.6 times as likely to be the best model (Supplementary Table 8). The SE on the estimate of BCI was greater than the coefficient estimate, indicating no measurable relationship. A hurdle model was not conducted for sumPAR due to insufficient sample size.

Only one model supported the presence of sumALK in chick feathers. The top ranked model included planning area

TABLE 6 Summary of model results from an information theoretic approach to assess relationships among polycyclic aromatic hydrocarbons (PAHs) and independent variables for brown pelican adults and chicks sampled from breeding colonies in the northern Gulf of Mexico, 2013–2015.

	BCI	Home range size	Migration class	Planning area	Sex
Presence in adult blood					
ΣPAH			n/a		F > M
ΣPAR			n/a		F > M
ΣALK			n/a		F > M
Concentration in adult blood					
ΣPAH			n/a		F > M
ΣPAR			n/a	East > Central	
ΣALK	(−)	(−)	n/a	East > Central	F > M
Presence in adult feathers					
ΣPAH	(−)		Long > short, medium		
ΣPAR		(+)	Long > medium	Central > East	F > M
ΣALK	(−)				
Concentration in adult feathers					
ΣPAH					
ΣPAR			Long > short, medium		
ΣALK					
Presence in chick feathers					
ΣPAH		n/a	n/a	Central > East	n/a
ΣPAR		n/a	n/a		n/a
ΣALK		n/a	n/a	Central > East East > West	n/a
Concentration in chick feathers					
ΣPAH		n/a	n/a	East > Central East > West	n/a
ΣPAR		n/a	n/a		n/a
ΣALK		n/a	n/a		n/a

ΣPAH, sum of parent and alkylated PAHs while ΣPAR and ΣALK, sum of only parent or alkylated PAHs, respectively. Independent variables defined in Methods (BCI, body condition index). (−), a negative relationship between the independent and dependent variable and (+), a positive relationship between the independent and dependent variable. n/a, variable not assessed for that dependent variable. Blank cells indicate no relationship was found.

(**Supplementary Table 9**). The planning area model carried 70% of the AIC weight and was 3.5 times as likely to be the best model compared to the null model which ranked second. Birds in the CPA were 6 times more likely (90% CI for odds ratio = 0.9, 75.9) to have a PAH compared to birds in the EPA (coefficient estimate = 1.79 ± 1.29) while birds in the EPA were 2.6 times more likely (90% CI for odds ratio = 0.7, 11.1) to have a PAH compared to birds in the WPA (coefficient estimates = −0.98 ± 0.85). Although the model including BCI received support for the concentration of sumALK, it ranked second to the null model which was 1.9 times as likely to be the best model (**Supplementary Table 9**).

We found a negative correlation between sumPAH in chick feathers with PCV (tau = −0.54, $p = 0.002$). All other correlations were not significant ($P > 0.10$).

Summary of model results

Table 6 summarizes model results from both age classes and both tissues types. Among adults, sex and migration distance appeared as relevant variables for 50% of the dependent PAH variables. In all cases PAH levels were higher in females compared to males, and higher in longer distance migrants compared to short or medium distance migrants. BCI and planning area appeared as relevant variables for 25% of the dependent PAH variables. BCI was negatively related to PAHs in all cases while the relationship with planning area was inconsistent. Home range appeared as a relevant variable for 17% of the dependent PAH variables and had a positive relationship in one case but a negative relationship in the other case.

Discussion

We found that PAHs occurred frequently, and in high concentrations, in brown pelicans in the Gulf of Mexico, and that higher concentrations of PAHs were associated with adverse outcomes such as lower body condition (adults) and lower packed cell volume (chicks). Of the 24 PAHs assessed, 17 occurred at least once when data were pooled among all three sample sets (i.e., adult blood, adult feathers, chick feathers). Each of the eight alkylated PAHs was detected and six were found in all three sample sets. Nine of the 16 parent PAHs were detected although only three were detected in all three data sets. Although the PAH profile of brown pelicans in the northern GOM was diverse, suggesting a wide range of inputs, 2- and 3-ring PAHs were the most commonly detected, indicating that PAH uptake and sequestration was petrogenic in nature (McConnell et al., 2015). Lesser-ringed PAHs tend to be less carcinogenic compared to higher-ringed PAHs, however, carcinogenic activity tends to increase with the addition of alkyl groups (Abers and Loughlin, 2003). Therefore the adult and chick brown pelicans we sampled, which commonly presented with alkylated versions of two-ring PAHs, may be at risk from carcinogenic PAH exposure. We also found that pathways for exposure varied among age classes and tissue types: adult exposure was higher in females and was positively correlated with larger home ranges and longer migrations, while chick exposure was positively correlated with the region in which the colony was located. This suggests that PAH exposure in adults incorporates variation in individual behavior across the annual cycle, while PAH levels in chicks reflect conditions in and around the breeding site.

PAH profiles in adults

We analyzed concentrations of PAHs in both blood and feathers from adults to provide a complementary assessment of PAH exposure in the environment. Alkylated PAHs occurred more commonly and were measured at higher summed concentrations compared to parent PAHs in adult samples from both blood and feathers. The most common and concentrated PAHs in brown pelican samples were all two-ring alkylated PAHs present in crude oil (2,3,5-trimethylnaphthalene, 1,3-dimethylnaphthalene, and 2,6-dimethyl naphthalene), suggesting that exposure to oil and/or byproducts of oil may have been a substantial source

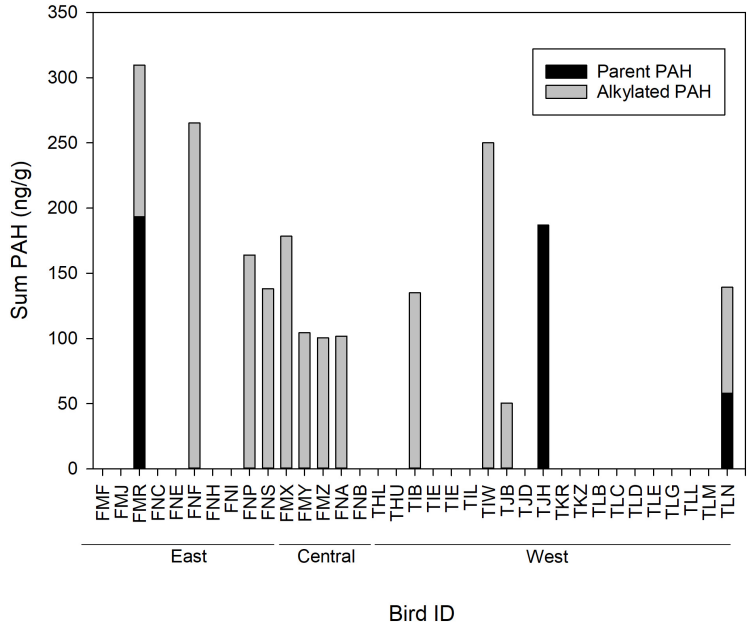

FIGURE 6
Concentration (ng g^{-1}; dry weight) of parent and alkylated polycyclic aromatic hydrocarbons (PAHs), and the sum of parent plus alkylated PAHs, in feather samples of brown pelican chicks in the northern Gulf of Mexico, 2014–2015. Bird ID, the three-letter combination from the field-readable plastic leg band applied to the chick. F, feather sample.

of PAH contamination for brown pelicans during this study. Alkylated PAHs are more abundant in crude oil, more persistent, and less prone to metabolization compared to parent PAHs (Seegar et al., 2015). Liu et al. (2012) found that alkylated PAHs had a stronger signature in crude oil, weathered oil, and oil mousse compared to parent PAHs. 2,6-dimethyl naphthalene was also identified as a known component of oil released during blowouts in the northern Gulf of Mexico (Seegar et al., 2015). This profile pattern indicates that exposure to oil and/or byproducts of oil may have been a substantial source of PAH contamination for brown pelicans during this study. Similar to the general profile we observed, alkylated PAHs also were more common compared to parent PAHs in blood samples of Common Loons (*Gavia immer*) and Northern Gannets (*Morus bassanus*) sampled during their non-breeding seasons in the northern Gulf of Mexico (Paruk et al., 2014, 2016; Champoux et al., 2020).

The presence or absence of PAHs in adult blood samples was most frequently predicted by sex, although this relationship was stronger for alkylated PAHs than for parent or the sum of all PAHs. Sex also had a positive effect on the increase in concentration of sumPAH and sumALK in adult blood samples. A dimorphic result based on sex could occur through differential resource selection by males and females with respect to habitat occupied and/or prey consumed (Seegar et al., 2015). In contrast, we did not observe a sex effect on either the occurrence of or concentration of PAHs in adult feathers. Given that blood reflects a relatively recent signature compared to feathers, our results indicate that during incubation and early chick-rearing, females are being exposed to PAHs more frequently or perhaps more recently than males but that over the course of the annual cycle exposure may be more similar.

A unique attribute of our samples was the opportunity to use tracking data to assess the extent of movement, as measured by home range size and migration strategy, on PAH exposure. Migration class predicted the occurrence and concentration of PAHs in adult feathers more frequently than the other independent variables we assessed. Three of the six model steps showed that longer distance migrants were more likely to be found with a PAH (sumPAH and sumPAR) and that the level of PAHs was likely to increase in that group (sumPAR). Most long-distance migrants in our study departed the northern Gulf and occupied wintering areas in the southern Gulf (i.e., Mexico EEZ), south Texas coast, Cuba, or the Pacific coast of Mexico (Lamb et al., 2018, 2020). Most long-distance migrants also attended distinct staging or molting sites separate from both their breeding and wintering areas, and approximately 75% of birds in our sample occupied ranges in the Louisiana Delta during the post-breeding period (Lamb et al., 2020). An investigation into potential sources of parent PAHs in molting and wintering areas may be warranted but likely would need to consider exposure to both pyrogenic sources (which could be quite varied) and petrogenic sources in these locations.

In contrast to migration class, home range during the breeding season rarely had an ecological relationship with PAH presence and was only related (positively) to the concentration of parent PAHs on adult feathers. Our results suggest that the extent of daily movement patterns (i.e., on the order of 10s of km) may not occur over a spatial scale that consistently experiences substantial heterogeneity in PAH availability. When considered with the results of migration class, our data suggest that PAH availability for adult brown pelicans is more heterogeneous at the larger spatial resolutions typical of migration (e.g., $\geq$100 km) compared to smaller spatial resolutions typical of home ranges (e.g., $\leq$100 km). Jodice et al. (2022) also did not report a consistent effect of home range size on measures of blood biochemistry and hematology in these same populations of brown pelicans.

We also assessed the relationship between PAH exposure and regional oil and gas development intensity, using the three jurisdictional planning areas of the U.S. Bureau of Ocean and Energy Management. The proximity to oil and gas activity is often used when predicting or assessing contaminant loads or the likelihood of risk exposure to marine wildlife (e.g., Nicholson et al., 2023), with an underlying assumption that exposure would increase with the level of oil and gas activity (i.e., highest in the central, intermediate in the west, and least in the east). During our study, however, planning area was never a strong predictor of the presence of PAHs in adult pelicans and when planning area was relevant, the results were not consistent with the background levels of oil and gas development among the regions. Similarly, Nicholson et al. (2023) found that proximity to active oil and gas activity was not a strong predictor of PAH levels in red snapper (*Lutjanus campechanus*) in the western Gulf. We detected a slightly higher concentration of sumPARs in the eastern planning area, the least developed with respect to oil and gas activity. This result was driven by two individuals from Florida, each with a high level of the parent PAH fluorene (n = 2 birds, 161.1 ng g^{-1} and 165.4 ng g^{-1}). The individuals from which these samples were collected were both nesting adults on Audubon Island, Florida, with home ranges centered in and proximate to bays surrounding Panama City, Florida, an area with substantial industrial, military, and agricultural development. Similarly, the three highest levels of sumALK occurred in birds nesting in the eastern planning area in Florida. The lack of a consistent relationship between PAHs and the level of oil and gas development within the planning areas agrees with our prior observation that breeding region is not a consistent predictor of pelican health parameters (Jodice et al., 2022). These results indicate that exposure to PAHs in pelicans is driven by a complex set of internal and external factors affecting exposure probability and cannot be distilled solely to the planning area (i.e., regional level of oil and gas development) within which individuals breed.

Of the health-related variables we assessed, the only relationship of significance was between the presence of PAHs in adult feathers and BCI. Individuals in poorer body condition were more likely to be found with a PAH, and it was more likely that the PAH was from the alkylated group. Paruk et al. (2016) found that in common loons sampled for PAHs during their non-breeding season in the northern Gulf of Mexico, individuals for which PAHs were detected were lighter in body mass compared to individuals for which PAHs were not detected. Similarly, Champoux et al. (2020) found that condition of northern gannets, as assessed through blood analytes, was negatively correlated with PAH concentration. Taken together, these results support the supposition that PAHs can result in sublethal effects within individuals (e.g., reduced body condition) even when concentrations are not at maximum levels.

The average concentration (i.e., excluding individuals where sumPAH = 0) of both parent (116.1 ± 61.8 ng g^{-1}) and alkylated (119.57 ± 55.6 ng g^{-1}) PAHs in blood samples from pelicans in this study appears to be higher compared to blood samples collected from other waterbirds from the Gulf of Mexico during a similar time frame. Paruk et al. (2016) reported means ± SEs for parent and alkylated PAHs in wintering common loons of 83.1 ± 15.2 and 15.2 ± 4.3, respectively. Champoux et al. (2020) reported means ± SEs for parent and alkylated PAHs in wintering northern gannets of 2.5 ± 2.4 and 11.1 ± 3.3 ng g^{-1}, respectively. Comparisons of PAH levels in feathers of adult pelicans from this study to other studies of PAH levels in feathers of free-ranging birds from this region are, to the best of our knowledge, unavailable at this time.

PAH profiles in chicks

Only feathers were assessed for PAHs in chicks. As with adults, however, each of the eight alkylated PAHs was detected in chicks but only three of the 16 parent PAHs were detected (and each only once). The most commonly occurring PAHs were the alkylated PAHs 2,3,5-trimethylnaphthalene, 1,3-dimethylnaphthalene, and 2,6-dimethyl naphthalene, the same common PAHs found in adult feathers. As with adults, 2- and 3-ring PAHs were the most commonly detected and therefore pelican chicks also may be at risk from carcinogenic PAH exposure (Abers and Loughlin, 2003). There were no PAHs found in chicks that were not also found in adults, suggesting that chicks represent a subset of the adult data although the profile and total concentration of PAHs of chicks are less diverse and lower, respectively, compared to adults.

Planning area irregularly and weakly predicted PAH levels in feathers of chicks. The presence of alkylated PAHs was more likely in the central planning area, which has a higher concentration of oil and gas activity compared to the western and eastern planning areas. Alkylated PAHs tend to be associated with petrogenic sources and may be more likely to occur from contact with recently foraging parents (Paruk et al., 2014; Seegar et al., 2015). The three highest concentrations of PAHs in chick feathers, however, were not recorded from the central planning area. These three high values occurred in birds sampled in Galveston Bay, Texas, and from the Florida Panhandle, suggesting that local factors may also be driving individual exposure to PAHs. For example, between 2004 and 2017, 17% of sediment samples from the Houston Ship Channel in Galveston Bay exceeded state standards for Pyrene[2] which was the second highest PAH we recorded in pelican chicks. The heterogeneity we observed in chick exposure suggests that specific and highly localized exposure events, such as consuming contaminated prey item or contacting contaminated material at or near nest sites, may substantially affect PAH levels over the relatively short time frame (i.e., weeks) of nestling development. Because of relative infrequency of PAHs in chick feathers, however, caution should be applied when interpreting these results.

Comparable data sets assessing PAH loads in chicks are, to the best of our knowledge, few in number. Fernie et al. (2018a) measured total PAH loads of 31–106 ng g^{-1} ww in muscle and feces of nestling tree swallows at oil sand sites. While not directly comparable the sumPAH values we measured (57.9–642.8 ng g^{-1} dry weight) appear to be substantially higher. Chick feathers likely represent contact transfer of PAHs from adults, deposition from aerial sediments, or contact with PAHs in ground/nesting material or prey (Fernie et al., 2018a,b; Perez-Umphrey et al., 2018). Fernie et al. (2018b) found that Tree swallow nestlings showed decreased growth rates and that reproductive success (% eggs that led to fledged chicks) was lower at oil sand sites with higher levels of

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PAHs compared to reference sites. Uptake and deposition of PAHs in the birds' muscle was related to diet and also higher at oil sand sites compared to reference sites (Fernie et al., 2018a). These results demonstrate that nest-bound chicks can accumulate detrimental levels of PAHs from the surrounding environment. Given that the route of contamination for PAHs on chick feathers is not entirely clear, we suggest that additional assessments be considered to determine if short- or long-term changes may occur in PAH loads dependent upon chick age or parental activity such as provisioning or brooding (i.e., activities that may enhance contact with chicks).

Correlates with other biomarkers

Exposure to contaminants such as PAHs can result in sublethal effects of varying severity. Fallon et al. (2018) identified several physiologic correlates of oiling in Brown Pelicans and other waterbirds following exposure to oiling including reduced PCV, reduced Hb, decreased counts of red blood cells, oxidative injury to erythrocytes, and formation of Heinz bodies. We assessed physiologic correlates of PAH exposure in two tissue types and two age classes. We found a positive correlation of PAH exposure with total proteins and with lymphocyte count in adults. An increase in total proteins or lymphocyte counts can occur with inflammation and infection (Newman et al., 2000; Grimaldi et al., 2015), both attributes of PAH exposure. Paruk et al. (2016, 2021) found positive correlations between PAH levels and total protein, and between PAH levels and counts of eosinophils, lymphocytes, and monocytes in common loons wintering in the Gulf of Mexico. Similarly, Champoux et al. (2020) found a positive correlation between lymphocyte count and PAH exposure in northern gannets wintering in the Gulf of Mexico.

We found that PCV was lower in chicks when PAH levels in their feathers were higher. A decrease in PCV can be indicative of sublethal physiological responses to environmental stressors. For example, Paruk et al. (2016) found a weak negative correlation between PCV and PAH exposure as measured in blood of adult common loons wintering in the Gulf of Mexico and Fallon et al. (2018) found decreased PCV in brown pelicans in areas exposed to oiling compared to reference areas. Each suggested that the decrease in PCV was symptomatic of environmental stressors related to exposure to PAHs and oiling. Given that feathers are more likely to represent longer term exposure, our results indicate that pelican chicks may be experiencing long term physiological stress correlated with exposure to PAHs. In contrast, we found that PCV was higher in adults when PAH levels in their feathers were higher. Pulmonary and renal diseases can also lead to relative increases in PCV and such diseases may occur with long term exposure to pollutants such as PAHs (Troisi et al., 2006; Jones, 2015; Bursian et al., 2017).

Conclusion

We assessed PAH loads in blood and feathers from adult brown pelicans during the breeding season, and from feathers of chicks of brown pelicans. Both alkylated and parent PAHs were commonly detected in both age groups and both sample types, indicating that both short- and long-term exposure (i.e., blood and feathers, respectively) are occurring for this species. The high level of oil and gas activity in waters of the northern Gulf of Mexico, as well as industrial, military, and agricultural activity in the coastal region, make it challenging to identify a primary source for PAH exposure although the higher frequency of alkylated PAHs in samples does suggest substantial exposure from a petrogenic source (Pereira et al., 2009; McConnell et al., 2015; Jesus et al., 2022). Our data provide reference levels for a time period following a major spill event, and further assessments conducted additional years out from the event (i.e., a time series) may also provide context for the stability of these initial levels (National Research Council [NRC], 2003). Long-term tracking of PAHs, as well as an assessment of sublethal effects of PAHs on pelicans, can also enhance our understanding of the persistence and effects of this contaminant in the northern Gulf. Recent studies also suggest that preen oil can also be used to assess PAH levels and assessing PAH levels through multiple matrices (e.g., blood, feathers, preen oil) may provide a more complete picture of contamination (Acampora et al., 2018). Lastly, the taxonomic diversity of marine avifauna assessed for PAH exposure in the northern Gulf remains narrow. Along with our study, PAHs have also been measured in free-ranging common loons and northern gannets, both species that migrate to the Gulf during their non-breeding seasons. Additional data from other marine avifauna that breed within the northern Gulf would provide stakeholders with a more complete view of PAH exposure, particularly if species representing different foraging modes and diets were included [e.g., gulls and terns (Laridae), or American oystercatchers (*Haematopus palliates*)].

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: Data on PAH levels and individual attributes from this research are available as a data release through the U.S. Geological Survey at https://doi.org/10.5066/P94GBJ4G (Lamb et al., 2019).

Ethics statement

This animal study was reviewed and approved by Clemson University Animal Care and Use Committee.

Author contributions

PJ: conceptualization, methodology, formal analysis, resources, writing (original and reviews), visualization, supervision, project administration, and funding acquisition. JL: conceptualization, methodology, validation, formal analysis, investigation, data collection and management, sample preparation, writing (original and reviews), supervision, and project administration. YS: validation, data collection and management, sample preparation,

formal analysis, writing (original and reviews), and visualization. CP: methodology, laboratory analysis, and writing (original and reviews). 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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Supplementary material

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Body condition and corticosterone stress response, as markers to investigate effects of human activities on Adélie penguins (*Pygoscelis adeliae*)

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Introduction: In Antarctica, there is growing concern about the potential effect of anthropogenic activities (i.e., tourism, research) on wildlife, especially since human activities are developing at an unprecedented rate. Although guidelines exist to mitigate negative impacts, fundamental data are currently lacking to reliably assess impacts. Physiological tools, such as circulating corticosterone levels, appear promising to assess the potential impact of human disturbance on Antarctic vertebrates.

Methods: In this study, we compared the body condition, and the physiological sensitivity to stress (i.e., basal and stress-induced corticosterone level) of adult and chick Adélie penguins between a disturbed and an undisturbed area (i.e., 2 colonies located in the middle of a research station exposed to intense human activities and 2 colonies located on protected islands with minimal human disturbance).

Results: We did not find any significant impact of human activities on body condition and corticosterone levels in adults (incubating adults, brooding adults). In chicks, there were significant inter-colony variations in stress-induced corticosterone levels. Specifically, the chicks from the disturbed colonies tended to have higher stress-induced corticosterone levels than the chicks from the protected areas although this difference between areas was not significant. In addition, and independently of human disturbance we also found significant differences in adult body condition, and chick corticosterone level between colonies.

Discussion: Overall, our study suggests that this species is not dramatically impacted by human activities, at least when humans and penguins have cohabited for several decades. Our results support therefore the idea that this species is likely to be tolerant to human disturbance and this corroborates with the persistence of Adélie penguin colonies in the middle of the research station. However, our results also suggest that chicks might be more sensitive to human disturbance than adults and might therefore potentially suffer from human disturbance. Our study also suggests that specific individual and environmental variables outweigh the potential minor impact of human disturbance on these variables. Combining corticosterone with complementary stress-related physiological markers, such as

heart rate, may strengthen further studies examining whether human disturbance may have subtle detrimental impacts on individuals.

KEYWORDS

seabird, *Pygoscelis adeliae*, human activity, stress response, stress-induced corticosterone, basal corticosterone, disturbance, Antarctica

1. Introduction

In recent years, there has been increasing concern about the potential effect of anthropogenic activities on Antarctic wildlife (Tin et al., 2014). Antarctica is often perceived as a pristine wilderness area unaffected by human activities. However, tourism activities have developed to an unprecedent scale over the last decade (IAATO ATCM Information Papers, 2022). Similarly, research activities have increased, including the creation of several new stations on the Antarctic continent (Chown et al., 2012). Importantly, human activities are predicted to continue to grow in the following decades (Woehler et al., 2014; Bender et al., 2016). Therefore, there is an urgent need to propose management policies regulating anthropogenic activities, to develop sustainable Antarctic tourism, and to limit the impact of anthropogenic activities on Antarctic wildlife (Tin et al., 2014; Coetzee et al., 2017; Pertierra et al., 2017).

Despite this urgent need, we currently lack basic data to reliably assess the impact of anthropogenic activities on wildlife (Tin et al., 2014). Surprisingly few studies have examined the impact of anthropogenic activities on wild vertebrate species such as seals and seabirds (reviewed in Coetzee and Chown, 2016). However, these species may be particularly vulnerable to anthropogenic activities in the Antarctic for several reasons. Firstly, these species have not coevolved with human presence or terrestrial predators and their behavioral and physiological systems have not necessarily been selected to cope with terrestrial disturbance. Secondly, seabirds and seals are iconic species, often considered a "must-see" during tourist expeditions (Tin et al., 2016). Among Antarctic species, they are therefore probably the most exposed to tourist activities. Finally, these species often breed on rocky coastlines and islands, which are also most suitable to establish research stations. Therefore, they may be directly impacted by the settlement of new Antarctic stations (Micol and Jouventin, 2001; Woehler et al., 2014).

Assessing the impact of human presence and activities on Antarctic vertebrates can be challenging. Having not coevolved with humans, they are often "tame" and do not necessarily flee in response to human presence. Therefore, they are often considered relatively insensitive to human presence. Coetzee and Chown (2016) recently reported in a meta-analysis that anthropogenic activities do not have consistent and significant effects on seabird and seal behavior. However, despite this apparent insensitivity there is increasing evidence that these species may perceive anthropogenic activities as a threat or a stressor even if they do not behaviorally react. For example, human presence was associated with an increase in heart rate in several sub-Antarctic and Antarctic seabird species without behavioral signs of stress being evident (Weimerskirch et al., 2002; de Villiers et al., 2006; Ellenberg et al., 2013).

Assessing the impact of anthropogenic activities on wild vertebrates warrants the use of alternative and additional tools, such as physiological parameters, which can reflect individual level of stress better than behavioral components (i.e., the concept of "Conservation Physiology", see Walker et al., 2005; Cockrem et al., 2006; Wikelski and Cooke, 2006). For example, there is growing interest in the use of "stress hormones" in vertebrates to assess the influence of biotic and abiotic factors (Busch and Hayward, 2009; Dickens and Romero, 2013). In response to a stressor the Hypothalamus-Pituitary-Adrenals (HPA) axis is activated, resulting in a rapid release of glucocorticoids into the bloodstream (Wingfield et al., 1998). This increase in circulating glucocorticoid levels mediates important physiological and behavioral changes, which aims to restore homeostasis (McEwen and Wingfield, 2003; Romero et al., 2009). Although circulating glucocorticoid levels generally return to baseline levels when the stressor ends, they can theoretically stay elevated during a prolonged period if homeostasis is not restored. Consequently, ecophysiologists have used baseline glucocorticoids levels to test whether a given individual, or population, is "stressed" (reviewed in Busch and Hayward, 2009; Dickens and Romero, 2013). In addition, the sensitivity of the HPA axis to a standardized restraint stress protocol (i.e., stress-induced glucocorticoid levels) is also used to test individual responses to a stressor (Wingfield et al., 1992; Angelier and Wingfield, 2013; Dickens and Romero, 2013). This stress-induced protocol allows us to understand whether a given individual, or population, could become habituated (low stress-induced glucocorticoid levels) or sensitized (high stress-induced glucocorticoid levels) to stress in a context of chronic anthropogenic disturbance (Angelier and Wingfield, 2013). Therefore, using elevated circulating glucocorticoid levels as a stress indicator has been proposed and successfully implemented as a physiological tool to monitor anthropogenic disturbance effects on wild vertebrates (Walker et al., 2005; Cockrem et al., 2006; Wikelski and Cooke, 2006; Busch and Hayward, 2009; but see Dickens and Romero, 2013).

We sought to assess the impact of anthropogenic activities on an Antarctic bird species widely used as an "ecosystem sentinel", the Adélie penguin (*Pygoscelis adeliae*). To do so we examined individual body condition and corticosterone levels (the main avian glucocorticoid) among penguin colonies located both within protected areas and in the close vicinity of a research station. In the protected areas human presence is absolutely limited to research purpose and access is restricted to a small number of scientists per breeding season. In contrast, other colonies are located in the middle of a research station with daily anthropogenic activities including vehicles, frequent human presence, maintenance work and the use of helicopters for transporting cargo and people.

If anthropogenic activities have a detrimental effect on Adélie penguins, we predict that penguins from the disturbed area will have a lower body condition (prediction 1a), higher baseline corticosterone levels (prediction 1b), and higher stress-induced corticosterone levels (prediction 1c) if they become hyper-sensitive to stress, relative to

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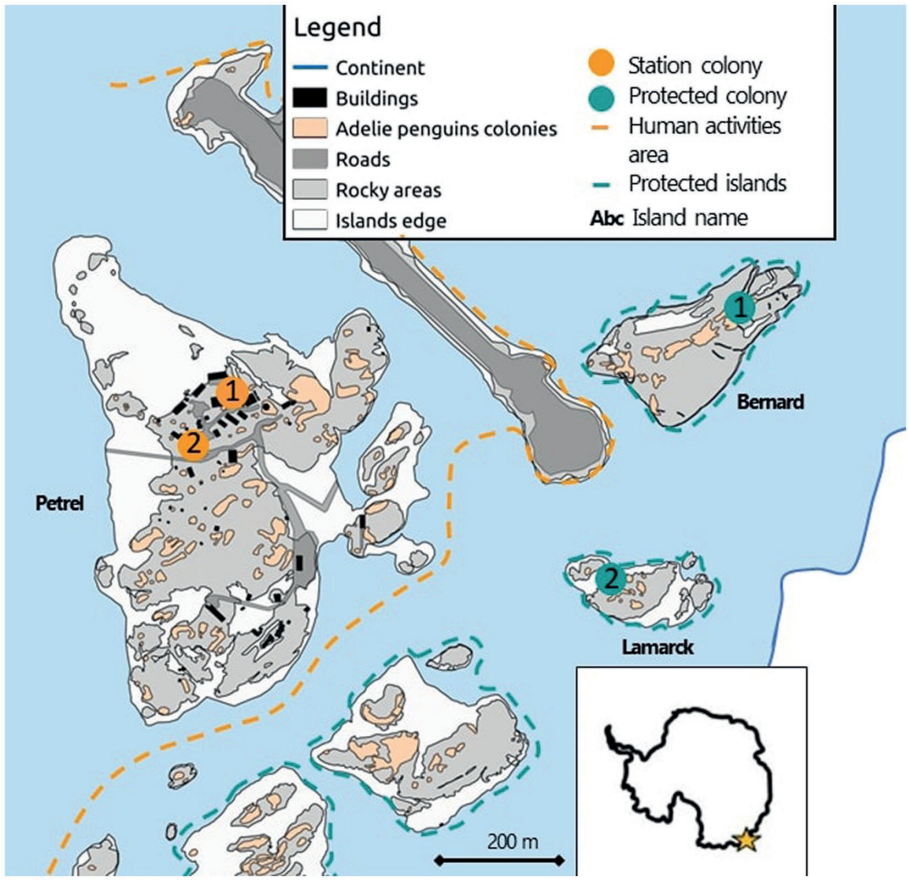

FIGURE 1
Study area in Adelie Land, East Antarctica. Representation of the disturbed (orange) and undisturbed (green) areas with sampled colonies highlighted with large colored dots.

penguins from undisturbed areas. Alternatively, if Adélie penguins have habituated to anthropogenic activities, we predict that penguins from the disturbed area will have a similar body condition (prediction 2a), similar baseline corticosterone levels (prediction 2b), and lower stress-induced corticosterone levels (prediction 2c), relative to penguins from protected areas. Finally, we predict that this effect of anthropogenic activities on corticosterone levels may be exacerbated during specific stages (i.e., early life) during which penguins may be more sensitive to disturbance (prediction 3).

2. Methods

2.1. Adélie penguins and study sites

This study was carried out at Pointe Géologie (66°400'S, 140°010'E) in Adélie Land, Antarctica, between November 2015 and February 2016. Breeding Adélie penguins were captured during the incubation stage (November 24 to December 4 for males; December 8 to December 11 for females) and the early chick-rearing period (December 31 to January 7). During these stages both parents alternate foraging trips at sea with incubating or brooding periods at the colony. A total of 127 incubating (78 males and 49 females) and 92 chick-rearing (43 males and 50 females) penguins were captured. In addition, 54 chicks (27 males and 27 females) were captured after thermal emancipation when they are left alone at the colony (end of January-beginning of February).

All penguins were captured from four colonies: two located within the station area considered as a "disturbed area", each hereafter termed a "station colony"; and two were located on protected islands considered as "undisturbed area", each hereafter termed a "protected colony" (Figure 1). The station colonies (station colony 1 and 2) are located in the middle of the Dumont d'Urville research station – here, birds are breeding nearby to the helicopter landing platform (10 to 50 m proximity) and are located within 1–2 m from paths or roads frequently used by people and vehicles. Noisy human activities related to the daily station operations are thus very frequent, however there is no tourism in the area. In contrast, the protected colonies (Bernard Island, protected colony 1; Lamarck Island, protected colony 2) both belong within an Antarctic Specially Protected Area (ASPA) where human activities are strictly limited to scientific research purposes. There are no paths nor roads, vehicles or recreational activities are prohibited, and helicopters cannot overfly these islands. The access permitted to scientists is restricted to small groups (2–3 persons) only a few times per breeding season. Sample size was evenly distributed among colonies (in the order of station colony 1, 2 and protected colony 1, 2; sample size is: incubating adults: 30, 33, 32, 31; rearing adults: 23, 23, 23, 23; chicks: 15, 14, 13, 12).

TABLE 1 Summary of the tested explanatory variables:

Dataset	Response variable	Fixed effects tested
Incubating adults	A. Flipper length	[Area or Colony] × Sex
Chick-rearing adults		[Area or Colony] × Sex
Chicks		[Area or Colony] × Sex
Incubating adults	B. Body condition	[Area or Colony] × Sex
Chick-rearing adults		[Area or Colony] × Sex
Chicks		[Area or Colony] × Sex
Incubating adults	C. Basal corticosterone	[Area or Colony] × Sex × Body condition
Chick-rearing adults		[Area or Colony] × Sex × Brood size + Body condition × Sex × Brood size
Chicks		[Area or Colony] × Sex × Body condition
Incubating adults	D. Stress-induced corticosterone	[Area or Colony] × Sex × Body condition
Chick-rearing adults		[Area or Colony] × Sex × Brood size + Body condition × Sex × Brood size
Chicks		[Area or Colony] × Sex × Body condition

2.2. Handling procedures, body measurements, and standardized stress protocol

All birds were captured by hand. A hood was immediately placed over the head and individuals were sampled for blood (0.5 mL of blood drawn from the tarsal vein within 3 min of capture) to obtain basal corticosterone levels (Romero and Reed, 2005; Angelier et al., 2010). Flipper length was measured using a ruler (±1 mm) and weight using a digital scale (±10 g). Body condition was calculated as the residuals from the linear regression between mass and flipper length (adults: $F_{1,218} = 20.02$, $p < 0.001$; chicks: $F_{1,49} = 20.99$, $p < 0.001$). Birds were then restrained for 15 min (standardized stress protocol, see Wingfield et al., 1992) and a second blood sample collected to measure stress-induced corticosterone levels. Birds were marked with a dye spot (Porcimark®) before being released near their nest to avoid recapturing the same individual twice.

2.3. Corticosterone assay and molecular sexing

Sex determination was performed by molecular sexing at the CEBC ('Service d'Analyses Biologiques') as described in Marciau et al. (2023). Plasma concentrations of corticosterone were determined in 50 µL samples by radio-immunoassay following procedures described in Lormée et al. (2003). Inter-assay and intra-assay precisions were, respectively, 8.00 and 12.34%. Corticosterone lowest detectable concentration was 0.28 ng/mL.

2.4. Statistical analysis

To examine the effect of anthropogenic activities on Adelie penguins in this region we tested models for four response variables: two morphometric, (A) flipper length and (B) body condition, and two physiological, (C) basal and (D) stress-induced corticosterone levels. All statistical analyses were performed using the R software (version 4.2.1 R Core Team, 2022). Linear mixed effect models and generalized least squares models were performed with the *nlme* package (Pinheiro et al., 2023) and model selection with the *MuMIn* package (Bartoń, 2022).

To first examine the appropriate random effect structure, we considered a relatively full linear mixed model (LMM) fitted using REML with all covariates included (Table 1, main fixed effect being Area) and including a hierarchical random effect with Colony nested within Area. However, only two colonies per area could not support estimation of this random effects structure and resulted in singularity errors. We secondly considered including only Colony as a random effect and used AIC and likelihood ratio tests (LRTs) to compare models with i. the nested random effect Area/Colony, ii. Colony only as a random effect, or iii. no random effect. In all cases, the AIC and LRTs provided no support for including a random effects structure and further analyses (detailed below) were developed using generalized least squares models fitted using ML estimation.

Our primary aim was to investigate for effects between disturbed (station) and undisturbed (protected) areas acknowledging our sampling was limited to two colonies per area and other potential influential factors (stage, sex etc.). To determine the best fixed-effects structure we therefore proceeded with a model selection approach using the *dredge()* function–to generate a model selection table of models with all possible subsets of terms – with models ranked using AIC_c and Akaike weights (AIC_w). In this model selection procedure, we considered Area OR Colony, excluding models containing both these terms (Table 1). This approach investigates disturbance effects between areas while acknowledging that effects may not manifest identically across colonies.

Following this workflow, we tested successively models for the four response variables, fitting separate models for the three stages in each case, i.e., incubating adults, rearing adults, chicks (12 final models in total, Table 1). We present results from the best-ranked model based on AIC_c and AIC_w criterion. In general, this approach follows the scientific rule of parsimony in selecting the simplest parameterization supported by the available data. However, for disturbance studies the burden of proof for demonstrating no effect (accepting a null hypothesis) is necessarily higher. In a conservative approach, we therefore also present and discuss the second-ranked models when needed, i.e., when AIC_w are very similar.

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TABLE 2 Summary of final best-ranked models for (A) flipper length, (B) body condition, (C) basal corticosterone, and (D) stress-induced corticosterone.

	Dataset	Figure	Response variable	Terms in the best-ranked model	Coefficient Estimate ± SE	*F*-value	*p*-value
A1	Inc. adults	S1	Flipper	**– Sex (male)**	3.70 ± 1.18	9.86	**0.002**
A2	Rear. adults	S1	Flipper	**– Sex (male)**	4.87 ± 1.14	18.19	**<0.001**
A3	Chicks	S1	Flipper	**– Sex (male)**	0.54 ± 0.13	15.4	**<0.001**
B1	Inc. adults		Body condition	**– Sex (male)**	−0.89 ± 0.15	78.26	**<0.001**
				– Colony		0.27	0.922
				– Colony × Sex	Detail **Supplementary Table S1**	4.04	**0.009**
B2	Rear. adults		Body condition	**– Sex (male)**	0.29 ± 0.07	15.39	**<0.001**
B3	Chicks		Body condition	– Null			
C1	Inc. adults	2	Basal corticosterone	– Sex		0.41	0.127
				– Body condition	−0.41 ± 0.12	11.45	**0.002**
C2	Rear. adults	2	Basal corticosterone	**– Sex (male)**	0.25 ± 0.12	1.75	**0.048**
				– Body condition	−0.34 ± 0.16	5.87	**0.033**
				– Brood size	0.28 ± 0.11	5.91	**0.017**
C3	Chicks	2	Basal corticosterone	**– Colony**	Detail **Supplementary Table S2**	4.80	**0.005**
D1	Inc. adults	3, 4	Stress-induced corticosterone	– Sex		0.63	0.07
				– Body condition	−6.83 ± 1.70	16.21	**<0.001**
D2	Rear. adults	3, 4	Stress-induced corticosterone	– Sex		<0.001	0.900
				– Body condition	−5.02 ± 1.66	12.27	**<0.001**
				– Brood size (2)	6.37 ± 1.59	4.47	**0.037**
				– Brood size (2) × sex (male)	−8.49 ± 2.33	13.26	**<0.001**
D3	Chicks	3, 4	Stress-induced corticosterone	**– Sex (male)**	−5.66 ± 2.20	21.66	**<0.001**
				– Body condition		1.99	0.1
				– Colony		7.3	**<0.001**
				– Colony × Sex	Detail **Supplementary Table S3**	4.73	**0.006**

Significant terms ($p < 0.05$) are in bold.

3. Results

3.1. Determinants of flipper length

For all stages examined, i.e., incubating and chick-rearing adults and chicks, the best ranked model for flipper length included only sex. In all cases males had significantly longer flippers than females (Table 2; Supplementary Figure S1). For incubating adults, the mean predicted flipper length for males was 192.13 ± 0.73 and for females was 188.43 ± 0.93 (Supplementary Figure S1a). For chick-rearing adults the predicted flipper length for males was 192.23 ± 0.84 and females was 187.36 ± 0.78 (Supplementary Figure S1a). For chicks predicted flipper length for males was 18.37 ± 0.10 and females was 17.83 ± 0.10 (Supplementary Figure S1b).

3.2. Determinants of body condition

For incubating adults the best ranked model included only sex, with males having a significantly lower body condition than females (F value = 78.26, value of p <0.001, Table 2; male predicted value = −0.26 ± 0.05, female predicted value = 0.41 ± 0.06). However, the model selection procedure showed the second-ranked model to be similarly weighted (AIC weight ratio was only 1.13: $AIC_{w1} = 0.40$, $AIC_{w2} = 0.36$). In this second model, Colony and a Colony x sex interaction effect were retained (F-value = 4.04, value of $p = 0.009$, Table 2). Females from protected colony 2 (Lamarck Is) presented a significantly lower condition (predicted value = 0.14 ± 0.12) than females from protected colony one (Bernard Is; predicted value = 0.58 ± 0.12) and from the station colony 1 (predicted values = 0.55 ± 0.12; contrasts detailed in Supplementary Table S1). For adults in chicks rearing, the best ranked model included only sex. Males had a significantly higher body condition than females ($p < 0.001$, male predicted value = 0.16, female predicted value = −0.14, Figure 2). For chicks the best-ranked model was the null model, indicating body condition did not vary in relation to sex or Area/Colony.

3.3. Determinants of basal corticosterone levels

For incubating adults the best ranked model included both body condition and sex, however this model had a similar weight to the second-ranked model excluding sex (i.e., AIC weight ratio was only 1.14: $AIC_{w1} = 0.28$, $AIC_{w2} = 0.25$) and the sex difference was not

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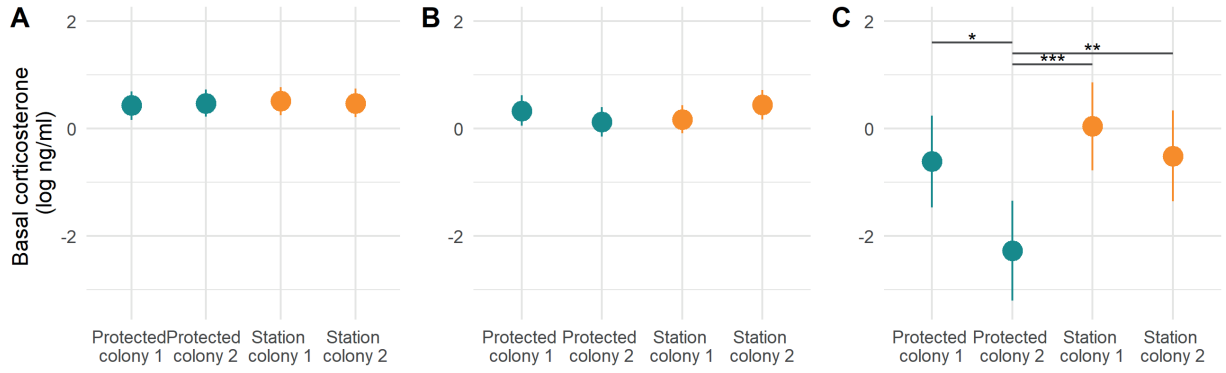

FIGURE 2
Model results for basal corticosterone levels (log) in relation to the colony in incubating adults **(A)**, rearing adults **(B)**, and chicks **(C)**. Predicted mean values ± SE are plotted. Asterisks denote statistical significance between two groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$) based on contrast analyses (presented in Supplementary Table S2).

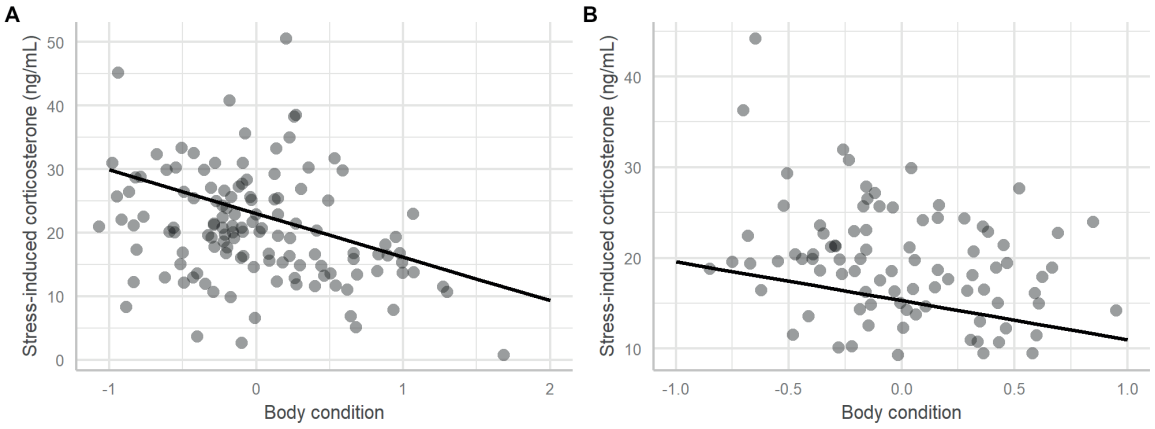

FIGURE 3
Stress-induced corticosterone in relation to the body condition, in incubating adults **(A)** and rearing adults **(B)**. The dots represent the raw data and the regression line is extracted from the fitted model.

significant (Table 2). Individuals in poorer condition had higher basal levels of corticosterone (coefficient estimate = −0.41 ± 0.12; *F*-value = 11.45, value of $p = 0.002$, Table 2). For chick-rearing adults the best ranked model included body condition, brood size, and sex. Individuals in poorer condition tended to have higher basal levels of corticosterone (coefficient estimate = −0.34 ± 0.16, *F*-value = 5.87, value of $p = 0.033$, Table 2). Individuals rearing 2 chicks had higher basal corticosterone levels than individuals rearing a single chick (*F*-value = 5.91, value of $p = 0.017$, Table 2; predicted value for single chick individual = 1.28 ± 1.08, two chicks = 1.70 ± 1.08). Males had higher basal corticosterone levels than females (*F*-value = 1.75, value of $p = 0.048$, Table 2; predicted value for females = 1.28 ± 1.08, males = 1.65 ± 1.09). For chicks the best ranked model included only the Colony term (*F*-value = 4.80, value of $p = 0.005$, Table 2). Chicks from protected colony 2 (Lamarck Is) presented lower basal corticosterone levels (Table 2; Figure 2, contrasts detailed in Supplementary Table S1) while the other three colonies were not different (predicted value for station colony 1 = 1.04 ± 1.52; station colony 2 = 0.60 ± 1.54; protected colony 1 = 0.54 ± 1.54; protected colony 2 = 0.10 ± 1.60).

3.4. Determinants of stress-induced corticosterone levels

For incubating adults the best ranked model included body condition and sex, but it did not include the terms colony or area. Birds in poorer body condition had higher stress-induced corticosterone levels (*F*-value = 16.21, value of $p < 0.001$, Table 2; Figure 3A) and there was weak evidence that males tended to have lower stressed-induced corticosterone levels than females (Table 2; predicted value for males = 19.37 ± 1.02, for females = 22.77 ± 1.35).

For chick-rearing adults the best ranked model included body condition, brood size, sex and the interaction between brood size and sex, but it did not include the terms colony or area. Again, individuals in poorer condition had higher levels of stress-induced corticosterone (*F*-value = 12.27, $p = <0.001$, Table 2; Figure 3B). Females with a higher brood size had higher levels of stress-induced corticosterone (*F*-value = 13.26, $p = <0.001$, Table 2; predicted value for females with one chick = 16.24 ± 1.15, two chicks = 22.71 ± 1.13), but this was not the case for males. The model selection procedure showed the second-ranked model to be similarly weighted (i.e., AIC weight ratio was only 1.10). In

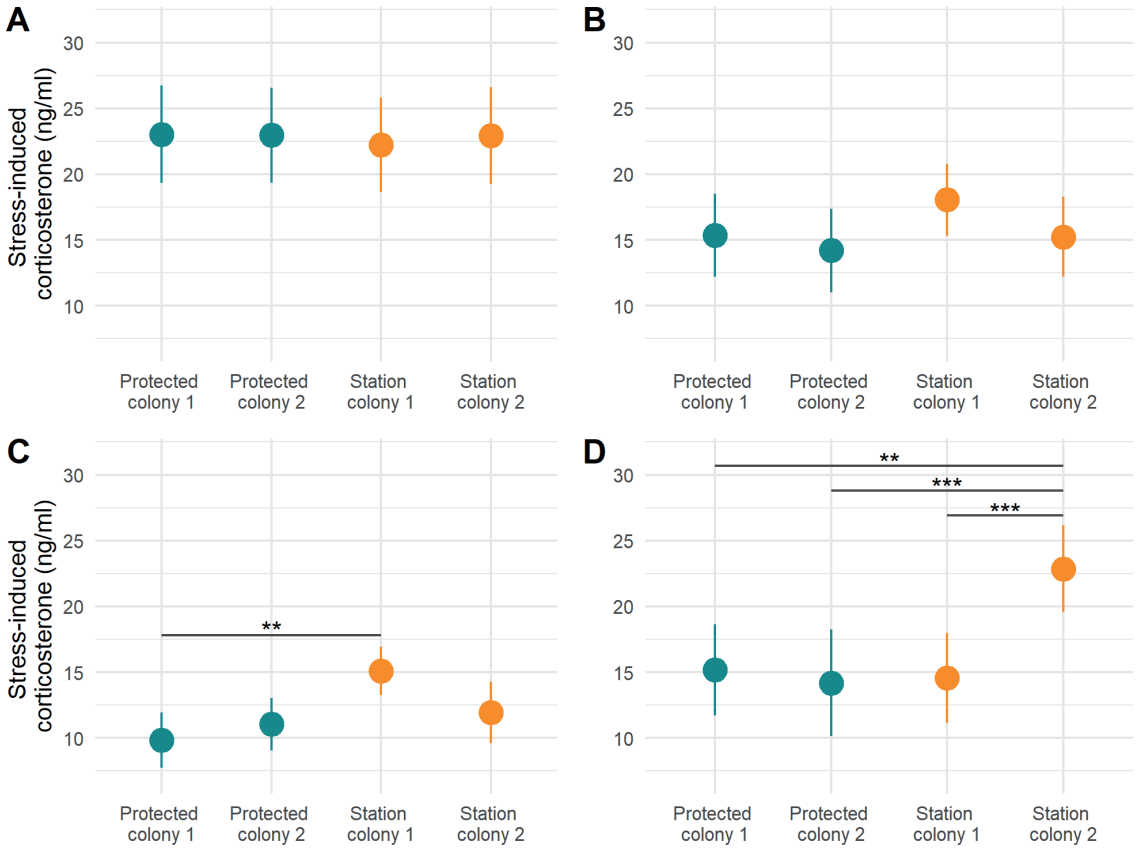

FIGURE 4
Stress-induced corticosterone in relation to the colony in incubating adults **(A)**, rearing adults **(B)**, male chicks **(C)**, and female chicks **(D)**. Predicted mean values ± SE are plotted. Asterisks denote statistical significance between two groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$) based on contrast analyses (presented in Supplementary Table S3).

this second model the area term was retained indicating lower stress-induced corticosterone in the undisturbed area, but this effect was not significant (coefficient estimate = −1.69 ± 1.19, *t*-value = −1.43, value of $p = 0.16$; predicted value for station colonies = 20.44 ± 0.83, protected colonies = 18.75 ± 0.83). Incubating and chick-rearing adults did not present any significant differences in stress-induced corticosterone levels between colonies (Figures 4A and B).

For chicks the best ranked model included body condition, colony, sex and the interaction between sex and colony. The colony-level effects showed male chicks from station colony 1 had a higher level of stress-induced corticosterone than chicks from the two protected colonies ($p = 0.009$ and 0.069; Table 2; Figure 4C; contrasts detailed in Supplementary Table S3) and female chicks from station colony 2 presented higher levels of stress-induced corticosterone than the three other colonies (all $p < 0.001$; Table 2; Figure 4D; contrasts detailed in Supplementary Table S3).

4. Discussion

4.1. What impact of human activities on body condition and corticosterone levels?

Contrary to our hypotheses, there was no difference in either body condition or corticosterone levels between the disturbed and undisturbed areas for breeding adult Adelie penguins. In chicks, the individuals from some disturbed colonies, but not others, had higher corticosterone levels that the chicks from undisturbed colonies, indicating that there was no strong and consistent difference in corticosterone level and body condition between disturbed and undisturbed areas (see Table 3). Our study therefore suggests that human activities have no major effect on the reproduction of this species in our study sites. Supporting this interpretation, the Adélie penguin population on the Pointe Géologie archipelago has been increasing over the last 10 years despite the human activities on this site (Barbraud et al., 2020). Indeed, this Adélie penguin population had increased even during major building construction (Micol and Jouventin, 2001). Similarly, other studies have found no evidence that human disturbance effects reproductive success and population trends in several sites of western Antarctica (Carlini et al., 2007; Lynch et al., 2010; Villanueva et al., 2014; but see Woehler et al., 1994; Giese, 1996).

The lack of difference in body condition between the disturbed and undisturbed areas in any group (breeding adults or chicks) suggests that human activities have no detrimental effect on energy expenditure or acquisition. Nonetheless, human presence can induce increased heart rate in some Antarctic birds including Adélie penguins (e.g., Culik et al., 1990; Weimerskirch et al., 2002; de Villiers et al., 2006; Ellenberg et al., 2013) which may be associated with higher energy expenditure, and a quicker depletion of energy reserves in fasting animals (e.g., Groscolas et al., 2010). In addition, human presence can affect foraging efficiency of breeding penguins if this delays departure from the colony or return from the sea (French et al., 2019). Body condition is a crucial

determinant of breeding success in Adélie penguins (Vleck and Vleck, 2002). First, nest desertion occurs when adults reach a low threshold in body condition (Spée et al., 2010; Thierry et al., 2013). Second, chick's body condition is thought to be the main determinant of fledging success and post-fledging survival (Ainley et al., 2018). Because we did not find any evidence of an effect of human activities on body condition, our study suggests that Adélie penguins at DDU are tolerant to interactions with humans and this could explain why colonies were able to persist in the middle of the station, perhaps due to individuals being habituated to this level of human activity.

Adult baseline and stress-induced corticosterone levels did not differ between the disturbed and undisturbed areas. We had hypothesized that human disturbance would be the main determinant of corticosterone levels (predictions 1b, 1c, 2b, and 2c), but instead corticosterone levels were mainly affected by body condition and brood size. The similarity in both baseline and stress-induced corticosterone levels between disturbed and undisturbed areas suggest that the functioning of the HPA axis was not affected by human disturbance in these colonies.

In contrast, there were variations in chick basal and stress-induced corticosterone levels that were consistently (but not exhaustively) in the direction of higher corticosterone levels in some of the disturbed colonies (i.e., sensitization). The best model retained the colony term rather than the area (i.e., level of disturbance) but because the colonies with the highest corticosterone levels were within the disturbed areas there is some support for a weak and inconsistent disturbance effect. This inter-colony variability in corticosterone levels shows the necessity of replication because only one disturbed and one protected colony could have led to misleading conclusions. Previous studies have reported that human activities did influence corticosterone levels in penguins and other species (reviewed in Dantzer et al., 2014), for example, and according to our predictions 1b and 1c, Ellenberg et al. (2007) reported Yellow-eyed penguins (*Megadyptes antipodes*) had higher baseline and stress-induced corticosterone levels and lower reproductive performance in disturbed areas. Similarly, Müllner et al. (2004) reported that tourist activity was associated with increased stress-induced corticosterone levels, lower body mass, and reduced survival in hoatzin juveniles (*Opisthocomus hoazin*). In contrast, other studies reported that individuals have lower stress-induced corticosterone levels or fecal corticosterone levels in response to human disturbance in penguins species (Barbosa et al., 2013; Scheun et al., 2021).

We predicted a dampened stress response (prediction 2c) because Adélie penguins may have habituated to human disturbance in the disturbed area (Rich and Romero, 2005; Cyr and Romero, 2009). Habituation enables individuals to avoid a detrimental state of chronic stress (Angelier and Wingfield, 2013). Adult Magelanic (*Spheniscus magellanicus*) penguins had lower stress-induced corticosterone levels in response to frequent tourism activities Walker et al. (2006) and this habituation was not associated with any apparent fitness costs. Other studies have also reported no significant effect of human disturbance on plasma (Walker et al., 2005; Villanueva et al., 2012) or fecal corticosterone levels (Ozella et al., 2017; Lynch et al., 2019). In our disturbed site, penguins live in the middle of the station (and have done for 60 years), but human activities do not seem to represent a threat to them. Adélie penguins have not co-evolved with terrestrial predators, and so adults and chicks may not perceive human presence a threat, and not modulate their corticosterone stress response. Altogether, these studies suggest that the response of the HPA axis to human disturbance (e.g., sensitization, habituation, or no effect) may be a key determinant of the ability of wild vertebrate populations to coexist with humans (Angelier and Wingfield, 2013).

The sensitivity of individuals to human disturbance may depend on life-history stages. For example, Magellanic penguin chicks are more sensitive to disturbance than adults because, contrary to adults, chicks do not show a dampened stress response in response to human presence, but even show increased stress-induced corticosterone levels during their first day of life (Walker et al., 2006). We therefore predicted that Adélie penguins may be more sensitive during specific stages (prediction 3). Despite only weak support and an inability to separate this effect from confounding factors (sex, colony), we observed this effect in chicks which presented higher levels of corticosterone (although not in all colonies even in the disturbed area). In addition, at our study site, a recent study reported that Adélie penguin chicks from the disturbed area had shorter telomeres relative to those from the undisturbed area, suggesting some hidden physiological costs of growing in disturbed areas at this stage (Caccavo et al., 2021). Further studies are therefore required to fully evaluate the behavioral and physiological impact of human disturbance on Adélie penguin chicks (possibly at different stages of development), and on their probability to recruit into the population.

Different penguin species may have different sensitivity to human disturbance. Humboldt penguin (*Spheniscus humboldti*) appears much

TABLE 3 Synthesis of main findings from the four markers examined in this study.

Body size (Flipper)	• *Sex effect*: in all stages, **males** were larger than females.
Body condition	• *Sex effect*: in incubating adults, **females** were generally in better condition at the exception of the females from Lamarck. • Inversely, in chick rearing adults, **males** were generally in better condition. • In chicks, no variation of body condition was evident
Basal corticosterone	• *Sex effect*: **males** presented higher corticosterone levels in rearing adults only • *Body condition*: individuals with a **better body condition** presented lower corticosterone levels, in incubating adults only (trend in chick-rearing adults) • *Brood size*: individuals with **2 chicks** presented higher corticosterone levels than individuals raising only one chick (in chick rearing adults) • *Colony*: chicks from the **protected colony 2** had lower corticosterone levels
Stress induced corticosterone	• *Sex effect*: **female** chicks presented higher corticosterone levels • *Body condition*: individuals with a **better body condition** presented lower corticosterone levels in adults (and male chicks) • *Brood size*: chick rearing females with **2 chicks** presented higher corticosterone levels than the ones raising only one chick • *Colony*: generally, chicks from **station colonies** had higher corticosterone levels (but see details in contrast table and figures).

more sensitive to human disturbance than the Magellanic penguin (Ellenberg et al., 2006). The history of co-habitation of a penguin species or population with humans and terrestrial predators may however modulate its sensibility to human disturbance (see Bricher et al., 2008; Villanueva et al., 2012; Viblanc et al., 2012; Pichegru et al., 2016 for some examples). The specific history of Antarctic penguins could explain why Adélie penguins appear quite tolerant to human disturbance (at least at our study site) because they have not co-evolved with terrestrial predators or human presence until very recently. It is however important to note that a few other studies have found that human presence can affect breeding success of this species in other areas (Woehler et al., 1994; Giese, 1996; Bricher et al., 2008), suggesting that human activities may be detrimental to this species in other populations or maybe under specific environmental circumstances (e.g., low food availability). Although we did not find any difference in our variables of interest between the disturbed and undisturbed areas, it is also important to note that Adélie penguins may benefit to some extent from human presence because buildings can provide shelters against inclement weather and predators such as skuas (some penguins nest under buildings, another sign of high tolerance to human activities). Finally, we cannot exclude that the impact of human disturbance on penguin physiology may vary between populations. For example, declining populations of Magellanic penguins showed chronic stress when exposed to tourism while increasing and stable populations did not (Palacios et al., 2018).

4.2. Inter-colony and individual heterogeneity in body condition and corticosterone levels

In addition to the comparison between the disturbed and undisturbed areas, we also investigated how our variables of interest varied between the four colonies, allowing us to document important variations in body condition and corticosterone levels at a finer scale. Given that differences in body condition or corticosterone levels between the four colonies were not driven by a strong and consistent effect of human disturbance, indicating the importance of other environmental or individual characteristics. All of the penguins breeding in this region forage in the same zones (Michelot et al., 2020), so food availability should not differ between the four studied colonies. Inter-colony differences in corticosterone levels are also unlikely to be linked to brood size as it did not differ between colonies. Similarly, body condition is unlikely to explain these differences in corticosterone levels between colonies because body condition did not dramatically vary between colonies. Abiotic factors, such as slope, orientation, exposition, and predation risk could explain the differences between colonies in our study (Patterson et al., 2003; Bricher et al., 2008; Schmidt et al., 2021), especially if penguins select their breeding site (i.e., colony) according to their individual quality or age. However, we did not find any important difference in body size or body condition between colonies.

Our study demonstrates that sensitivity to abiotic and biotic factors may vary between females and males. Adélie penguins do not have strong sexual dimorphism (Jennings et al., 2016) but breeding female and male Adélie penguins may forage in different areas (Widmann et al., 2015; Lescroël et al., 2020) and may differ in their parental expenditure (Beaulieu et al., 2009; Colominas-Ciuró et al., 2017). There were also differences in chick corticosterone levels between sexes, and sex-dependent differences in corticosterone levels between colonies. Although the origin of these differences remains unclear, they suggest that female chicks may be more sensitive to environmental constraints, and possibly human disturbance than male chicks (Jennings et al., 2016).

4.3. Conclusion

By studying complementary variables (body condition, corticosterone levels and breeding performance) at several colonies in two areas with contrasting levels of human disturbance, our study provides evidence that breeding Adélie penguins were not detrimentally affected by human activities, at least at the adult stage. Rather, in adults, baseline and stress-induced corticosterone levels were primarily influenced by body condition, sex, and brood size. In chicks, there was some support for higher corticosterone levels in the colonies located in the vicinity of the station relative to the colonies located in protected areas. Adélie penguins at Dumont D'Urville station seem to be quite tolerant to human presence, and this may result from the specific history of this species, which has not co-evolved with humans until very recently. Future studies should now investigate complementary and promising stress-related physiological markers, such as heart rate (Weimerskirch et al., 2002; Viblanc et al., 2012; Schaefer and Colombelli-Négrel, 2021) or telomere length (Caccavo et al., 2021; Salmon and Burraco, 2022), to further assess the influence of multiple human disturbance stimuli on Adelie penguins (Nimon et al., 1995; Ellenberg et al., 2013).

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by French Regional Ethical Committee in animal experimentation (No. 84), permit number APAFIS#2111–2,015,092,414,273,394 v4.

Author contributions

FA and TR designed the study. FA, TP, and TR conducted the fieldwork. FA, CM, and SB analyzed the data. CP and CR conducted the laboratory analyses. CB and KD provided additional data from the P109. CM and FA wrote the manuscript with input from TP, YR-C, MAH, SB, CP, TR, CR, KD, CB, and AK. 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

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Circulating corticosterone predicts near-term, while H/L ratio predicts long-term, survival in a long-lived seabird

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Assessing stress in wild populations is important in many ecological and conservation contexts because the physiological responses of individuals to stressors can be used to identify at-risk populations and the ability to respond appropriately to stressors is related to individual quality and fitness. Yet, one of the great challenges in ecophysiology is linking physiological measures in wild animal populations with changes in individual fitness. Here, we examined two indices of stress, namely, circulating baseline corticosterone concentration ([Cort]) and the heterophil:lymphocyte (H/L) ratio, in a long-lived seabird, the Nazca booby (*Sula granti*) and their relationship with current individual state and subsequent survival and residual and lifetime reproductive success. [Cort] was related to sex, age, and current reproductive effort in in that males, older birds, and birds currently engaged in a breeding attempt birds had higher [Cort]. [Cort] was negatively associated with survival to the next breeding season. The H/L ratio was not associated with the current state of birds but predicted cohort-specific long-term survival. Lifespan and reproductive performance are correlated in Nazca boobies; therefore, our results suggest that the H/L ratio may be useful as an indicator of overall fitness, while [Cort] can be used to predict current or near-term fitness in this species. We further propose the H/L [or neutrophil/lymphocyte (N/L)] Ratio-Fitness Hypothesis, which posits that this ratio is repeatable within individuals and are negatively associated with fitness. This hypothesis needs to be tested in Nazca boobies and other species, and when supported by empirical evidence, then these ratios could be a powerful monitoring tool for assessing population health or identifying at-risk populations.

KEYWORDS

seabird, *Sula granti*, stress response, glucocorticoids, allostasis, H/L ratio, survival, lifetime reproductive success

1. Introduction

Assessing stress in wild populations is important in many ecological and conservation contexts because the physiological responses of individuals to stressors can be used to identify at-risk populations (Wikelski and Cooke, 2006; Fefferman and Romero, 2013), and the ability to respond appropriately to stressors is related to individual quality and fitness (Romero et al., 2009; Angelier et al., 2010). Environmental and anthropogenic disturbances activate the vertebrate neuroendocrine hypothalamic–pituitary–adrenal (HPA) axis, resulting in the production and release of glucocorticoids (GCs). Elevated GCs act within a relatively short time frame to help vertebrates cope with the immediate stressor by

modulating energy allocation, metabolism, behavior, and immune function (Romero, 2004; Martin, 2009). Thus, GCs are a primary mediator of vertebrate allostasis or the ability of individuals to cope with changing environments (McEwen and Wingfield, 2003). Therefore, baseline GCs should be an indicator of how well an individual copes with its environment when individuals are sampled under the same conditions (Bonier et al., 2009a).

Many hypotheses have been proposed to explain the relationship between baseline GCs and fitness (Schoenle et al., 2018); however, three non-mutually exclusive hypotheses have emerged as the broadest (Breuner and Berk, 2019). The "Cort-Fitness Hypothesis" predicts that individuals with elevated baseline GCs have low fitness (integrating survival and reproductive success), because baseline GCs increase with environmental challenges, and fitness decreases with increasing environmental challenges (Bonier et al., 2009a). While this hypothesis is supported by the literature, a major exception often occurs during reproduction, giving rise to the "Cort-Adaptation Hypothesis" (Bonier et al., 2009a). This modified version of the Cort-Fitness Hypothesis predicts that when the environmental challenge is associated with reproduction, elevated baseline GCs correlate with increased reproductive success because high-quality individuals can increase their allostatic load (indicated by baseline GCs) beyond those of low-quality individuals. Finally, the "Cort-Tradeoff Hypothesis" represents the long-standing view that GCs mediate tradeoffs between reproduction and survival; thus, elevated GCs will increase survival but decrease reproductive success (Breuner and Berk, 2019). Originally applied to stress-induced GCs, this hypothesis has since expanded to include baseline GCs (Schoenle et al., 2018).

Life-history strategies appear to be a major determinant of the effects of GCs on fitness, with short-lived species generally providing support for the Cort-Adaptation Hypothesis and long-lived species supporting the Cort-Tradeoff Hypothesis (Bókony et al., 2009; Hau et al., 2010; Breuner and Berk, 2019). However, few studies have tested these hypotheses in long-lived animals, because long-term (i.e., > 10 years) data sets on lifetime reproductive success and survival can be challenging to obtain. Long-lived species, such as seabirds, are largely able to escape adult mortality due to predation. Because of this, they demonstrate a shift toward adult self-maintenance and an extended reproductive lifespan and away from short-term reproductive effort (Apanius et al., 2008). Thus, environmental stressors (e.g., low food availability) typically result in decreased reproductive success, but no change in adult survival until stressors become severe or prolonged (Kitaysky et al., 2007). Previous work in seabirds has revealed that GC secretion is an indicator of nutritional stress during the breeding season, as it increases with challenging environmental conditions and declining food supply (Kitaysky et al., 2010; Satterthwaite et al., 2010; Will et al., 2020; Shimabukuro et al., 2023) and decreases with experimental supplemental feeding (Schultner et al., 2013). Nutritional stress, and thus GC secretion, then negatively predicts reproductive success (Kitaysky et al., 2007) and adult survival when breeding in poor food conditions (Kitaysky et al., 2010; Satterthwaite et al., 2010) in long-lived seabirds, in general supporting the Cort-Fitness Hypothesis.

Although GCs are frequently measured in wild animals, their collection and interpretation pose many challenges. Because GCs increase rapidly in circulation in response to stressors, collection of baseline GCs is extremely time-sensitive (typically requiring collection within 3 min of disturbance for birds) (Romero and Reed, 2005). Moreover, "normal" GC concentrations vary by breeding state, season, age, sex, time of day, and recent social interactions, as well as exposure to stressors (Romero and Wingfield, 2016). Finally, low baseline GC concentrations could indicate a low level of HPA axis activation or suppression of the HPA axis due to acclimation to a stressor, chronic exposure to long-term stressors (Johnstone et al., 2012), or increasing age (Heidinger et al., 2006, 2008).

Given these difficulties in obtaining and interpreting baseline GCs, ecologists increasingly have turned to leucocyte profiles, specifically the heterophil/lymphocyte ratio (H/L) in birds and reptiles, or the neutrophil/lymphocyte (N/L) ratio in fish, amphibians, and mammals, as a measure of stress in wild animals. Leucocytes are redistributed to different areas of the body in response to elevated GCs. Specifically, lymphocytes leave circulation (i.e., lymphopenia), while heterophils or neutrophils enter circulation (i.e., heterophilia or neutrophilia Dhabhar et al., 1996; Davis et al., 2008). This redistribution of leucocytes is slower than changes in circulating GC concentrations, sometimes occurring up to 24h after stressor exposure (Davis and Maney, 2018). Thus, the H/L(N/L) ratio is easier to obtain for wild populations because sampling is far less time-sensitive than for GC concentrations. Although both circulating GCs and H/L(N/L) ratios typically change after exposure to an acute stressor, these indices may not be interchangeable because they are not always correlated at baseline due to the differences in the timing of the onset of these changes (reviewed by Davis and Maney, 2018). This had led some authors to argue that these metrics reflect different types of stressors (Müller et al., 2011) or that leucocyte profiles are better suited for assessing chronic stress (Davis and Maney, 2018) while GCs may be better for assessing current acute stressors (Romero and Wingfield, 2016; Davis and Maney, 2018). In addition, the H/L ratio may be an important evolutionary and life-history trait in birds because a phylogenetic analysis found a negative relationship between the H/L ratio and lifespan at the order and superfamily level (Minias, 2019).

In this study, we examined baseline corticosterone (the primary avian GC) and the H/L ratio in a long-lived seabird, the Nazca booby (*Sula granti*; Anderson and Apanius, 2003) during the early breeding season. We evaluated the relationships between these indices of stress, current individual state, and subsequent survival and reproductive success to test the Cort-Fitness, Cort-Adaptation, and Cort-Tradeoff hypotheses.

2. Materials and methods

2.1. Field site and sample collection

Adult Nazca boobies were sampled in the breeding colony at Punta Cevallos, Española, and Galápagos Islands, Ecuador from 24 November to 5 December 2009 (see Apanius et al., 2008 for a detailed description of the site). Nazca boobies breed from

October to May or June of the following calendar year (Huyvaert and Anderson, 2004); therefore, sampling was performed early in the 2009–2010 breeding season. Birds at Punta Cevallos have been banded and monitored for survival since 1984. Monitoring of the reproductive success of banded individuals began in 1992. This continuing effort has allowed the accumulation of detailed longitudinal data on survival and reproduction in this species (e.g., Tompkins and Anderson, 2019, 2021) because site fidelity is essentially 100% (Huyvaert and Anderson, 2004).

Adult birds were caught and restrained by hand from 0300h to 0520h, which is the period of the highest circadian corticosterone concentration and when birds are least likely to be disturbed by external events (Tarlow et al., 2003a). Blood samples (1–2 ml; ≤1% of body weight) were collected by brachial venipuncture, allowed to clot at ambient temperature for 2–4 h in 1.5-ml microfuge tubes, and then centrifuged at 6000 rpm for 5 min. The serum was then transferred to a clean 1.5-ml cryovial and frozen in the field in liquid nitrogen. Samples were transported from the field in liquid nitrogen and then stored at −80°C until laboratory analysis. Sampling time from the moment the bird was captured was recorded. If sampling took longer than 3 min, then any further blood collection was put into a second 1.5-ml microfuge, labeled as ">3 min", and treated as described previously. At the time of sampling, one drop of fresh blood was used to make blood smears on microscope slides that were air-dried and fixed in methanol in the field (Fudge, 2000). Smears were kept at ambient temperature until laboratory processing.

We sampled birds across all age classes present in the colony, but particularly focused on birds that fledged during the 2002–2003 breeding season, which were 7 years old in 2009–2010. Young Nazca boobies return to the breeding colony between the ages of 2 and 7 years (Maness and Anderson, 2013; Champagnon et al., 2018). Thus, the 2002–2003 cohort was sampled toward the beginning of their reproductive years, with relatively little opportunity for the selective disappearance of low-quality adults to limit their representation in the sample. The adult sex ratio is male-biased in the study colony (Maness et al., 2007) due to the lower survival probability of females during the juvenile period (Maness and Anderson, 2013). Efforts were made to sample equal numbers of males and females.

2.2. Sample processing

Total bound and unbound circulating corticosterone concentration ([Cort]) was measured by quantitative competitive enzyme immunoassay (Enzo Life Sciences/Assay Designs, Cat. No. ADI-901-097), validated for use with Nazca booby serum for accuracy, precision, cross-reactivity, and parallelism in measurements (Grace et al., 2011). Serum was used directly in the enzyme immunoassay as described by Grace and Anderson (2014), and samples were run in duplicate. All corticosterone assays were performed by JKG and samples were part of a larger sampling effort, which were all analyzed simultaneously. For all samples in this larger dataset, the efficiency of immunoassay in measuring known quantities of corticosterone (using the supplied corticosterone standard diluted in stripped chicken serum) averaged 100% (SD = 6.6, N = 23). The immunoassay detection limit was 0.078 nmol/L, and average intra- and inter-assay coefficients of variation were 3.4% (SD = 0.5%, N = 190) and 6.2% (SD = 1.3%, N = 30), respectively. Because the primary antibody in the assay did not cross-react to a significant degree with other circulating steroids, all measures are called "Cort" measurements. Blood smears were stained using a Hemacolor® Staining Kit (EMD Millipore Corp., product code 65044), following manufacturer protocols. Stained slides were viewed at 400X magnification to estimate the total white blood cell count (TWBC; Fudge, 2000). Smears were then viewed at 1000X magnification for differential blood cell counts following Fudge (2000). All blood cell counts were performed by MRH.

2.3. Statistical analyses

[Cort] and H/L measurements were examined for outliers using the Dixon outlier range statistic, which identifies the most extreme value at the upper or lower limit as an outlier if D/R > 0.3, where D = |extreme value – next nearest value| and R is the range of all values (Dixon, 1983). Reference intervals for [Cort] and the H/L ratio in Nazca boobies were calculated with MedCalc Statistical Software (version 20.216, Ostend, Belgium) using non-parametric estimation when the sample size was large and the robust method when the sample size was small (Geffré et al., 2011). Adult Nazca boobies were categorized by their breeding status when sampled as follows: non-breeder (no breeding attempt in 2009–2010), pre-breeder (started a breeding attempt after sampling), incubating an egg, brooding a chick, breeder that failed at the egg stage, and breeder that failed at the chick stage. The effect of age, sex, sampling date, and breeding status on [Cort] or the H/L ratio was determined with general linear models.

Short-term effects of [Cort] on survival probability in the year following sampling and long-term effects of both [Cort] and H/L ratio on average annual survival probability over the next 11 years (2009–2010 to 2021–2022) were evaluated by fitting data from an annual band-resight survey (BRS) to capture–mark–recapture (CMR) models. CMR models estimated apparent annual survival probabilities from individual recapture histories while controlling imperfect detection (Lebreton et al., 1992) and were used to control detection probabilities that were less than one in Nazca boobies (Townsend et al., 2007; Champagnon et al., 2018; and lower in females). Recapture histories covered all breeding seasons from 2009–2010 to 2021–2022 except 2020–2021 (details of the BRS in Huyvaert and Anderson, 2004; Champagnon et al., 2018).

Effects of [Cort] and H/L ratio on survival were modeled separately because of a difference in sample size (N = 565 birds for [Cort], N = 85 birds for H/L ratio). Initially, previous results were used to construct a base model: female Nazca boobies have slightly lower annual survival than males do (Champagnon et al., 2018; Tompkins and Anderson, 2019), and each sex shows actuarial senescence starting in the late teens. Sex differences in the rate and timing of actuarial senescence are slight (Tompkins and Anderson, 2019), and, given our small sample size, we started with a base model predicting survival by sex (a two-level factor) and age (a continuous variable), but not their interaction. Age effects were

modeled using a threshold function allowing slope estimates to change (e.g., become steeper) after age 16 (following Tompkins and Anderson, 2019). Detection probabilities were sex-specific. [Cort] or H/L ratio was added as a continuous predictor of annual survival probability to our base model, and their performance was evaluated using AICc-based model comparison (Burnham and Anderson, 2010). For the [Cort] model set, a third candidate model was included, fitting an interaction between [Cort] and a two-level factor differentiating the 2009–2010 to 2010–2011 interval from all other years. This interaction tested the hypothesis that [Cort] predicts survival to the next breeding season but does not affect survival over subsequent intervals.

Initially, analyses were run on data from the 2002–2003 cohort ($N = 285$ birds with [Cort] measurements, 38 birds with H/L measurements). Analyses were then repeated on data from all cohorts combined ($N = 565$ birds with [Cort] measurements, 85 birds with H/L measurements).

Models were constructed using a logit link function and fit using the program MARK (White and Burnham, 1999) to generate maximum-likelihood estimates of survival and detection parameters. We ran MARK through the RMark interface (v.3.0.0; Laake, 2013) in R (v.4.2.2; R Core Team, 2022). We evaluated the overall fit of our base model on data from the [Cort] data subsets (all cohorts combined vs. the 2002–2003 cohort only) using the median ĉ method in program MARK; goodness-of-fit testing was performed on the base model, and not on our most complex model, because individual covariates, like [Cort] and H/L ratio, are not allowed using the median c method. Estimated overdispersion parameters (ĉ) were close to 1 for the base models (for [Cort]), suggesting a reasonable fit (ĉ = 1.09 [95% CI: 1.08–1.10] and ĉ = 1.20 [95% CI: 1.05–1.46] for all cohorts vs. the 2002–2003 cohort data subsets). We mean-centered [Cort] before analysis.

Effects of [Cort] and H/L ratio on reproductive performance were evaluated using "lifetime" reproductive success (LRS), calculated as the sum of offspring produced through the 2019–2020 breeding season. Although 50% of the 2002–2003 cohort were still alive in the final year of the study (age 17 in 2019–2020), reproductive senescence begins in the mid-teens (Anderson and Apanius, 2003; Tompkins and Anderson, 2019), and offspring production through age 17 is highly correlated with lifetime reproductive success in older cohorts (males: Pearson's r = 0.96, d.f. = 1,504, $P < 0.01$; females: Pearson's r = 0.97, d.f. = 1,202, $P < 0.01$; data from cohorts 1984–1987 and 1992–1995).

Nazca boobies raise at most one offspring per breeding season (Humphries et al., 2006). Reproductive success for each parent, in each breeding season, was assigned based on daily monitoring of banded offspring late in the breeding cycle. Rarely, a nestling/fledgling will die after monitoring ends but before reaching independence and we adjusted reproductive success based on the recovery of offspring bands/carcasses during the following two breeding seasons (following Tompkins and Anderson, 2021). Offspring raised in 2007–2008 were excluded from lifetime reproductive success estimates because reproductive success was not monitored for some study colony subsections. We excluded 17 boobies with [Cort] measurements from analyses (6% of the total) because they occupied a colony subsection that was not monitored for reproductive success in 2017–2018 through 2019–2020 (location information from the annual band resight survey identified these individuals) for a final sample size of 268 birds in the 2002 cohort (38 for the H/L ratio) and 557 birds in all cohorts combined (85 for the H/L ratio).

As was done for survival, the effects of [Cort] or H/L ratio on reproductive performance were assessed separately, first for members of the 2002–2003 cohort and then for the full dataset. Effects of [Cort] and H/L ratio on lifetime reproductive success for the 2002–2003 cohort were evaluated in R using linear models (LMs) with a Gaussian error distribution and an identity link function. Lifetime reproductive success for the 2002–2003 cohort was predicted by sex (a two-level factor) and either the H/L ratio or [Cort]. The magnitude of slope estimates and span of 95% CIs relative to zero were used to evaluate support for [Cort] and the H/L ratio as predictors of lifetime reproductive success. Finally, because lifetime reproductive success is a count variable, we verified that our results were unchanged when fit with a GLM with Poisson errors and a log link function (Zuur et al., 2009). The same approach was used for the data from all cohorts combined, except using linear mixed models (LMMs; using package lme4; Bates et al., 2015) and including cohort as a random intercept in addition to the fixed effects (sex and either [Cort] or the H/L ratio).

The baseline [Cort] and H/L ratio sampled in the 2009–2010 breeding season may not reflect individual state earlier in life, and we repeated our reproductive performance analyses using summed reproductive success from 2009–2010 onward as the response variable ("residual reproductive success") using GLMs (for the 2002–2003 cohort) and GLMMs (for all cohorts) with Poisson errors and a log link function. Cohort was included as a random intercept, accounting for cohort-level differences in residual reproductive success due to the age when stress indices were measured or to other factors. Finally, we verified that including individuals with incomplete LRS information (still alive at the end of the study) did not affect our results by repeating our evaluation of stress effects on reproductive performance (for LRS and residual reproductive performance) using data restricted to cohorts 1994–1995 and earlier ($N = 158$ for [Cort], $N = 35$ for the H/L ratio). A few members (8%) of the 1994–1995 cohort were still alive in 2019–2020 (and even lower percentages for the 1992–1993 and 1993–1994 cohorts), but successfully producing an offspring at age 25 or older is extremely rare (only four cases in our long-term data).

3. Results

The 2009–2010 breeding season was a poor year for reproductive success in the colony at Punta Cevallos. In 27 years of regular nest monitoring by our group, only two other breeding seasons, which were years of strong El Niño Southern Oscillation warm events (Clifford and Anderson, 2001; Champagnon et al., 2018), had lower reproductive success (Figure 1). This indicates that the birds in our colony experienced greater-than-usual environmental stressors associated with reduced food availability and/or prey quality (Champagnon et al., 2018) at the time of sampling.

One outlier was identified for baseline [Cort] and was subsequently removed for measurement of reference intervals. However, we had no indication that this value was due to sampling

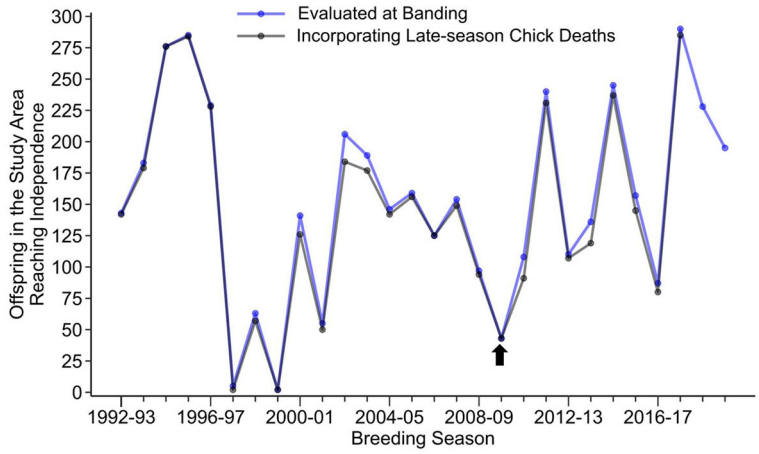

FIGURE 1
Reproductive success, as indicated by the production of an independent offspring, of Nazca booby breeding pairs located in an area of the colony designated as the "Study-Area", where all nests are monitored regardless of the banding status of the parents (Huyvaert and Anderson, 2004). Only the 1997–1998 and 1999–2000 breeding seasons had lower reproductive success than the year of sample collection in the current study (2009–2010, indicated by the arrow). Production of independent offspring was assessed at two different time points during the breeding season, at the time the chick was banded (blue line), which was typically done at the 1% down developmental stage (Maness et al., 2011), and at a later time that would include late-season chick deaths (blue line; Tompkins and Anderson, 2021).

or analytical error, so we retained this sample for further analyses. Tests were run with and without this sample to see whether the results changed and, as none did, the results we present included this value. The reference interval (RI) for baseline [Cort] as measured by the non-parametric method was 25.66 (90% CI = 24.87–28.7) – 278.63 (90% CI = 258.12–295.91) nmol/L. In total, 14 birds had elevated [Cort] (i.e., above the upper RI limit) and 14 birds had values below the lower RI limit. Those with high [Cort] were 2.5 times more likely to be in older age classes, and those with low values were 4.7 times more likely to be female and 11.2 times more likely to be in younger age classes (odds ratio 95% CI = 1.0–7.6; 1.8–11.2; and 4.8–26.3, respectively). Comparison of our baseline [Cort] with other published studies of sulids revealed that our values were higher than most, except for oiled and breeding northern gannets (*Morus bassanus*) (Table 1).

No outliers were identified for the H/L ratio or individual cell count. In total, 11 slides were counted twice to assess the reliability of the white blood cell count. Intraclass correlation coefficients were calculated using two-way random-effects models (ICC; Liljequist et al., 2019) and were TWBC ICC = 0.93 (95% CI = 0.74–0.98), lymphocyte count ICC = 0.93 (95% CI = 0.73–0.98), heterophil count ICC = 0.88 (95% CI = 0.57–0.97), eosinophil count ICC = 0.85 (95% CI = 0.46–0.97), basophil count ICC = 0.85 (95% CI = 0.45–0.96), and monocyte count ICC = 0.78 (95% CI = 0.19–0.94). The RI for the H/L ratio as estimated by the robust method ranged from 0.15 (90% CI = 0.13–0.18) to 1.15 (90% CI = 0.99–1.39). RIs for TWBC and individual cell types are available in Supplementary Table 1. Three birds had low H/L ratios, and two had high ratios. The sample size of birds falling outside the RI limits for the H/L ratio was too small to reliably calculate odds ratios for group membership. Our H/L ratio values were lower than values reported in other studies of sulids (Table 2). However, most studies did not report variability measures in this index or did not calculate the H/L ratio from their differential white blood cell counts (Table 2), which makes comparisons difficult. We also had a substantially larger sample size than the other studies (Table 2).

The baseline [Cort] and H/L ratio were natural log-transformed to meet normality assumptions, with 1 added to the H/L ratio before log transformation to avoid negative values for ratios that were < 1.0. Sampling time was >3 min for 24 birds; however, all samples were collected within 5 min of capture. The mean baseline [Cort] was not different between birds sampled in ≤ 3 min (Ln(mean) = 4.50; 95% CI = 4.45–4.55) and those sampled in 3–5 min (Ln(mean) = 4.63; 95% CI = 4.38–4.87; $F_{1,27} = 0.99$, $P = 0.32$); therefore, all samples were combined for all analyses.

3.1. Effects of the current state on stress indices

Model ranking (Burnham et al., 2011) of baseline [Cort] included two top additive models: sex, age, and reproductive state and reproductive state and age (Table 3). The relative importance of the variables included in the top models was as follows: reproductive state = 0.87, age = 0.87, and sex = 0.52. Males had a higher baseline [Cort] than females; birds that were currently engaged in a breeding attempt had a higher baseline [Cort] than other groups; and age was positively associated with baseline [Cort] (Figures 2A–C). Models including sex, reproductive state, age, and date and additive combinations of these predictors did not explain more variation in the H/L ratio than the null model (Supplementary Table 2). No association between baseline [Cort] and H/L ratio (Pearson's $r = -0.08$; $P = 0.54$) or between [Cort] and any white blood cell type count (data not shown; $P > 0.45$) was found.

TABLE 1 Baseline [Cort] values (converted to standard international (SI) units if needed) and sample sizes (N) reported in studies of sulids.

Species	N	Baseline [Cort] (SI: nmol/L)	State	Study
Nazca Booby *(Sula granti)*	576	107.5 (± 63.9 SD)	Breeding season	This study
	222	45.3 (± 41.0 SD)	Breeding	(Grace and Anderson, 2014
	80	2.9 – 8.7	Non-breeding and moon phase	(Tarlow et al., 2003a)
	42	14.4 – 23.1	Non-breeding	(Tarlow et al., 2003b)
Blue-footed Booby *(Sula nebouxii)*	140	28.8 – 77.9	Breeding season	(Wingfield et al., 1999)
Red-footed Booby *(Sula sula)*	22 22	14.4 – 37.5 11.5 – 100.94	Breeding in El Niño Breeding in La Niña	(Wingfield et al., 2018)
Australasian Gannet *(Morus serrator)*	8	8.91 (± 1.5 SE)	Breeding	(Cockrem et al., 2016)
Northern Gannet *(Morus bassanus)*	58	115.4 – 187.5	Oil exposed and Breeding	(Franci et al., 2014)

Breeding season refers to birds that were sampled during a breeding season, and the breeding status of birds was either unknown or included both breeding and non-breeding birds.

TABLE 2 The H/L ratio, sample size (N), state at sampling, total white blood cell count (TWBC), heterophil count, and lymphocyte count of other studies in adult sulids.

Species	N	State	TWBC (10^3/uL)	Heterophil (10^3/uL)	Lymphocyte (10^3/uL)	H/L	Study
Nazca Booby *(Sula granti)*	86	Breeding season	6.32 ± 2.68	1.63 ± 0.93	3.74 ± 1.62	0.46 ± 0.22	This study
	30	Breeding season	17.55 ± 3.33	5.94 ± 1.29	5.17 ± 1.23	1.19 ± 0.32	(Tucker-Retter et al., 2021)
	24	Non-breeding	9.40 ± 3.50	4.39 ± 1.34	3.23 ± 1.33	1.36	(Padilla et al., 2006)
Blue-footed Booby *(Sula nebouxii)*	26	Breeding (↑ effort)	–	–	–	2.30	(González-Medina et al., 2015)
	26	Breeding (↓ effort)	–	–	–	1.20	
	26	Breeding (control)	–	–	–	1.19	
Red-footed Booby *(Sula sula)*	31	Breeding	4.54 ± 1.06	2.29 ± 0.65	2.78 ± 0.70	0.82[a]	(Lewbart et al., 2017)
	18	Non-breeding	10.30 ± 4.70	3.72 ± 1.72	5.64 ± 1.80	0.66	(Padilla et al., 2006)
	35	Breeding	9.91 ± 2.69	5.73 ± 2.18	3.26 ± 1.41	1.76	(Work, 1996)
Brown Booby *(Sula leucogaster)*	37 (F)	Breeding	–	–	–	0.73	(Dehnhard and Hennicke, 2013)
	34 (M)	Breeding	–	–	–	1.33	
	35 (F)	Breeding	10.33 ± 3.09	7.23 ± 2.23	1.22 ± 0.60	5.93[a]	(Work, 1999)
	35 (M)	Breeding	8.60 ± 2.94	6.19 ± 2.15	0.96 ± 0.47	6.45[a]	
Northern Gannet *(Morus bassanus)*	27	Breeding	4.81 ± 1.82	2.79 ± 1.06	0.78 ± 0.40	4.07 ± 1.78	(Malvat et al., 2020)
Cape Gannet *(Morus capensis)*	46	Breeding	–	–	–	3.05 ± 1.35	(Moseley, 2010)

F indicates females, while M indicates males.

[a]This study did not report the H/L ratio, so we calculated this value from reported mean values for heterophils and lymphocytes. Our calculated value should differ from values calculated in individual birds but should be relatively close to the true population value.

Breeding season refers to birds that were sampled during a breeding season and the breeding status of birds was either unknown or included both breeding and non-breeding birds.

3.2. Effects of stress indices on subsequent survival

For the 2002–2003 cohort, baseline [Cort] did not influence survival: the base model outperformed all others (Table 4A) and coefficient estimates describing the effect of [Cort] on annual survival probability immediately after the study ($\beta = -0.77$ [95% CI: −2.74–1.21]) and in later years ($\beta = -0.03$ [95% CI: −0.34–0.29]) were not distinct from zero. Although a candidate model including baseline [Cort] falls < 2 ΔAICc units from the top model (Table 4), this does not indicate support for the predictor: when a candidate model is identical to the top model except for the addition of one predictor, that model will be < 2 ΔAICc units from the top simply because the penalty for the additional complexity is low (2 AIC units) and not because the additional predictor is supported (Burnham and Anderson, 2010).

TABLE 3 Model ranking of potential predictors of baseline [Cort] in adult Nazca boobies showing the model tested, the change in Akaike information criterion corrected for small sample sizes (ΔAICc), the model weight (ω_i), number of predictors in the model (K), the residual sums of squares of the model (RSS), and evidence ratio (Burnham et al., 2011).

Model	ΔAICc	ω_i	K	RSS	Evidence ratio
Sex + Age + State	0.00	0.523	5	201.5	1.00
State + Age	0.83	0.345	4	203.8	1.52
Sex + State	4.18	0.065	4	206.5	8.09
State	5.69	0.030	3	209.5	17.24
Date	6.96	0.016	3	210.5	32.44
Age	7.94	0.010	3	211.4	53.12
Sex + Age	8.01	0.010	4	209.7	54.85
Sex	12.08	0.001	3	214.9	420.68
β	12.59	0.001	2	217.1	542.42

Predictors are as follows: the sex of the bird (Sex) as determined by voice (Maness and Anderson, 2013), its age in years at the time of sampling (Age), the sampling date (Date), and current reproductive state (State). The null model is indicated by β.

In contrast, the H/L ratio negatively affected annual survival probabilities ($\beta = -2.80$ [95% CI: −4.86 – −0.74], Figure 3, Table 4B). Lower average annual survival probabilities for individuals with high H/L ratio (Figure 3) accumulated across the 11-year period of our study to exert large effects on expected lifespan (Figure 4). We did not attempt to distinguish between immediate and long-term effects of the H/L ratio on annual survival probability because of our small sample size.

When data from all sampled cohorts were included, [Cort] appeared as a predictor within the best-supported model and interacts with a two-level factor distinguishing effects on survival in the year after the study from those affecting all subsequent years (Table 4, Figure 5). [Cort] negatively affected the probability of survival in the year after the study ($\beta = -0.87$ [−1.71 – −0.05]) but not beyond ($\beta = -0.04$ [95% CI: −0.23–0.15]; Table 4C). In contrast with results from data restricted to the 2002–2003 cohort, the H/L ratio was not an important predictor of survival when data for all sampled cohorts were combined (Table 4D), although a negative slope estimate was maintained ($\beta = -0.30$ [95% CI: −1.50–0.91]).

3.3. Effects of stress indices on reproduction

Because 2009–2010 was a poor year for reproductive success in the entire colony (Figure 1), few sampled birds produced a fledgling (54 out of 576 individuals with baseline [Cort] measured, a 9% success rate). Therefore, we could not assess the effect of [Cort] or H/L ratio on the current reproductive effort.

Neither baseline [Cort] nor the H/L ratio predicted lifetime reproductive success for the 2002-2003 cohort ($\beta = -0.01$ [95% CI: −0.52–0.49] and $\beta = -1.32$ [95% CI: −5.37-2.72], respectively). Visual inspection of residual plots did not indicate a lack of fit, and results were identical using Poisson GLMs. Extending the data to include all sampled cohorts also showed no effect of [Cort] ($\beta = 0.08$ [95% CI: −0.26–0.42]) or H/L ratio on lifetime reproductive success ($\beta = -1.24$ [95% CI: −3.63–1.24]).

As with lifetime reproductive success, the baseline [Cort] and H/L ratio did not predict residual reproductive success for the 2002–2003 cohort or for all cohorts combined (coefficients reported in Supplementary Table 3). Finally, restricting the data to cohorts with (nearly) complete information on lifetime and residual reproductive success (cohorts 1994–1995 and earlier) did not affect our results: across all versions of our analyses, we document no association between stress indices and reproduction (Supplementary Table 3).

4. Discussion

4.1. Corticosterone

In our study, the baseline [Cort] reflected the current state of sampled birds in that males, older birds, and actively breeding birds had higher [Cort] than females, younger birds, and currently non-breeding birds (Figure 2). Increased baseline GCs during reproductive events are relatively well documented in birds and other vertebrates (reviewed in Bonier et al., 2009a) including Nazca boobies (Grace and Anderson, 2014), and in other long-lived seabirds when food is scarce (Kitaysky et al., 2007, 2010; Shimabukuro et al., 2023). This finding supports one tenet of the Cort-Adaptation Hypothesis that predicts a positive correlation between allostatic load (measured by baseline GCs) and reproductive activities (Bonier et al., 2009a). However, the three predictors associated with baseline [Cort] (sex, age, and current reproductive state) in our study only explained approximately 7% of the variability in [Cort]. Several studies have shown that nutritional stress raises circulating [Cort] in seabirds and is more strongly associated with [Cort] than reproduction alone (Kitaysky et al., 2007, 2010; Schultner et al., 2013; Will et al., 2020; Shimabukuro et al., 2023). Thus, the challenging environmental conditions in the sampling year (Figure 1) may have elevated baseline [Cort] of all birds (Table 1) overwhelming trends between [Cort] and other indicators of allostatic load (Sorenson et al., 2017). Indeed, Nazca boobies at our study site had a higher baseline [Cort] in March during the 2009–2010 breeding season than in March of the 2008–2009 season (see Supplementary Material 1 of Grace and Anderson, 2014), indicating that nutritional stress was probably quite high.

Regarding survival and reproductive success, we found mixed support for the Cort-Fitness hypothesis and no support for the Cort-Adaptation or Cort-Tradeoff hypotheses. The baseline [Cort] negatively predicted the immediate survival of birds in our study, supporting the Cort-Fitness hypothesis (Bonier et al., 2009a) and contradicting expectations of the Cort-Tradeoff hypothesis (Breuner and Berk, 2019). Birds with elevated [Cort] were less likely to survive to the following year when all cohorts were considered, and the effect of age at the time of sampling was controlled (Figure 5), similar to findings in other seabirds (Kitaysky et al., 2007; Schultner et al., 2013). This effect was not seen when considering only the 2002–2003 cohort, although the sign and

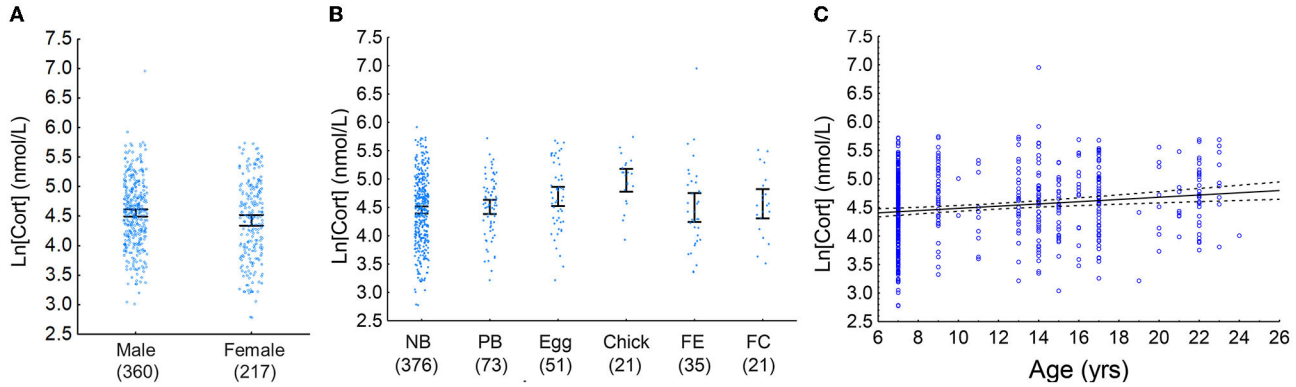

FIGURE 2
(A) Mean male and female baseline [Cort]. Males had a higher mean [Cort] than females (partial eta^2 = 0.011). Samples sizes are in parentheses below the x-axis, blue circles indicate individual data points, and error bars are 95% confidence intervals. **(B)** Mean baseline [Cort] of adult Nazca boobies in various reproductive states at the time of sampling. NB, non-breeders; PB, pre-breeders; Egg, incubating an egg, Chick, incubating a small chick; FE, nesting attempt failed at the egg stage; FC, nesting attempt failed after eggs had hatched. Sample sizes are in parentheses below the x-axis, blue circles indicate individual data points, and error bars are 95% confidence intervals. Birds that were currently breeding (Egg and Chick) had higher baseline [Cort] than all other groups (partial eta^2 = 0.037). **(C)** Baseline [Cort] increased with increasing age in Nazca boobies (partial eta^2 = 0.024). Blue circles are individual data points, the black line is the line of best fit, and the dashed lines are 95% confidence intervals.

TABLE 4 **Candidate models evaluating the H/L ratio and [Cort] as predictors of Nazca booby survival (S), ranked by AICc.**

Data: The 2002–03 cohort						
	Recapture	Survival	K	AICc	ΔAICc	ω_i
A.	p(Sex)	S(Sex + Age + (Age − 16)$_+$)	6	2,060.40	0.00	0.69
	p(Sex)	S(Sex + Age + (Age − 16)$_+$ + Cort)	7	2,062.39	1.99	0.25
	p(Sex)	S(Sex + Age + (Age − 16)$_+$ + Cort*Time$_{2009,>2009}$)	9	2,065.23	4.83	0.06
	Recapture	Survival	K	AICc	ΔAICc	ω_i
B.	p(Sex)	S(Sex + Age + (Age − 16)$_+$ + H/L)	7	269.55	0.00	0.92
	p(Sex)	S(Sex + Age + (Age − 16)$_+$)	6	274.40	4.85	0.08
Data: All cohorts						
	Recapture	Survival	K	AICc	ΔAICc	ω_i
C.	p(Sex)	S(Sex + Age + (Age − 16)$_+$ + Cort*Time$_{2009,>2009}$)	9	4,028.47	0.00	0.81
	p(Sex)	S(Sex + Age + (Age − 16)$_+$)	6	4,032.17	3.69	0.13
	p(Sex)	S(Sex + Age + (Age − 16)$_+$ + Cort)	7	4,033.51	5.04	0.07
	Recapture	Survival	K	AICc	ΔAICc	ω_i
D.	p(Sex)	S(Sex + Age + (Age − 16)$_+$)	6	595.58	0.00	0.71
	p(Sex)	S(Sex + Age + (Age − 16)$_+$ + H/L)	7	597.40	1.82	0.29

Detection rates (p) were sex-specific in all candidate models.and"(Age$_{ij}$ − T$_1$)$_+$" is the product of Age$_{ij}$ (minus the threshold age, 16) and a logical function equal to 1 when Age$_{ij}$ > 16, and 0 otherwise. "Time$_{2009,>2009}$" is a two-level factor separating the year after stress sampling from all others. The number of parameters (K) and Akaike weights (ω_i) are included.

magnitude of the coefficient estimate are similar in the two datasets (−0.77 vs. −0.87). The larger confidence intervals surrounding the estimate for the 2002–2003 cohort alone probably reflect the extremely low mortality rates for 7-year-old boobies (Anderson and Apanius, 2003; Tompkins and Anderson, 2019).

However, baseline [Cort] was not associated with long-term survival or lifetime reproductive success in our study species, which contradicts the predictions of all three hypotheses. Relationships between baseline [Cort] and reproductive success are well studied in birds but with varying conclusions. Most studies, predominantly in passerine birds, support the Cort-Adaptation hypothesis (e.g., Ouyang et al., 2011; Burtka et al., 2016) or the Cort-Tradeoff Hypothesis (Bókony et al., 2009; Hau et al., 2010), while some of those in seabirds support the Cort-Fitness Hypothesis (e.g., Kitaysky et al., 2007), but others have found no relationship between [Cort] and reproductive success (e.g., Schoenle et al., 2017), like our own study. This may, in part, be due to relationships with reproductive success being specific

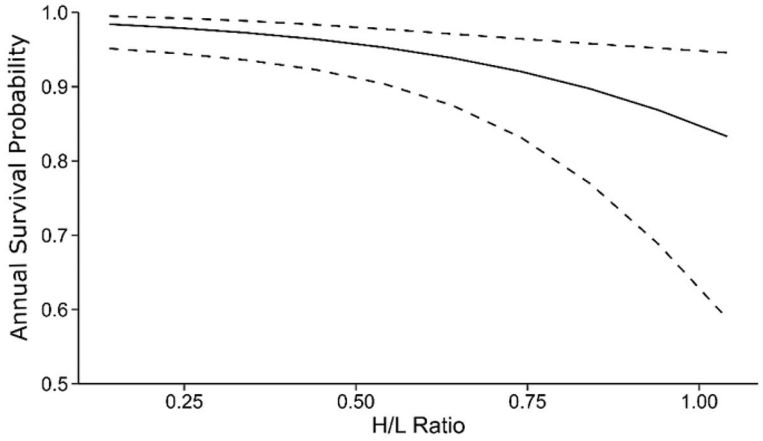

FIGURE 3
Negative effects of the H/L ratio on annual survival probability of the 2002–2003 (focal) cohort. Dashed lines show the 95% CI around the model-estimated relationship. Predictions are from the best-supported model in Table 1. Age was set to 10, and sex was set to female for plotting (effects of the H/L ratio are not sex-specific). Note that the lower boundary of the y-axis is at 0.5.

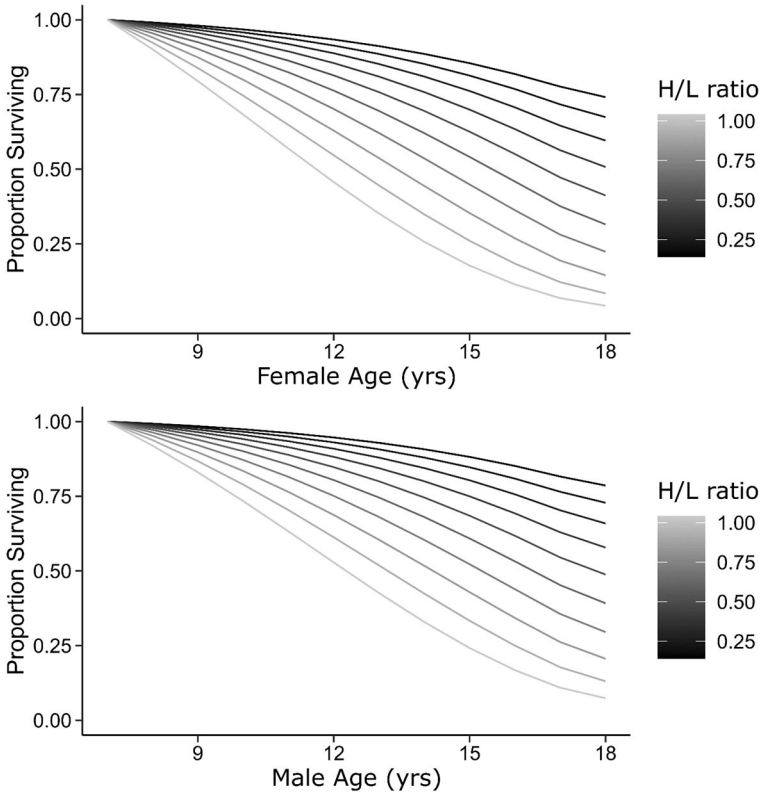

FIGURE 4
Survivorship curves for females **(top)** and males **(bottom)** of the 2002–2003 cohort as a function of the H/L ratio. Curves begin at age 7, the age at which the H/L ratio was sampled. Lines show predicted values from a model including age (as a threshold function), sex, and the H/L ratio as predictors of annual survival probability (see Methods).

to the reproductive stage at the time of [Cort] measurement (e.g., incubation vs. chick-rearing; Bonier et al., 2009b; Fischer et al., 2020). Most studies also only examine reproductive success during a single breeding season (e.g., Ouyang et al., 2011; Burtka et al., 2016; Schoenle et al., 2017; Fischer et al., 2020) and not lifetime reproductive success, which may be a more comprehensive indicator of fitness and a stronger test of these hypotheses. Although we were unable to evaluate short-term reproductive success in this study, our finding that [Cort] predicted short-term survival in conjunction with the results of these previous studies suggests that baseline [Cort] reflects current nutritional stress (Kitaysky et al., 2007, 2010; Shimabukuro et al., 2023) and

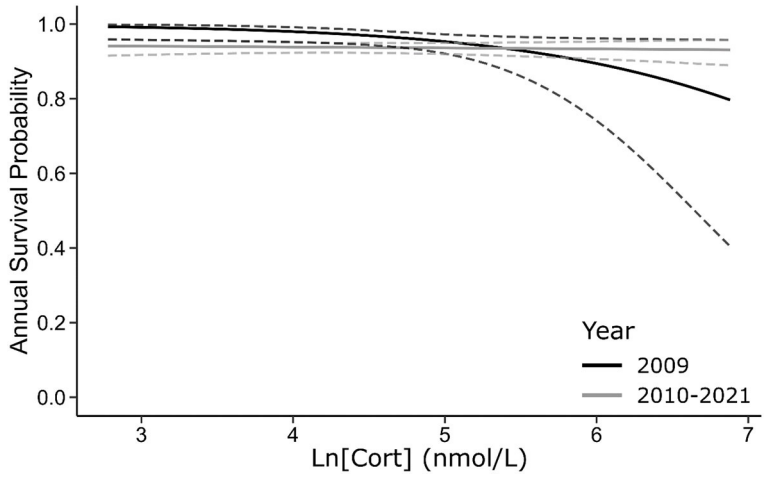

FIGURE 5
For all sampled cohorts, baseline [Cort] negatively covaries with the probability of surviving from 2009–2010 to the following year (black line) but has no effect on annual survival probability over subsequent intervals (gray line). Dashed lines are 95% CI.

thus correlates with short-term proxies of fitness, but may correlate less well with long-term indicators of fitness. Future studies in other species should investigate relationships between [Cort] and both short-term and lifetime reproductive success to evaluate this hypothesis.

Together, our results suggest that the baseline [Cort] reflects the current, transient physiological state of Nazca boobies (i.e., current reproductive activities and immediate survival) and does not reflect long-term differences in individual quality or fitness. This finding concurs with previous work indicating that GCs reflect current acute stressors such as environmental conditions (Kitaysky et al., 2007, 2010; Satterthwaite et al., 2010; Schultner et al., 2013; Romero and Wingfield, 2016; Davis and Maney, 2018; Shimabukuro et al., 2023). Moreover, the year-to-year repeatability of baseline [Cort] is low in our study species after controlling for other environmental factors (i.e., sex, reproductive status, mass, and year) (Grace and Anderson, 2014), providing no support for an assumption of the Cort-Fitness and Cort-Adaptation hypotheses that baseline [Cort] reflects inherent, repeatable differences in the overall quality of individuals in a population (e.g., Angelier et al., 2010). Instead, Grace and Anderson (2014) found that maximum stress induced [Cort] was highly repeatable in Nazca boobies, thus this metric could be a better indicator of individual quality differences than baseline [Cort] in our study species. In addition, maximum acute stress-induced [Cort] in seabirds may reflect the long-term nutritional state of birds (Kitaysky et al., 2007), reflecting differences in foraging ability or long-term reduction in food quantity or quality (Champagnon et al., 2018).

4.2. H/L ratio

In contrast to [Cort], the H/L ratio was a strong negative predictor of long-term survival for the 2002–2003 cohort but did not reflect the current state in our study, as it had no association with sex, age, or reproductive activities. The effect of the H/L ratio on survival was not apparent when all cohorts were considered. This is perhaps due to the selective disappearance of low-quality phenotypes from older cohorts, which would leave a biased subset of relatively low H/L ratio individuals at the time of sampling (e.g., Nussey et al., 2008; Bouwhuis et al., 2009). Although we found no relationship between the H/L ratio and age in this study (Supplementary Table 2), the power of this analysis was low ($\beta = 0.10$) due to the low number of older birds in the dataset.

Longevity has been found to be an important component of lifetime reproductive success in many species (Brown, 1988; Newton, 1989; Oring et al., 1991; Kruuk et al., 1999; Heidinger et al., 2021) including Nazca boobies (Townsend et al., 2007). However, we did not find any relationship between the H/L ratio and reproductive success. This may be because lifetime reproductive success was probably incomplete for our youngest and largest cohort. The number of individuals that fledged from the colony in 2002–2003 indicates that this was a good year for fledging success in the colony (Figure 1). Fledglings produced during this breeding season were heavier, larger, had faster nestling growth rates, and had higher juvenile survival rates than other cohorts (Maness and Anderson, 2013). These favorable rearing conditions likely influence the fitness of this cohort (e.g., Van de Pol et al., 2006) and could influence when these individuals begin to senesce. The 2002–2003 cohort was 17 years old during the last year of the nest monitoring effort (2019–2020). These birds should be entering reproductive senescence (Anderson and Apanius, 2003; Tompkins and Anderson, 2019), but 50% were still alive, so our lifetime reproductive success estimate may be incomplete in this cohort. Further monitoring of the reproductive success of this focal cohort is needed to determine whether the H/L ratio corresponds with complete lifetime reproductive success, as would be expected when the H/L ratio reflects the individual quality. If true, then the H/L ratio would seem to be a good indicator of fitness in Nazca boobies, in a similar manner to the Cort-Fitness hypothesis, at least when it is measured in young individuals. Alternatively, the H/L ratio may indicate differential investment in the innate and acquired arms of the immune system (Martin et al., 2006; Martin, 2009; Brace

et al., 2017), creating a tradeoff between survival probabilities and reproductive success similar to that of the Cort-Tradeoff hypothesis. However, this tradeoff is not supported by our data because we found no relationship between the H/L ratio and lifetime or residual reproductive success in the focal cohort or in the entire dataset.

Few studies have attempted to correlate the H/L or N/L ratios with fitness in animal populations. The H/L ratio is associated with aspects of fitness in selectively bred domestic chickens (*Gallus gallus*), for which low H/L ratios were associated with greater egg production, heavier eggs with higher fertility and hatchability, and heavier chicks with lower mortality rates (Al-Murrani et al., 2006). In wild animals, studies that have attempted to link the H/L or N/L ratio and fitness have been short-term or used proxies for fitness-like body condition (e.g., Xuereb et al., 2012) male ornamentation (e.g., Lebigre et al., 2012) or chick weight (e.g., Parejo and Silva, 2009). If the H/L ratio reflects inherent individual quality, then the H/L ratio should be repeatable from year to year after controlling for confounding environmental variables, similar to the Cort-Fitness Hypothesis. This possibility needs to be assessed in Nazca boobies, but evidence from a truncation selection experiment (using the 99% confidence interval for H/L ratio to select breeders) showed that the H/L ratios are heritable in domestic chickens (Al-Murrani et al., 2006), suggesting individual repeatability in birds. Similarly, the N/L ratio was found to be repeatable in three populations of wild roe deer (*Capreolus capreolus*) over an 8- or 9-year interval (Carbillet et al., 2019). Further studies are needed to assess both the repeatability of individual H/L and N/L ratios and their correlates with fitness in animal populations.

5. Conclusion

One of the great challenges in ecophysiology is linking physiological measures in wild animal populations with changes in individual fitness. Here, we assessed two indicators of stress in a wild long-lived bird, namely, baseline [Cort] and H/L ratio, to determine whether these metrics corresponded with individual performance. [Cort] corresponded with the current conditions and activities (e.g., breeding) that the animal was engaged in (Figure 2) and predicted near-term survival probability (Figure 5). The H/L ratio predicted long-term survival in our youngest focal cohort (Figures 3, 4). Our results suggest that the H/L ratio may be a good indicator of long-term fitness in Nazca boobies because survival and reproductive performance are known to correlate in this species (Townsend et al., 2007). Our results also support findings from other seabirds (e.g., Kitaysky et al., 2007, 2010) that suggest that baseline [Cort] may be a good indicator of the current state and near-term performance. We propose a corresponding hypothesis to the Cort-Fitness Hypothesis: the H/L(N/L) Ratio-Fitness Hypothesis. This hypothesis predicts that H/L or N/L ratios reflect the underlying quality of an individual, such that lower values predict higher fitness. An assumption of the hypothesis is that these ratios should be repeatable across years. This hypothesis needs to be tested in this and other species and, if supported by empirical evidence, then these ratios could be a powerful monitoring tool for assessing population health or identifying at-risk populations. Continued monitoring of reproductive success in study birds that are still alive and assessment of the repeatability of the H/L ratio is needed to demonstrate the applicability of this hypothesis in our study system.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: ZSR Library, http://hdl.handle.net/10339/102187.

Ethics statement

This animal study was reviewed and approved by Wake Forest University Institutional Animal Care and Use Committee. This study was permitted under Wake Forest University IACUC (protocol A08-029) and Galápagos National Park, and adheres to NIH and the Ornithological Council's standards for animal use in research.

Author contributions

TM: conceptualization, supervision of lab analyses, data analysis, writing, and manuscript preparation. JG: supervision of fieldwork, sample collection, performed baseline [Cort] analysis, writing, and manuscript preparation. MH: performed blood cell counts, data analysis, and manuscript preparation. ET: data analysis, writing, and manuscript preparation. DA: conceptualization, supervision, writing, and manuscript preparation. 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

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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.2023.1172904/full#supplementary-material

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Blood analytes of hawksbill sea turtles (*Eretmochelys imbricata*) from Florida waters: reference intervals and size-relevant correlations

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Assessments of health variables in wild animal populations have evolved into important tools for characterizing spatiotemporal population trends and fitness, effects of stressors, diseases, and ecosystem health. Blood as a sample matrix can be obtained fairly non-invasively in the field, with preservation and sample processing techniques that allow for readily available routine and advanced diagnostic testing of blood. For wild-caught hawksbill sea turtles (*Eretmochelys imbricata*) foraging in southeastern Florida, USA, the objectives of this study were to (1) establish reference intervals for hematological and 24 plasma biochemical analytes, (2) determine length-and body condition-specific relationships with blood analytes, and (3) determine how water temperature influenced plasma biochemical analytes. Reference intervals were established for clinically normal juvenile (n=26) and subadult (n=39) hawksbills, with descriptive data reported for adult turtles (n=3). Although subadults (mainly captured at Palm Beach County) were heavier and larger with greater body depth, juveniles (mainly captured at Monroe County) had a higher body condition index. Positive length-specific correlations were identified for packed cell volume, eosinophils, aspartate aminotransferase, phosphorus, cholesterol, glutamate dehydrogenase, total protein, albumin, and globulins, with negative correlations including alkaline phosphatase, creatine kinase, calcium, calcium to phosphorus ratio, and glucose. Subadults had less frequent morphological features of red blood cell regeneration compared to juveniles. These findings provide insight into life-stage class differences regarding hematopoiesis, antigenic stimulation, somatic growth, dietary shifts, nutritional status, osmoregulation, metabolism, physical activity or stress levels, and possible habitat differences. Life-stage class is the likely driver for the observed blood analyte differences, in addition to influences from water temperature. The data herein offer baseline information for a snapshot in time for critically endangered hawksbills inhabiting the Florida reef system and for answering individual-and population-relevant questions of relevance to conservation and population management.

KEYWORDS

health assessment, hematology, marine turtle, plasma biochemistry, physiology, protein electrophoresis, somatic growth

1. Introduction

Six sea turtle species are known to occur in waters off the east and west coasts of Florida, USA (Foley et al., 2003; Eaton et al., 2008). After olive ridleys (*Lepidochelys olivacea*), the critically endangered (Mortimer and Donnelly, 2008) hawksbill sea turtle (*Eretmochelys imbricata*) is the rarest species to occur in Florida (Meylan and Redlow, 2006; Mortimer and Donnelly, 2008), with this aggregation being the second most northern of the Atlantic Ocean population (Meylan et al., 2011; Wood et al., 2013). The Atlantic hawksbill population appears to be genetically distinct from the Pacific population (Wood et al., 2013). Hawksbills are believed to occupy the Florida reef system due to warm water transport by the Florida current from the Gulf of Mexico and the Caribbean (Meylan and Redlow, 2006; Blumenthal et al., 2009). Although hawksbill nesting in Florida is uncommon (Meylan and Redlow, 2006), juvenile and subadult life-stage classes are known to inhabit Florida's waters year-round (Wood et al., 2013, 2017). The Florida Continental Reef Tract provides refuge for hawksbills and is home to numerous sponges and octocoral species on which these animals forage (Moyer et al., 2003; Banks et al., 2008; Wood et al., 2017). Hawksbill turtles in Florida waters originate from Mexico and the Caribbean *via* the Florida Current and/or Gulf Stream, recruiting from oceanic to neritic habitats predominantly in the Florida Keys (i.e., Monroe County) as small juveniles. It is apparent that some move along the Southeast Florida Continental Reef Tract to eventually reach their northern terminus in Palm Beach County, Florida, as they advance through their subadult life-stage (Wood et al., 2013). Then, upon reaching sexual maturity, the young adults undertake reproductive migrations out of Florida, and are only seen transiently thereafter.

Despite the known occurrence of these animals in Florida, no assessment of overall health using blood analytes has occurred for this aggregation to date. Health assessments have been conducted for wild-caught hawksbills from Oman (nesting adults) (Alkindi et al., 2002), Brazil (nesting adults) (Goldberg et al., 2013), the Persian Gulf (nesting adults) (Ehsanpour et al., 2015), Australia (immature foraging) (Whiting et al., 2014), the Galápagos Archipelago (immature and adult foraging) (Muñoz-Pérez et al., 2017), Mexico (nesting adults) (Salvarani et al., 2018), Belize (immature) (Crooks et al., 2023) and in rehabilitating hawksbills from the United Arab Emirates (juvenile) (Hampel et al., 2009; Caliendo et al., 2010). Reference intervals using American Society for Veterinary Clinical Pathology (ASVCP) guidelines (Friedrichs et al., 2012) have only been established for one wild-caught hawksbill population in Australia (Whiting et al., 2014); however, several other hawksbill studies report measures of central tendency and full range of values (Goldberg et al., 2013; Ehsanpour et al., 2015; Salvarani et al., 2018). Establishment of blood analyte reference intervals in sea turtles is important, as various stressors that sea turtles face continue to intensify; therefore, health assessments are becoming increasingly valuable to conservation efforts as they provide baseline data for future population health and fitness comparisons in the face of environmental changes, increasing stressors, and potential disease outbreaks (Deem et al., 2001; Aguirre and Lutz, 2004; Wikelski and Cooke, 2006; Cooke and O'Connor, 2010; Deem and Harris, 2017; Reséndiz and Lara-Uc, 2018; Mashkour et al., 2020; Page-Karjian et al., 2020; Perrault et al., 2020), in addition to improving clinical decision-making in individual animals undergoing veterinary care (Delgado et al., 2011).

Coral cover in the Florida Continental Reef Tract, where hawksbills are known to inhabit, is declining at alarming rates due to climate change, disease, and deteriorating water quality (Ruzicka et al., 2013; Voosen, 2019; Neely et al., 2021); therefore, establishing baselines of hawksbill health variables is necessary to understand physiological responses of animals and future population and ecosystem challenges that may result from habitat degradation or loss. For wild-caught juvenile and subadult hawksbills foraging in southeastern Florida, USA, the objectives of this study were to (1) establish reference intervals for hematological and 24 plasma biochemical analytes, (2) determine length-and body condition-specific relationships with blood analytes, and (3) determine how water temperature influenced plasma biochemical analytes.

2. Materials and methods

2.1. Ethical procedures

This study was reviewed and authorized by the National Marine Fisheries Service (NMFS) [Permit #22988], Florida Fish and Wildlife Conservation Commission (FWC) [Marine Turtle Permits #021 and #077], Florida Keys National Marine Sanctuary [Research Permit #175], and University of Florida's Institutional Animal Care and Use Committee (IACUC) [#201706823]. All handling and sampling procedures of sea turtles were performed according to NMFS and FWC regulations (NMFS SEFSC, 2008).

2.2. Capture technique, morphometrics, and sample collection, processing, and analysis

Hawksbill turtles were hand-captured during snorkel and/or scuba surveys between Jun 2017 and Oct 2020 along the 2–30 m deep nearshore reefs of the Southeast Florida Continental Reef Tract from Jupiter, Florida, USA (26.987°N, −80.033°W) to Key West, Florida, USA (23.673°N, −82.981°W) (Figure 1). The water temperature was recorded at the time and depth of each capture with a Sherwood Wisdom 2 (Sherwood Scuba®, Santa Ana, CA) dive computer while on scuba, and/or the vessel's onboard surface water temperature gauge when snorkeling in water less than 9 m deep. Primarily, two counties in Florida were utilized for this study including Palm Beach (towns of Jupiter and West Palm Beach) and Monroe (towns of Islamorada and Key West), which are known foraging areas of this species in Florida (Meylan and Redlow, 2006; Wood et al., 2013, 2017). One turtle was captured in Miami, Florida, USA (Miami-Dade County) (25.690°N, −80.085°W). This individual was included in analyses of the Monroe County turtles due to the close proximity of the two locations. Turtles captured at depths exceeding 10 m (>1 atm) were brought to the surface at a rate of ≤9 m/min. Turtles were transferred to a research vessel within 20 min of capture and examined for the presence of internal passive integrated transponder (PIT) tags.

Within 5–10 min of returning to the vessel, up to 5 mL of blood were collected from the dorsal cervical sinus using 20-gauge, 1.5″ needles and 7 mL lithium heparin vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA), following standardized safe blood volume withdrawal guidelines for sea turtles (NMFS SEFSC, 2008).

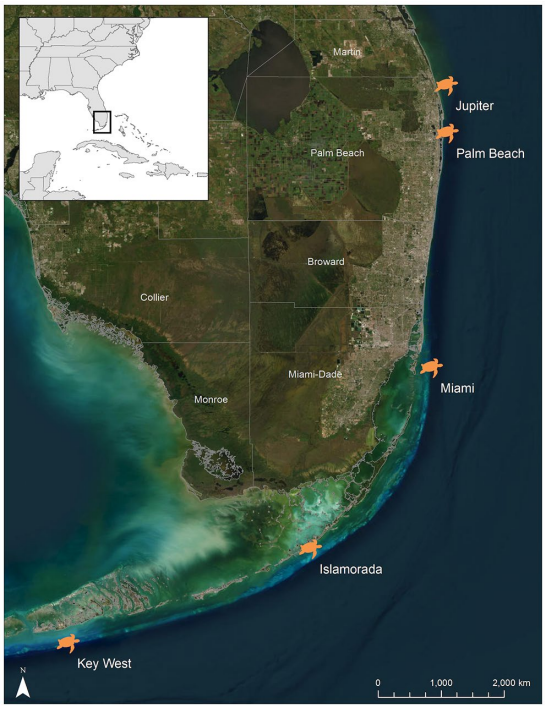

FIGURE 1
Locations of the five capture sites of hawksbill sea turtles (*Eretmochelys imbricata*) across Florida's, USA reef tract. Jupiter and Palm Beach are located in Palm Beach County, while Islamorada and Key West are located in Monroe County. One individual was captured off of a reef in Miami (Miami-Dade County). This individual was included in the Monroe County group for statistical analyses.

FIGURE 2
Photograph of a subadult hawksbill sea turtle (*Eretmochelys imbricata*) from Palm Beach County representative of the study turtles with associated normal epibiota coverage.

The sampling site was disinfected with alternating applications of povidone iodine and 70% isopropyl alcohol both before and after venipuncture. The vacutainers were then transferred to an insulated sleeve (i.e., bubble wrap) and placed on wet ice for up to 8 h until processing.

Following blood collection, standard (i.e., notch to tip) straight carapace length (SCL) and the maximum body depth (BD) of each subject were measured with aluminum calipers. Mass was estimated to the nearest kg using a 300 kg digital scale. Body condition index (BCI) was calculated (Bjorndal et al., 2000), applying a correction factor of 10,000. Physical examination included assessment of the presence and location of conspicuous epibiota (e.g., macroalgae, coral, barnacles, etc.; Figure 2) and/or physical abnormalities, which were diagrammed and photographed. Prior to release, Inconel tags (National Band and Tag Co., Newport, Kentucky, USA) were placed on the trailing edge of the second distal scale of each front flipper, and/or one PIT tag (Biomark, Inc., Boise, Idaho, USA) was injected into the right front shoulder in all turtles of sufficient size (>30 cm SCL). All turtles were released ~20 min after sample collection, at or near the capture site.

Upon return to the laboratory, whole blood was well-mixed and packed cell volume (PCV) was determined using the average of two microhematocrit tubes after centrifugation for 5 min at 7,500 *g* (12,000 rpm) using a ZipCombo microhematocrit centrifuge (LW Scientific, Lawrenceville, Georgia, USA). Blood films were prepared from well-mixed whole blood, air-dried, and stained with Wright-Giemsa (Harleco®, EMD Millipore, Billerica, Massachusetts, USA). Blood film evaluation included white blood cell (WBC) estimate (Weiss, 1984) using an eyepiece of 18 mm diameter, a 200 white blood cell differential including heterophils, lymphocytes, monocytes, eosinophils, and basophils, and morphological evaluation of red blood cells (RBC), WBCs, and thrombocytes. The heterophil:lymphocyte ratio was calculated.

The remaining whole blood was spun at 1350 *g* (3,300 rpm) in a Champian E33 centrifuge (Ample Scientific, LLC, Norcross, Georgia, USA) for 6–8 min. Plasma was separated from the RBC and WBC, placed in cryovials, and stored in a standard freezer (−20°C) until shipment on dry ice after 48 h to the University of Florida for storage in an ultralow freezer. After 8–434 d (mean ± SD = 139 ± 102 d), samples were then shipped to the University of Miami Avian and Wildlife Laboratory for biochemical analyses using an Ortho 250XR (Ortho Clinical Diagnostics, Rochester, New York, USA) dry slide chemistry analyzer. Biochemical analytes of interest included alkaline phosphatase (ALP), amylase, aspartate aminotransferase (AST), bile acids, blood urea nitrogen (BUN), calcium, phosphorus, chloride, cholesterol, creatine phosphokinase (CK), gamma-glutamyl transferase, glucose, glutamate dehydrogenase (GLDH), lipase, magnesium, potassium, sodium, triglycerides, and uric acid. The calcium:phosphorus ratio was calculated.

Plasma protein electrophoresis was conducted using the SPIFE 3000 system (Helena Laboratories, Inc., Beaumont, Texas, USA). Fractions of interest included albumin and total globulins. The albumin:globulin ratio was calculated.

3. Statistical analyses

Statistical analyses were performed using MedCalc® statistical software (version 19.6, Ostend, Belgium) and SPSS for Windows (version 27; Chicago, Illinois, USA). Measures of central tendency and range are reported for all blood analytes in Standard International (SI) units. Reference intervals (95% with associated 90% confidence intervals) were determined for sea turtles of juvenile and subadult life-stage classes that fit the inclusion criteria for the study (Moore et al., 2020), using parametric methods based on recommendations by Friedrichs et al. (2012) for sample sizes ≥20, but <40. Normality

was assessed using the Shapiro-Wilk test, while outliers were detected using the Dixon-Reed test. Outliers were subsequently removed and when necessary, logarithmic or Box-Cox transformations were employed to generate accurate reference intervals. Several blood analytes could not be normalized to fit a Gaussian distribution; therefore, reference intervals for those analytes were calculated using the robust method.

Spearman correlations were used to determine relationships between individual hematological and plasma biochemical analytes. Differences in SCL by capture location (Palm Beach v. Monroe) and BCI in juveniles and subadults were assessed using independent samples t-tests. Linear regression analysis was used to assess the relationship of SCL to mass (using log-transformed data). Stepwise backward multiple regression was used to determine the impacts of SCL, BCI, water temperature (independent variables) upon capture on blood analytes (dependent variables). Outliers were determined using Tukey's method and subsequently removed from analyses. Transformations were employed as necessary. To determine if polychromasia, anisocytosis, basophilic stippling, and number of immature RBCs/100 mature RBCs were influenced by life-stage class, Kruskal-Wallis with Dunn's post-hoc comparisons were used.

4. Results

4.1. Physical examination and morphometrics

A total of 68 hawksbills were captured from 5 Jun 2017–30 Oct 2020. Water temperatures were similar for both sites from May–October, when turtles were typically captured: 23.3–29.4°C (mean 27.4°C) in Palm Beach County and 26.7–29.4°C (mean 28.1°C) in Monroe County, respectively. Inclusion criteria for study animals included the absence of overt acute or debilitating external injuries, normal behavior, visibly adequate body condition, and absence of difficulties during blood withdrawal (Figure 2). Morphometric results by sampling location are reported in Table 1. Turtles of <50 cm SCL were considered juveniles ($n = 26$), those of 50–78 cm SCL were considered subadults ($n = 39$), and turtles >78 cm were considered adults ($n = 3$) (Boulon, 1994; Wood et al., 2017). Hawksbills from Palm Beach County were significantly heavier, had a significantly longer SCL (Figure 3), and a greater body depth than turtles from Monroe County ($p < 0.001$ in all cases; Table 1); turtles from Monroe County had significantly larger BCI ($p < 0.001$; Table 1). Log-transformed mass and SCL strongly correlated ($r^2 = 0.989$; $p < 0.001$; Figure 4). Using a t-test (t (60) = −2.378; $p = 0.021$), we found that juveniles had a significantly higher BCI (mean ± SD: 1.16 ± 0.10; range: 0.97–1.39) than subadults (mean ± SD: 1.10 ± 0.10; range: 0.94–1.32), and overall that BCI tended to decrease with SCL ($y = -0.002x + 1.24$; $r^2 = 0.079$; $p = 0.027$; $n = 62$).

Small patches of red and/or brown algae (Figure 2), particularly on the posterior carapace, were ubiquitous, while small patches of calcareous algae were less common. Fire coral colonies (*Millepora* sp.) were occasionally found on larger individuals. Fourteen turtles (20%) had dented or chipped marginal scutes; seven (10%) had at least one scalloped hind limb; one (1%) had a partially amputated (~50%) hind limb; and two (3%) had minor front flipper damage. All described injuries/abnormalities were completely healed and were not expected to impact blood analytes. All turtles appeared to be in robust condition as observed by the thickset nature of the soft tissues surrounding the neck and flippers and BCI scores. No fibropapilloma tumors or acute or healed injuries specific to shark predation were observed.

All turtles were considered to be clinically normal and fit the inclusion criteria for this study as they were all active and alert, had robust subjective body condition scores (Tristan and Norton, 2017), minimal epibiota, and minor injuries or shell abnormalities that most likely did not impact blood analytes.

4.2. Reference intervals

Mild (1+) hemolysis was present in seven samples, while mild (1+) lipemia was present in one sample. Neither degree of interferences is thought to affect dry chemistry analyses (Andreasen et al., 1997; Stacy and Innis, 2017; Stacy et al., 2019). Measures of central tendency and range of the measured blood analytes are reported in SI units for all three life-stage classes (juvenile, subadult, and adult; Tables 2–4, respectively; conventional units are reported in Supplementary Tables S1–S3), while reference intervals, due to the sufficient number of data points, are described for juveniles and subadults only (Tables 2, 3). Results of morphological evaluation of RBC, WBC, and thrombocytes are shown in Table 5.

Spearman correlations of hematological and biochemical analytes from all study turtles identified several statistically significant correlations of biological relevance: bile acids and AST ($r_s = 0.363$; $p = 0.032$; $n = 35$), GGT and AST ($r_s = 0.310$; $p = 0.010$; $n = 68$), GGT

TABLE 1 Morphometric data of in-water assessed hawksbill sea turtles (*Eretmochelys imbricata*) from Florida, USA.

	Palm Beach (5 Jun 2017–30 Oct 2020)				Monroe (21 Jul 2017–20 Sep 2020)						
Measurement	Mean±SD	Median	Range	*N*	Mean±SD	Median	Range	*N*	*t*	df	*p*
Mass (kg)	27 ± 11	28	11–48	35	9 ± 6	7	3–22	27	−8.378[a]	54.2	<0.001
SCL (cm)	63.3 ± 9.4	63.9	46.9–84.4	40	40.8 ± 8.5	39.8	27.4–56.2	28	−10.073	66	<0.001
BCI	1.09 ± 0.09	1.09	0.94–1.32	35	1.18 ± 0.09	1.15	1.03–1.39	27	3.901	60	<0.001
Body depth (cm)	23.3 ± 3.8	23.4	16.4–31.1	36	15.1 ± 3.2	14.4	9.8–21.2	28	−9.206	62	<0.001

[a]Welch's t-test was used as variances were not homogenous.

Individual metrics for each county are included. Sampling dates are included parenthetically next to the capture location. Statistical differences between sites, determined using independent samples t-tests, are also indicated. BCI, body condition index; df, degrees of freedom; SCL, standard straight carapace length; SD, standard deviation.

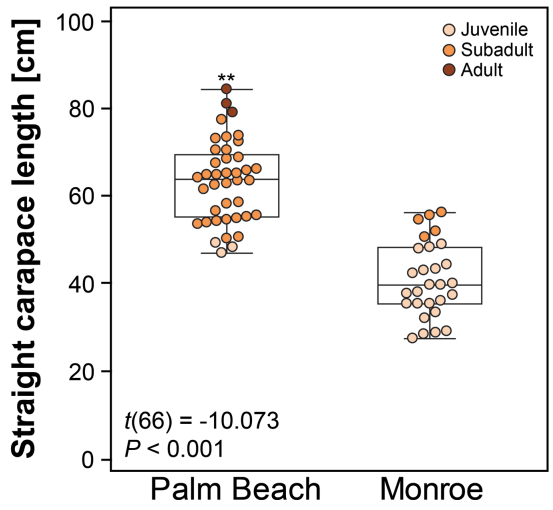

FIGURE 3
Straight carapace length of hawksbill sea turtles (*Eretmochelys imbricata*) from Palm Beach and Monroe Counties, Florida, USA. The central box represents the lower to upper quartiles, with the middle line representing the median. The vertical lines extend from the minimum to maximum values. Asterisks above the Palm Beach box-plot indicate a significant difference between the two groups at $p<0.050$.

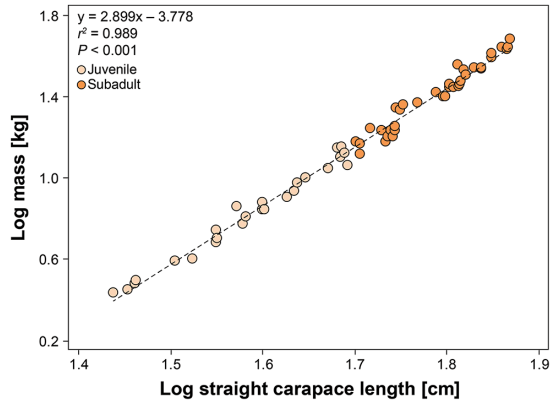

FIGURE 4
Relationship between log-transformed mass (kg) and standard straight carapace length (cm) in in-water assessed hawksbill sea turtles (*Eretmochelys imbricata*) from Florida, USA. Adults are not shown as they were too large to weigh. The equation for the line-of-best-fit is shown.

and cholesterol ($r_s=0.288$; $p=0.017$; $n=68$), GLDH and AST ($r_s=0.709$; $p<0.001$; $n=35$), magnesium and phosphorus ($r_s=0.369$; $p=0.002$; $n=68$), uric acid and phosphorus ($r_s=0.369$; $p=0.002$; $n=68$), uric acid and magnesium ($r_s=0.325$; $p=0.007$; $n=68$), uric acid and sodium ($r_s=0.328$; $p=0.007$; $n=68$), triglycerides and GGT ($r_s=0.318$; $p=0.008$; $n=68$), and triglycerides and glucose ($r_s=0.359$; $p=0.003$; $n=68$). There were no correlations between CK and AST ($r_s=0.117$; $p=0.340$; $n=68$) or CK and phosphorus ($r_s=0.158$; $p=0.197$; $n=68$).

Blood film review data (Table 5) showed that polychromasia and anisocytosis significantly differed by life-stage class ($H(2)=6.347$; $p=0.042$ for both categories), with subadults having a lower occurrence of both in comparison to juveniles ($p=0.035$ for both categories). Basophilic stippling also significantly differed by life-stage class ($H(2)=7.406$; $p=0.025$), with subadults having a lower occurrence in comparison to juveniles ($p=0.020$). Lastly, number of immature RBCs/100 mature RBCs significantly differed by life-stage class ($H(2)=7.209$; $p=0.027$), with subadults having a lower occurrence of immature RBCs in comparison to juveniles ($p=0.033$).

4.3. Influence of straight carapace length, body condition, and water temperature upon capture on blood analytes

SCL showed significant positive relationships with PCV, eosinophils, AST, phosphorus, cholesterol, GLDH, total protein, albumin, and globulins and significant negative relationships with ALP, CK, calcium, calcium:phosphorus ratio, and glucose. BCI showed a significant negative relationship with PCV. Water temperature showed significant positive relationships with AST, cholesterol, GGT, glucose, lipase, triglycerides, and albumin. Complete statistical results are shown in Table 6.

5. Discussion

This study reports hematology and plasma biochemical blood analyte data for wild-caught hawksbills during four years of field work in the Atlantic Ocean, and evaluated influences of intrinsic (length, body depth, BCI) and extrinsic factors (water temperature), with life-stage class being identified as the most relevant factor for the observed blood analyte differences. The data herein provide an important baseline framework and insight into the physiology of the critically endangered hawksbill sea turtle from the reef system of southeastern Florida during a defined time frame. This work will provide a springboard for future assessments on fitness and stressor effects on this population and allows for comparisons to other geographical populations.

5.1. Physical examination and morphometrics

A thorough external physical examination is an essential component of sea turtle health assessment studies as it provides important information on body condition, activity level, mentation, physical abnormalities, and evidence of trauma or disease (Deem and Harris, 2017; Tristan and Norton, 2017; Page-Karjian and Perrault, 2021). These findings are endpoints needed for defining inclusion criteria in wild animal studies. In fact, some health assessment studies are solely based on physical examination and morphometric measurements (Maulida et al., 2017). All hawksbills captured during the study time frame fit the inclusion criteria for the study. The lack of overt injuries in hawksbills leading to exclusion of some of the study animals contrasts with other sea turtle species. For example, two of 36 immature Kemp's ridleys (*Lepidochelys kempii*) from Georgia, USA were excluded due to abnormal shell formation or monofilament entanglement (Perrault et al., 2020).

TABLE 2 Measures of central tendency, range, and reference intervals (with 90% confidence intervals for upper and lower limits) for hematological and plasma biochemical data (including protein electrophoresis) in Standard International units for in-water, juvenile (25–49.9cm standard straight carapace length) hawksbill sea turtles (*Eretmochelys imbricata*) from Florida, USA.

Analyte	Mean±SD	Median	Range	*n*	RI	LRL 90% CI	URL 90% CI
Hematology							
Packed cell volume [L/L]	0.30 ± 0.05	0.30	0.22–0.38	11	–	–	–
Immature RBC/100 mature RBC	6 ± 2	7	3–9	8	–	–	–
White blood cells [x 10^9/L]	8.35 ± 2.60	8.10	4.80–12.10	8	–	–	–
Heterophils [x 10^9/L]	4.33 ± 1.82	4.30	1.60–6.80	8	–	–	–
Immature heterophils [x 10^9/L]	0	0	0	8	–	–	–
Lymphocytes [x 10^9/L]	3.36 ± 0.78	3.00	2.40–4.40	8	–	–	–
Heterophil:lymphocyte ratio	1.26 ± 0.37	1.34	0.57–1.61	8	–	–	–
Monocytes [x 10^9/L]	0.53 ± 0.24	0.55	0.19–0.82	8	–	–	–
Eosinophils [x 10^9/L]	0.14 ± 0.13	0.17	0–0.31	8	–	–	–
Basophils [x 10^9/L]	0.04 ± 0.05	0.03	0–0.12	8	–	–	–
Biochemistry							
Alkaline phosphatase [µkat/L]	0.85 ± 0.28	0.85	0.32–1.39	26	0.38–1.34	0.23–0.55	1.17–1.49
Amylase [µkat/L]	11.59 ± 4.41	11.91	4.24–18.45	26	4.33–18.85	1.84–6.83	16.35–21.34
Aspartate aminotransferase [µkat/L]	2.37 ± 1.09	2.10	1.24–6.05	26	1.17–4.11[e]	0.94–1.45[e]	3.31–5.09[e]
Bile acids [µmol/L]	–	2.1	<0.5–5.0[a]	10	–	–	–
Blood urea nitrogen [mmol/L]	31.0 ± 4.9	30.0	21.8–41.4	26	23.0–39.0	20.2–25.7	36.2–41.7
Calcium [mmol/L]	2.4 ± 0.3	2.4	1.7–3.2	26	1.9–2.9	1.7–2.0	2.7–3.1
Phosphorus [mmol/L]	1.9 ± 0.3	2.0	1.4–2.5	26	1.5–2.4	1.3–1.6	2.2–2.6
Calcium:phosphorus ratio	1.26 ± 0.30	1.20	0.85–1.98	26	0.77–1.76	0.60–0.94	1.59–1.92
Chloride [mmol/L]	123 ± 4	123	115–134	26	116–130	114–118	128–132
Cholesterol [mmol/L]	–	1.63	<1.17–3.81	26	<1.17–3.08	<1.17	2.64–3.52
Creatine phosphokinase [µkat/L]	23.53 ± 15.25	22.34	5.93–88.84[b]	26	8.45–33.38	4.07–12.83	29.01–37.76
Gamma-glutamyl transferase [µkat/L]	–	<0.08	<0.08–0.35	26	–[d]	–[d]	–[d]
Glucose [mmol/L]	6.0 ± 0.9	6.0	3.9–7.7	26	4.6–7.4	4.1–5.1	6.9–7.9
Glutamate dehydrogenase [µkat/L]	1.05 ± 0.57	0.83	0.47–2.13	10	–	–	–
Lipase [µkat/L]	–	0.35	<0.02–1.22	26	<0.02–1.20[f]	<0.02–0.06[f]	0.82–1.69[f]
Magnesium [mmol/L]	3.6 ± 0.5	3.5	3.0–4.8	26	2.9–4.3[e]	2.8–3.2[e]	4.1–4.7[e]
Potassium [mmol/L]	4.6 ± 0.5	4.6	3.8–5.5	26	3.7–5.5	3.4–4.0	5.2–5.8
Sodium [mmol/L]	158 ± 5	159	148–167	26	151–165	148–153	163–168
Triglycerides [mmol/L]	1.22 ± 0.43	1.17	0.49–2.15	26	0.51–1.94	0.27–0.76	1.69–2.18
Uric acid [mmol/L]	–	0.04	<0.01–0.05	26	0.01–0.05[e]	<0.01–0.02[e]	0.05–0.06[e]
Protein electrophoresis							
Total protein [g/L]	33 ± 6	32	22–43	26	23–43	20–26	39–46
Albumin [g/L]	12.9 ± 2.0	13.1	7.0[c]–17.3	26	10.0–16.3	9.1–10.9	15.4–17.3
Total globulins [g/L]	19.4 ± 4.9	18.9	11.4–27.2	26	9.8–28.9	7.1–12.6	26.2–31.7
Albumin:globulin ratio	0.73 ± 0.22	0.66	0.45–1.27	26	0.41–1.20[e]	0.35–0.48	1.03–1.40

[a]5.0 µmol/L was an outlier. The next highest value was 3.4 µmol/L.
[b]88.84 µkat/L was an outlier and was removed from calculation of reference intervals. The next highest value was 31.26 µkat/L.
[c]7.0 g/L was an outlier and was removed from calculation of reference intervals. The next lowest value was 10.5 g/L.
[d]Reference intervals for gamma glutamyl transferase could not be calculated as the majority of values (54%) fell below detection limits.
[e]Reference intervals were calculated using logarithmic transformations, as data were non-normal.
[f]Reference intervals were calculated using Box-Cox transformations, as data were non-normal.
For analytes with $n < 20$, values are reported descriptively, as reference intervals cannot be calculated for sample sizes <20. For analytes with values below the limits of detection, only median and range are reported; values of half of the detection limit were then assigned to generate reference intervals. Parametric methods for sample sizes ≥20, but <40 were used to calculate reference intervals (Friedrichs et al., 2012), unless otherwise indicated in the footnotes. Normality was assessed using the Shapiro-Wilk test, while outliers were detected using the Dixon-Reed test. Three plasma samples had mild hemolysis (1+), while one sample had mild lipemia (1+), which are not considered to cause interference using dry chemistry analysis (Andreasen et al., 1997; Stacy and Innis, 2017; Stacy et al., 2019). CI, confidence interval; LRL, lower reference limit; RI, reference interval; SD, standard deviation; URL, upper reference limit.

BCI of all hawksbills in this study ranged from 0.94–1.39 (mean ± SD: 1.13 ± 0.10), which is similar to two aggregations of hawksbills from Puerto Rico (means of 1.16 and 1.18) (Diez and van Dam, 2002) and the Bahamas (mean ± SD: 1.17 ± 0.08; range: 1.05–1.41) (Bjorndal and Bolten, 2010), but higher than hawksbills from Indonesia (mean ± SD: 1.06 ± 0.07; range: 0.92–1.14) (Maulida et al., 2017), and lower than hawksbills from the Monito cliff wall in Puerto Rico (mean: 1.24) (Diez and van Dam, 2002) and the Cayman Islands (Little Cayman mean ± SD: 1.25 ± 0.17; Grand Cayman mean ± SD: 1.24 ± 0.18) (Blumenthal et al., 2009). Norton

TABLE 3 Measures of central tendency, range, and reference intervals (with 90% confidence intervals for upper and lower limits) for hematological and plasma biochemical data (including protein electrophoresis) in Standard International units for in-water, subadult (50–78cm standard straight carapace length) hawksbill sea turtles (*Eretmochelys imbricata*) from Florida, USA.

Analyte	Mean±SD	Median	Range	*n*	RI	LRL 90% CI	URL 90% CI
Hematology							
Packed cell volume [L/L]	0.38 ± 0.05	0.38	0.27–0.48	27	0.30–0.46	0.27–0.33	0.43–0.49
Immature RBC/100 mature RBC	3 ± 3	2	0–11	16	–	–	–
White blood cells [×10^9/L]	8.42 ± 1.94	8.15	5.30–12.30	16	–	–	–
Heterophils [×10^9/L]	3.91 ± 0.98	3.80	2.50–6.40[a]	16	–	–	–
Immature heterophils [×10^9/L]	0	0	0	16	–	–	–
Lymphocytes [×10^9/L]	3.63 ± 1.19	3.55	1.50–6.00	16	–	–	–
Heterophil:lymphocyte ratio	1.18 ± 0.49	1.04	0.58–2.73[b]	16	–	–	–
Monocytes [×10^9/L]	0.50 ± 0.27	0.51	0.12–1.20[c]	16	–	–	–
Eosinophils [×10^9/L]	0.34 ± 0.14	0.34	0.05–0.62	16	–	–	–
Basophils [×10^9/L]	0.08 ± 0.13	0	0–0.44	16	–	–	–
Biochemistry							
Alkaline phosphatase [μkat/L]	0.70 ± 0.22	0.68	0.25–1.29	39	0.35–1.05	0.25–0.45	0.95–1.15
Amylase [μkat/L]	13.73 ± 4.17	12.94	8.73–34.05[d]	39	9.03–17.35	7.85–10.20	16.17–18.52
Aspartate aminotransferase [μkat/L]	2.32 ± 0.92	1.97	1.05–5.43	39	1.10–3.59[e,i]	0.97–1.30[e,i]	2.99–4.29[e,i]
Bile acids [μmol/L]	–	2.3	<0.5–9.2[f]	21	<0.5–4.9[j]	<0.5–0.9[j]	3.8–6.2[j]
Blood urea nitrogen [mmol/L]	30.3 ± 6.6	31.4	10.7–40.7	39	19.1–38.5[j]	11.4–23.6[j]	36.7–40.2[j]
Calcium [mmol/L]	2.2 ± 0.3	2.2	1.8–3.1	39	1.9–2.7[i]	1.8–2.0[i]	2.5–2.8[i]
Phosphorus [mmol/L]	2.2 ± 0.3	2.1	1.5–3.0	39	1.6–2.7	1.5–1.8	2.5–2.8
Calcium:phosphorus ratio	1.05 ± 0.19	1.01	0.71–1.53	39	0.73–1.37	0.64–0.82	1.28–1.46
Chloride [mmol/L]	122 ± 5	122	112–134	39	113–131	111–116	128–133
Cholesterol [mmol/L]	–	1.99	<1.17–5.59	39	<1.17–3.53[e]	<1.17[e]	2.95–4.02[e]
Creatine phosphokinase [μkat/L]	19.12 ± 11.94	18.02	3.62–80.74[g]	39	6.95–28.06	3.96–9.94	25.07–31.05
Gamma glutamyl transferase [μkat/L]	–	<0.07	<0.08–0.50[h]	39	<0.08–0.18[e]	<0.08[e]	0.15–0.23[e]
Glucose [mmol/L]	5.2 ± 0.6	5.2	4.0–7.1	39	4.4–6.2[i]	4.2–4.6[i]	5.9–6.5[i]
Glutamate dehydrogenase [μkat/L]	1.98 ± 1.55	1.16	0.45–5.69	23	0.48–4.94[i]	0.31–0.74[i]	3.22–7.57[i]
Lipase [μkat/L]	–	0.17	<0.02–0.80	39	0.03–0.53[j]	0.02–0.05[j]	0.40–0.68[j]
Magnesium [mmol/L]	3.7 ± 0.4	3.7	2.5–4.6	39	3.0–4.4	2.8–3.2	4.2–4.6
Potassium [mmol/L]	4.4 ± 0.5	4.5	3.7–5.4	39	3.7–5.2	3.5–3.9	5.0–5.4
Sodium [mmol/L]	157 ± 5	156	148–178	39	147–165[e]	144–151[e]	162–168[e]
Triglycerides [mmol/L]	0.97 ± 0.40	0.89	0.36–2.21	39	0.32–1.62	0.14–0.51	1.44–1.81
Uric acid [mmol/L]	–	0.04	<0.01–0.08	39	0.01–0.07[e]	<0.01–0.02[e]	0.06–0.07[e]
Protein electrophoresis							
Total protein [g/L]	36 ± 4	36	27–43	39	29–43	27–31	41–45
Albumin [g/L]	14.7 ± 1.9	14.0	11.8–20.3	39	11.0–18.4	10.1–11.9	17.6–19.3
Total globulins [g/L]	20.8 ± 3.4	20.1	13.8–28.5	39	14.2–27.4	12.6–15.7	25.9–29.0
Albumin:globulin ratio	0.75 ± 0.15	0.74	0.47–1.25	39	0.52–1.06[h]	0.47–0.56	0.98–1.16

(Continued)

TABLE 3 (Continued)

[a]6.4×10^9 cells/L was an outlier. The next highest value was 5.1×10^9 cells/L.
[b]2.73 was an outlier. The next highest value was 1.50.
[c]1.20×10^9 cells/L was an outlier. The next highest value was 0.78×10^9 cells/L.
[d]34.05 µkat/L was an outlier and was removed from calculation of reference intervals. The next highest value was 19.79 µkat/L.
[e]Data for aspartate aminotransferase, cholesterol, gamma glutamyl transferase, sodium, and uric acid could not be normalized; therefore, the robust method was used to generate reference intervals.
[f]9.2 µmol/L was an outlier and was removed from calculation of reference intervals. The next highest value was 5.2 µmol/L.
[g]80.74 µkat/L was an outlier and was removed from calculation of reference intervals. The next highest value was 31.76 µkat/L.
[h]0.50 µkat/L was an outlier and was removed from calculation of reference intervals. The next highest value was 0.32 µkat/L.
[i]Reference intervals were calculated using logarithmic transformations, as data were non-normal.
[j]Reference intervals were calculated using Box-Cox transformations, as data were non-normal.
For analytes with $n < 20$, values are reported descriptively, as reference intervals cannot be calculated for sample sizes <20. For analytes with values below the limits of detection, only median and range are reported; values of half of the detection limit were then assigned to generate reference intervals. Parametric methods for sample sizes ≥20 but <40 were used to calculate reference intervals (Friedrichs et al., 2012), unless otherwise indicated in the footnotes. Normality was assessed using the Shapiro-Wilk test, while outliers were detected using the Dixon-Reed test. Three plasma samples had mild hemolysis (1+), while one sample had mild lipemia (1+), which are not considered to cause interference using dry chemistry analysis (Andreasen et al., 1997; Stacy and Innis, 2017; Stacy et al., 2019). CI, confidence interval; LRL, lower reference limit; RI, reference interval; SD, standard deviation; URL, upper reference limit.

and Wyneken (2015) suggest that BCI scores of <1.00 are indicative of emaciation in sea turtles, and since all turtles in the present study were considered robust based on visual evaluation, BCI scores should be specifically developed for individual sea turtle species, geographical locations/populations, and life-stage classes (Nishizawa and Joseph, 2022).

There is evidence that hawksbill turtles migrate during their development and growth from small juveniles in the Florida Keys along the Southeast Florida Continental Reef Tract to subadults in their northern range in Palm Beach County, Florida (Wood et al., 2013). The observed statistically significant difference between capture sites based on SCL and thus differentiating juveniles mainly captured in Monroe County vs. subadult and adult turtles mainly captured at Palm Beach County supports this further. The very small overlap of SCL (Figure 3) between juveniles and subadults at both capture sites also substantiates that secondary recruitment of juveniles from the Keys to the north occurs in this population.

BCI was significantly higher in hawksbills from Monroe County compared to those from Palm Beach County, despite turtles from Palm Beach County having higher SCL, mass, and body depth. These trends are driven by life-stage class distribution between the two sites, as juveniles had a significantly higher BCI than subadults, and more juveniles (23 of 28 total turtle captures; 82%) were caught in Monroe compared to Palm Beach County (3 of 40 total turtle captures; 8%). These morphometrical data trends in hawksbills suggest fast somatic growth rates in juvenile hawksbills since smaller juveniles had higher BCI potentially indicating greater food intake. A comparison of forage items of the two Florida hawksbill aggregations from this study has not been reported, therefore it is unknown if dietary or habitat-related differences between the Palm Beach County and Monroe County sites have influenced the results. Differences in BCI by capture locations and by carapace length have previously been reported in green turtles (*Chelonia mydas*) (Diez and van Dam, 2002; Peig and Green, 2010; Lamont and Johnson, 2021). It is likely that dissimilarities in BCI of sea turtles are mainly driven by differences in environmental conditions, population density, disease prevalence, and/or forage availability (Bjorndal et al., 2000; Diez and van Dam, 2002; Labrada-Martagón et al., 2010; Rossi et al., 2019; Lamont and Johnson, 2021).

5.2. Hematology

PCV is considered an indicator of overall fitness (Stamper et al., 2005). Based on data from captive animals, Frair (1977) suggested that hawksbills have an overall lower PCV than other sea turtle species (range: 0.17–0.42 L/L) and attributed this to dissimilarities in size and growth. In foraging hawksbills from Australia and nesting hawksbills from Brazil, PCV ranged from 0.12–0.41 L/L and 0.34–0.40, respectively (Goldberg et al., 2013; Whiting et al., 2014). The PCV of all hawksbills in the current study ranged from 0.22–0.48 L/L. Mild anemia in sea turtles is defined as PCV ranging from 0.19–0.25 L/L, with severe anemia occurring in patients with PCV ≤0.12 L/L (Stacy and Innis, 2017); therefore, it seems that wild-caught juvenile hawksbills may have naturally occurring lower PCV (Frair, 1977; Whiting et al., 2014), under due consideration that lymph contamination during blood sampling cannot always be reliably excluded in sea turtles (Stacy and Innis, 2017). Of note, the methodology of obtaining PCV data should be considered, as some studies report discrepancies between hematocrit (Hct) data (using automated analyzers) and the manually obtained PCV (*via* spun capillary tubes), with Hct data being considered inaccurate (i.e., lower) *via* automated methods in sea turtles (Muñoz-Pérez et al., 2017; Stacy and Innis, 2017).

While the ranges of PCV established in this and other studies overlap, it also appears that there may be life-stage class differences in this species. For example, we observed a strong positive relationship between PCV and SCL in Florida hawksbills, indicating that RBCs presumably increase in length, width, and volume as turtles mature and oxygen demands increase with longer dive times as described in other studies (Frair, 1977; Stamper et al., 2005; Perrault et al., 2016; Stacy et al., 2018). This trend of increasing PCV with turtle size has been documented in six of the seven sea turtle species (Frair, 1977; Wood and Ebanks, 1984; Casal et al., 2009; Rousselet et al., 2013; Perrault et al., 2016; Stacy et al., 2018).

In comparison to subadults, juvenile hawksbills had higher incidences of RBC polychromasia (i.e., increased basophilic color), anisocytosis (i.e., variation in RBC size), and basophilic stippling (i.e., punctate basophilic cytoplasmic inclusions), and a greater proportion of immature RBC in relation to total mature RBC. These morphological features of RBC are often associated with active erythroid production (Stacy et al., 2011), an observation also described in younger turtles of other sea turtle species and reptiles in general (Fleming et al., 2020; Perrault et al., 2020; Stacy and Harr, 2020; Perrault et al., 2022).

TABLE 4 Measures of central tendency and range for hematological and plasma biochemical data (including protein electrophoresis) in Standard International units for in-water, adult (>78cm straight carapace length) hawksbill sea turtles (*Eretmochelys imbricata*) from Florida, USA.

Analyte	Mean±SD	Median	Range	*n*
Hematology				
Packed cell volume [L/L]	0.36 ± 0.02	0.36	0.34–0.38	3
Immature RBC/100 mature RBC	2 ± 2	3	0–4	3
White blood cells [×10^9/L]	6.67 ± 2.15	5.90	5.00–9.10	3
Heterophils [×10^9/L]	3.40 ± 0.75	3.30	2.70–4.20	3
Immature heterophils [×10^9/L]	0	0	0	3
Lymphocytes [×10^9/L]	2.60 ± 0.95	2.10	2.00–3.70	3
Heterophil:lymphocyte ratio	1.35 ± 0.22	1.35	1.14–1.57	3
Monocytes [×10^9/L]	0.54 ± 0.41	0.41	0.20–1.00	3
Eosinophils [×10^9/L]	0.20 ± 0.08	0.20	0.12–0.27	3
Basophils [×10^9/L]	0	0	0	3
Biochemistry				
Alkaline phosphatase [μkat/L]	0.53 ± 0.20	0.65	0.30–0.65	3
Amylase [μkat/L]	18.12 ± 2.69	17.54	15.78–21.06	3
Aspartate aminotransferase [μkat/L]	1.89 ± 0.42	2.10	1.40–2.17	3
Bile acids [μmol/L]	–	<0.5	<0.5–2.4	2
Blood urea nitrogen [mmol/L]	27.7 ± 8.4	27.1	19.6–36.4	3
Calcium [mmol/L]	2.0 ± 0.3	2.1	1.7–2.3	3
Phosphorus [mmol/L]	2.0 ± 0.03	2.0	1.9–2.0	3
Calcium:phosphorus ratio	1.03 ± 0.17	1.07	0.85–1.19	3
Chloride [mmol/L]	122 ± 4	120	119–127	3
Cholesterol [mmol/L]	2.50 ± 0.68	2.62	1.76–3.11	3
Creatine phosphokinase [μkat/L]	10.62 ± 2.72	9.55	8.60–13.71	3
Gamma-glutamyl transferase [μkat/L]	–	<0.08	<0.08–0.45	3
Glucose [mmol/L]	5.55 ± 0.60	5.38	5.05–6.22	3
Glutamate dehydrogenase [μkat/L]	0.52 ± 0.03	0.52	0.50–0.54	2
Lipase [μkat/L]	–	0.05	<0.02–0.10	3
Magnesium [mmol/L]	3.2 ± 0.7	3.4	2.4–3.7	3
Potassium [mmol/L]	4.3 ± 0.3	4.2	4.1–4.7	3
Sodium [mmol/L]	154 ± 3	153	152–158	3
Triglycerides [mmol/L]	1.62 ± 0.86	1.23	1.03–2.61	3
Uric acid [mmol/L]	0.02 ± 0.01	0.03	0.01–0.03	3
Protein electrophoresis				
Total protein [g/L]	39 ± 3	39	36–41	3
Albumin [g/L]	16.4 ± 0.8	16.1	15.8–17.3	3
Total globulins [g/L]	21.4 ± 2.3	20.6	19.6–23.9	3
Albumin:globulin ratio	0.82 ± 0.09	0.84	0.72–0.89	3

For analytes with values below the limits of detection, only median and range are reported. One plasma sample had mild hemolysis (1+), which is not considered to cause interference using dry chemistry analysis (Andreasen et al., 1997; Stacy and Innis, 2017; Stacy et al., 2019). SD, standard deviation.

Eosinophils in this study made up a small proportion (range: 0–8%) of the overall leukogram (<0.70 × 10^9 cells/L), which is much lower than in similarly sized wild-caught hawksbills from the Galápagos (Muñoz-Pérez et al., 2017). We also observed a significant positive relationship between SCL and absolute eosinophils. While the exact function of reptilian eosinophils has not been established, they are thought to aid in immune stimulation and phagocytosis of parasites (Stacy et al., 2011; Rousselet et al., 2013; Stacy and Innis, 2017; Stacy and Harr, 2020). Therefore, the observed correlation may be due to antigenic stimulation or influences from environmental factors (Stacy

et al., 2011), with increasing antigenic stimulation as turtles grow and age (e.g., increased incidence of spirorchid infection in larger turtles) (Deem et al., 2006; Casal et al., 2009; Innis et al., 2010).

5.3. Electrolytes and minerals

Although plasma electrolyte and mineral concentrations in hawksbill turtles from this study fell within normal ranges reported for other sea turtle species (Stacy and Innis, 2017), several physiologically relevant relationships with SCL were observed. Similar to juvenile loggerheads (*Caretta caretta*) in North Carolina (Kelly et al., 2015), phosphorus concentrations positively correlated with SCL in hawksbills, suggesting bone and/or somatic growth, given that there were no positive size-relevant correlations with CK, the main enzyme of muscle tissues (Stacy and Innis, 2017). The observed negative correlations of calcium and calcium to phosphorus ratio with SCL suggest differences in somatic growth rates as turtles grow and age (Bolten and Bjomdal, 1992), bone metabolism, or possibly osmoregulation (i.e., associated with changes in salt gland regulation with somatic growth). Hawksbill turtles have an oceanic-neritic development pattern and recruit back to nearshore environments at a small size (20–35 cm carapace length) (Bolten, 2003). Additionally, regional differences are a known major driver for somatic growth in the species (Bjorndal et al., 2016). In contrast to hawksbill SCL, phosphorus in oceanic-juvenile loggerheads correlated negatively (Stacy et al., 2018) and calcium to phosphorus ratio in immature Kemp's ridleys (Perrault et al., 2020) correlated positively with carapace length. Possible considerations for these differences include species, life stage-class, and dietary or habitat variations. The observed positive correlations of magnesium with phosphorus and uric acid with magnesium, phosphorus, and sodium suggest potential clinical utility of these analytes in the assessment of renal function in sea turtles, particularly in the presence of normal CK activities (Stacy and Innis, 2017).

5.4. Tissue enzyme activities

Tissue enzyme activities showed several metabolically important correlations with SCL and water temperature in this study. The positive correlations of AST and GLDH with SCL, with the absence of CK associations that would indicate release from muscle tissue, suggest that larger turtles with higher hepatic volume likely have greater tissue enzyme release into plasma, similar to hawksbills in the Persian Gulf and Brazil (Goldberg et al., 2013; Ehsanpour et al., 2015). The negative correlation of SCL with CK further supports the conclusions on mineral correlations, in that the observed correlations with SCL and calcium and phosphorus suggest bone growth differences in smaller turtles rather than growth of muscle tissue.

The negative correlation of ALP with SCL was unexpected, since ALP activities are known to be higher in younger, growing mammals (Allison, 2022); however, this tissue enzyme is widely distributed in sea turtle tissues and isoenzymatic activities of ALP in bone tissue have not been assessed to date in reptiles (Anderson et al., 2013; Petrosky et al., 2015; Adamovicz et al., 2019). The tissue enzymes AST, GGT, and lipase correlated positively with water temperature, which could be driven by higher physical activity or somatic growth rate in smaller turtles.

TABLE 5 Morphological evaluation of red blood cells (RBC), white blood cells, and thrombocytes for in-water, hawksbill sea turtles (*Eretmochelys imbricata*) of three life-stage classes from Florida, USA.

Morphological finding	Juveniles	Subadults	Adults
Polychromasia*	Mild: 100% (8/8)	Absent: 25% (4/16) Minimal: 31% (5/16) Mild: 44% (7/16)	Absent: 33% (1/3) Mild: 66% (2/3)
Anisocytosis*	Mild: 100% (8/8)	Absent: 25% (4/16) Minimal: 31% (5/16) Mild: 44% (7/16)	Absent: 33% (1/3) Mild: 66% (2/3)
Basophilic stippling*	Mild: 8/8 (100%)	Absent: 19% (3/16) Rare: 6% (1/16) Minimal: 38% (6/16) Mild: 38% (6/16)	Absent: 33% (1/3) Mild: 66% (2/3)
Immature RBC/100 mature RBC*	6 ± 2 (3–9)	3 ± 3 (0–11)	2 ± 2 (0–4)
Erythrocyte morphology	NSCF	NSCF	NSCF
Leukocyte morphology	NSCF	NSCF	NSCF
Thrombocytes	Adequate: 100% (8/8)	Adequate: 100% (16/16)	Adequate: 100% (3/3)

For immature RBC/100 mature RBC, mean±standard deviation is reported, with the range parenthetically. NSCF, no significant cytological findings. Asterisks denote statistically significant differences between juveniles and subadults at $p < 0.050$.

Activities of GLDH have rarely been reported in sea turtles (March et al., 2018) and the clinical utility of this enzyme is currently unknown (Stacy and Innis, 2017). Green turtles undergoing rehabilitation in Australia showed strong correlations between plasma GLDH and AST activities, suggesting the potential use as indicators for hepatocellular injury (March et al., 2018). This assumption is further supported by the observed correlations between GLDH and AST, but not CK in hawksbill turtles from this study. Additionally, in eastern box turtles (*Terrapene carolina carolina*), GLDH activities were highest in liver, followed by kidney and gall bladder, with activities in skeletal muscle being among the lowest of the ten tissues examined (Adamovicz et al., 2019). The observed positive correlations of GGT with AST and cholesterol, bile acids with AST, and triglycerides with GGT and glucose highlight the potential for the clinical utility of these analytes for the diagnosis of liver disorders in hawksbill turtles. As such, concurrently increased GLDH, GGT, and/or AST activities, increased triglycerides, cholesterol, and/or bile acids, along with normal CK could support the clinical diagnosis of liver abnormalities, as supported by general assumptions in clinical chemistry interpretations in sea turtles (Stacy and Innis, 2017).

5.5. Lipids

Plasma lipid concentrations in sea turtles often differ by life-stage class. Similar to green turtles and loggerheads (Hasbún et al., 1998; Labrada-Martagón et al., 2010; Delgado et al., 2011; Prieto-Torres

TABLE 6 Relationship of blood analytes to straight carapace length (SCL), body condition index (BCI), and water temperature upon capture of in-water, hawksbill sea turtles (*Eretmochelys imbricata*) from Florida, USA.

Analyte	Adj. r^2	*N*	*p*	Significant predictor(s)	*r*	*p*
Hematology						
Packed cell volume	0.63	36	<0.001	SCL	0.69	<0.001
				BCI	−0.56	<0.001
Eosinophils	0.27	22	0.007	SCL	0.55	0.007
Biochemistry						
Alkaline phosphatase	0.15	62	0.001	SCL	−0.41	0.001
Aspartate aminotransferase[a]	0.06	59	0.035	Water temperature	0.28	0.035
Creatine phosphokinase	0.12	61	0.004	SCL	−0.37	0.004
Calcium	0.15	62	0.001	SCL	−0.40	0.001
Phosphorus	0.07	62	0.022	SCL	0.29	0.022
Calcium:phosphorus ratio[a]	0.18	62	<0.001	SCL	−0.44	<0.001
Cholesterol	0.25	62	<0.001	SCL	0.37	0.003
				Water temperature	0.44	<0.001
Gamma-glutamyl transferase[a]	0.10	60	0.010	Water temperature	0.33	0.010
Glucose	0.39	62	<0.001	SCL	−0.56	<0.001
				Water temperature	0.41	0.001
Glutamate dehydrogenase[a]	0.16	32	0.013	SCL	0.44	0.013
Lipase[a]	0.20	62	0.001	Water temperature	0.43	0.001
Triglycerides	0.23	62	<0.001	Water temperature	0.46	<0.001
Protein electrophoresis						
Total protein	0.20	62	<0.001	SCL	0.46	<0.001
Albumin	0.27	62	<0.001	SCL	0.51	<0.001
				Water temperature	0.28	0.027
Globulins	0.09	62	0.011	SCL	0.32	0.011

[a]Log-transformed.
Stepwise backward multiple regressions were employed. Results of significant predictors are shown. adj, adjusted.

et al., 2013; Rousselet et al., 2013), positive correlations of plasma cholesterol and SCL were also observed in hawksbill turtles from this study. These observations suggest BCI differences due to life-stage class as observed in leatherbacks (*Dermochelys coriacea*), whereby foraging individuals had higher plasma lipids compared to nesting turtles (Harris et al., 2011), likely due to little or lack of foraging during the nesting season (Perrault et al., 2014).

5.6. Glucose

Glucose concentrations in hawksbills from Florida were similar in range to juveniles after successful rehabilitation and in adult nesting hawksbills (Caliendo et al., 2010; Goldberg et al., 2013), and were negatively correlated with SCL, a trend that has also been observed in loggerheads in captive care (Kakizoe et al., 2007; Rousselet et al., 2013). With the additional positive correlation with water temperature, considerations for higher plasma glucose in smaller turtles include higher activity (i.e., in warmer waters), comparatively increased stress level during capture and handling (i.e., smaller turtles often require a short duration chase before capture that is not typically required in larger turtles), or, given similar trends in captive loggerheads, differences in metabolic rates between life-stage classes (Goldberg et al., 2013; Rousselet et al., 2013). Specifically for water temperature, possible higher carbohydrate consumption during periods of higher temperatures is presumed to result in higher metabolic rate, as described in green turtles from Mexico and loggerheads from North Carolina, USA (Stamper et al., 2005; Labrada-Martagón et al., 2010; Kelly et al., 2015).

5.7. Proteins

Similar to other sea turtles, we found that SCL positively correlated with total protein, albumin, and globulins (Hasbún et al., 1998; Osborne et al., 2010; Delgado et al., 2011; Whiting et al., 2014; Kelly et al., 2015; Stacy et al., 2018; Perrault et al., 2020), trends that can be explained by nutritional, seasonal, and/or environmental differences (Frair and Shah, 1982), or as a result of immune system maturation due to antigenic stimulation as turtles grow and age (Innis et al., 2010; Osborne et al., 2010; Perrault et al., 2014). Larger hawksbills foraging in Honduras preferred poriferan prey (e.g., *Melophlus ruber*), with smaller individuals foraging mainly on algae

(Berube et al., 2012). Forage items of the two hawksbill aggregations in Florida from this study have not been reported and could also influence size-relevant correlations with plasma protein concentrations. Lastly, we found that temperature and albumin were positively correlated in hawksbills from this study possibly due to higher activity levels and increased food consumption during warming periods, a trend that has also been observed in Blanding's turtles (*Emydoidea blandingii*) from Illinois (Andersson et al., 2021).

6. Conclusion

This study reports blood analyte data from the critically endangered hawksbill sea turtle encompassing three life-stage classes inhabiting the Florida reef system and gives insight into basic physiological metrics for this population during the study period of 2017–2020. With the defined inclusion criteria used herein, the population was assessed as "clinically normal," thus offering to identify various aspects of sea turtle biology and physiology that are influenced by extrinsic and intrinsic factors. These data will support answering population-driven questions (e.g., future spatio-temporal comparisons, conservation management) in addition to their utility for individual animals (e.g., stranding, rehabilitation).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary materials. Further inquiries can be directed to the corresponding author.

Ethics statement

This study was reviewed and authorized by the National Marine Fisheries Service (NMFS) [Permit #22988], Florida Fish and Wildlife Conservation Commission (FWC) [Marine Turtle Permits #021 and #077], Florida Keys National Marine Sanctuary [Research Permit #175], and University of Florida's Institutional Animal Care and Use Committee (IACUC) [#201706823]. All handling and sampling procedures of sea turtles were performed according to NMFS and FWC regulations.

Author contributions

NIS, JRP, and LDW conceptualized the study and wrote the original manuscript. LDW led all fieldwork and sample collection. JRP analyzed the data. 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.2023.1199688/full#supplementary-material

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Framework for multi-stressor physiological response evaluation in amphibian risk assessment and conservation

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Controlled laboratory experiments are often performed on amphibians to establish causality between stressor presence and an adverse outcome. However, in the field, identification of lab-generated biomarkers from single stressors and the interactions of multiple impacts are difficult to discern in an ecological context. The ubiquity of some pesticides and anthropogenic contaminants results in potentially cryptic sublethal effects or synergistic effects among multiple stressors. Although biochemical pathways regulating physiological responses to toxic stressors are often well-conserved among vertebrates, different exposure regimes and life stage vulnerabilities can yield variable ecological risk among species. Here we examine stress-related biomarkers, highlight endpoints commonly linked to apical effects, and discuss differences in ontogeny and ecology that could limit interpretation of biomarkers across species. Further we identify promising field-based physiological measures indicative of potential impacts to health and development of amphibians that could be useful to anuran conservation. We outline the physiological responses to common stressors in the context of altered functional pathways, presenting useful stage-specific endpoints for anuran species, and discussing multi-stressor vulnerability in the larger framework of amphibian life history and ecology. This overview identifies points of physiological, ecological, and demographic vulnerability to provide context in evaluating the multiple stressors impacting amphibian populations worldwide for strategic conservation planning.

KEYWORDS

amphibian, conservation, risk assessment, stressor, physiology

1 Stressors

Multiple common sources of physiological stress contribute to the ubiquitous threats to amphibian populations worldwide, including disease, pollution, and habitat loss as well as combinations of these stressors (Stuart et al., 2004; Wake and Vredenburg, 2008; Foden et al., 2013; Grant et al., 2016; Green et al., 2020). Stressor impacts can be detected at the organismal level before long-term population decline is apparent. Habitat constraints are frequently observed as higher density resource competition inhibiting metamorphosis, recruitment, or reproductive success in some species (Harper and Semlitsch, 2007; Rittenhouse and Semlitsch, 2007). Disease transmission often presents as an immunological response prior to mass mortality (Ohmer et al., 2021). Pollution, likewise, can result in reduced reproductive success or growth in addition to mortality, and the chronic effects of these stressors can often be detected as systemic responses within the organism that precede impacts apparent at the population level (Thambirajah et al., 2019; Trudeau et al., 2020). Oxidative stress, compromised immunity, endocrine disruption, and altered metabolic activity are some physiological indications of perturbations in biological function that can lead to phenotypical impacts on individual fitness, with implications for population dynamics.

1.1 Habitat degradation

Habitat conversion, degradation, and fragmentation are the primary global causes of terrestrial biodiversity loss (Haddad et al., 2015; Newbold et al., 2015). Though global amphibian declines are linked to multiple stressors and their interactions, habitat loss typically plays an outsized role due to impacts on survival, gene flow, and dispersal (Sodhi et al., 2008). Spatial range, dispersal rates, or seasonal constraints influence population connectivity, and the abiotic conditions limiting habitat availability are projected to be less favorable in response to climate change (Sodhi et al., 2008; Funk et al., 2021). Warmer and drier conditions produced from changing climatic trends provide a direct thermal stressor and are expected to accelerate habitat loss of ephemeral wetlands (Blaustein et al., 2010; Lertzman-Lepofsky et al., 2020). Thermal stressors can geographically constrain or shift suitable aquatic (Duarte et al., 2012) and terrestrial (Hoffmann et al., 2021) ranges, particularly for cold-adapted species and microclimate-dependent life history stages with limited acclimation capacity (Frishkoff et al., 2015).

Sources of anthropogenic modifications linked to amphibian habitat loss are driven by deforestation and urbanization (Cordier et al., 2021). Continued fragmentation of amphibian populations based on their hydroregime dependency has demonstrated that periods of drought effectively isolate numerous endangered species (Zamberletti et al., 2018; Allen et al., 2020). Further, conservation of breeding wetlands is insufficient to overcome the challenges presented by anthropogenically or climatically modified habitats (Allen et al., 2020), particularly the anticipated reduction in temporary wetland inundation (Brice et al., 2022). Additionally, wetland protection depends on legal decisions that are subject to amendment or revision. Even with ample habitat available, environmental stochasticity increases variance in juvenile recruitment for species dependent on ephemeral wetlands (Greenberg et al., 2017), particularly for species with high dispersal rates and/or an energetically costly metamorphosis (Funk et al., 2021; Brooks and Kindsvater, 2022). Hydroperiod duration could have a greater impact on metapopulation persistence than pathogen or contaminant exposure (Smalling et al., 2019), specifically anomalous deluge events or multiple years of drought (Walls et al., 2013; Awkerman and Greenberg, 2022; cf. Moss et al., 2021).

1.2 Pathogens

In addition to the limitations of habitat availability, amphibian populations are also regulated by disease and predation. Many species require fish-free breeding ponds for sufficient reproductive success and are vulnerable to predation by aquatic insects (Ohba, 2011). Anuran species and life stages vary in inherent susceptibility and ecological likelihood of exposure to waterborne pathogens such as ranaviruses and *Batrachochytrium dendrobatidis* (*Bd*) (Haislip et al., 2011; Hoverman et al., 2011). *Bd*, the fungus responsible for chytridiomycosis, is found in cooler, lentic waterbodies (Spitzen-van der Sluijs et al., 2017), and prevalence is often highest among amphibian larvae, with later life stages more resistant to infection (Li et al., 2021). Amphibian response to chytridiomycosis often involves the complement system, in an immunological response to the pathogen, and is frequently detected through bacteria-killing assays (BKA; Rodriguez and Voyles, 2020). Ranavirus is often detected in amphibian communities with greater species diversity (Bienentreu et al., 2022). Pathogen effects can be exacerbated by transmission via more resilient invasive species that spread disease in addition to competing for the diminishing habitat of native species. For example, the American bullfrog (*Lithobates catesbeianus*) is a particularly invasive species that is less susceptible to ranavirus and chytridiomycosis-induced lethality, and therefore acts as an influential vector facilitating world-wide transmission of ranavirus (Hossack et al., 2023). The global trade and subsequent farming of this species for human consumption have resulted in the detection of ranavirus in native populations from previously uncontaminated regions such as those of Brazil and Mexico (Ruggeri et al., 2019 and Saucedo et al., 2019, respectively). International trade of the invasive *Xenopus laevis* has also contributed to the spread of chytridiomycosis (Fisher and Garner, 2020).

Amphibian species differ in their response to the fungal pathogen *Bd* with some species showing downregulation of cellular and metabolic functions and upregulation of adaptive immune gene response; however, such responses are ultimately insufficient to prevent high microbial infection loads (e.g., Eskew et al., 2018). Other species with more diverse dermal antimicrobial peptide communities showed minimal response to infection (Eskew et al., 2018). Lower temperatures may increase inflammation-related responses as opposed to warmer temperatures increasing

adaptive immune responses (Ellison et al., 2020). More bacterial reads, presumably from frog microbiomes, were found in populations with a history of ranavirus (Campbell et al., 2018). Differential impacts of changing climate on host and pathogen further complicate strategies to prevent transmission (Blaustein et al., 2012). It is likely that warming climates will impact viral loads, as observed in juveniles at warmer temperatures with less intense but persistent infections (Brunner et al., 2019), and bacteria-killing ability is reduced at higher temperatures in some species (Rodriguez and Voyles, 2020). Coinfection of ranavirus and chytrid in several endemic tadpoles underscores the importance of understanding the etiology and interactions of these pathogens for effective conservation of amphibians and other aquatic vertebrates (Warne et al., 2016).

1.3 Pesticides

Agricultural and residential pesticide use has also been implicated as a contributing factor in declining amphibian populations (Hayes et al., 2010; Brühl et al., 2011, 2013) with agriculture identified as the most common cause of extinction threats for amphibians and other terrestrial vertebrates (Munstermann et al., 2022). A meta-analysis of pesticide effects revealed moderate impacts on survival and decreased mass and relatively greater impacts from deformities not associated with phylogeny. Although contaminants of emerging concern were underrepresented in pollutant studies, pesticide effects were comparable with those of wastewater, less impactful compared to deicer effects, and relatively greater than those of metals and phosphorus compounds (Egea-Serrano et al., 2012). Additionally, transgenerational impacts, lethal and sublethal, have been demonstrated from exposure to environmentally relevant pesticide concentrations (Karlsson et al., 2021; Usal et al., 2021). The amphibian life cycle allows complex exposure dynamics in both aquatic and terrestrial environments, and recommended application rates of many pesticides result in high mortality from terrestrial exposure (Brühl et al., 2013), although terrestrial effects are less frequently documented. Indirect effects of pesticide use at lower concentrations than those toxic to amphibians potentially impact the full lifecycle of amphibians through reduction of resources, although aquatic food web effects are more frequently reported than terrestrial food web effects (Relyea and Diecks, 2008; Relyea, 2009). Overall, aquatic pesticide exposure can alter various endocrine functions important to development and reproduction and result in a variety of systemic impacts in amphibians (Thambirajah et al., 2019). A recent review of endocrine disruption by agrochemicals summarized changes in lipid and energy metabolism among fungicides; effects on metabolism, metamorphic success, and gonadal development for some herbicides; and reduced metamorphosis from fertilizer and other pesticide exposures (Trudeau et al., 2020). Evaluating the non-lethal effects of pesticides is complicated by timing of exposure and sample collection as well as tissue type, such that measured effects vary depending on species, mechanism of action, route of exposure, and the concentration of the compound (Rohr and McCoy, 2010; Glinski et al., 2021; Seim et al., 2022). Even with the abundance of scientific support correlating pesticide exposure to declining amphibian populations, it is unrealistic that impacts of pesticide exposure will be reversed, given the moderate generation times of most amphibians, the complexity of potential exposure based on their life cycle, and the substantial proportion of croplands in protected areas associated with continuing tradeoffs between food security and conservation (Vijay and Armsworth, 2021).

1.4 Stressor interactions

Uncertainty surrounding individual response, species vulnerability, and exposure regime complicates risk assessment determinations of multiple stressor impacts at the landscape level (Relyea and Hoverman, 2006). For instance, co-stressors such as heat, pesticides, and parasites impact amphibian immune responses and can have synergistic effects on fecundity and post-recruitment survival (Kiesecker, 2002, Thompson et al., 2022). When anthropogenic stressors and abiotic factors synergize, the immune system is challenged (Kiesecker, 2011), and early stress experienced during development can affect resilience in later life stages (Kohli et al., 2019; Le Sage et al., 2022). Disease susceptibility can increase following herbicide exposure (Rohr et al., 2013), and lower microbiota diversity, a common result of pesticide exposure, is associated with reduced parasite resistance (Knutie et al., 2017). Anticipating potential long-term effects in response to various stressors and their interactions, which can promulgate into subsequent life stages, challenges both establishing *in situ* causality from single stressors needed for tighter regulations and effective conservation management. Ultimately, ecological risk assessment is complicated not only by a deficit of toxicological data, but also a lack of ecological data to document changes in land use, species abundance and distribution, and disease transmission that are necessary for adaptive management approaches (Womack et al., 2022). Extrinsic stressors are presented in Table 1 along with physiological measurements of these effects, endogenous processes affecting the same biochemical pathways, and life stages in which departures from typical functions are detectable and/or problematic (Figure 2).

2 Lifestage-specific physiology

Effective adaptive conservation management strategies target vulnerable life stages and critical threats to wildlife populations. The biphasic life cycle of anuran amphibians makes them particularly vulnerable to extrinsic stressors because of their dependence on variable aquatic habitat resources as well as terrestrial environmental quality (Nolan et al., 2023). Their complex life history strategy and multiple potential drivers of population decline require a more nuanced approach to targeting spatial and temporal variability in stressors relative to life stage (Walls and Gabor, 2019; Awkerman et al., 2020). Distinguishing stage-specific endogenous variation in physiological processes enables anticipation of compromised physical condition in response to common stressors (Brooks and Kindsvater, 2022; Nolan et al., 2023). Here we focus on stressor impacts on transition between life

TABLE 1 Endogenous activity associated with transitional phases of the anuran lifecycle, exogenous stressors that alter biological processes, methods to assess organismal effects, and potential demographic impacts. ↑ upregulation or increased expression; ↓ downregulation or decreased expression.

Stage	Systemic response	Endogenous activity	Exogenous stressors	Assessment methods	Demographic endpoint
Embryolarval development	Metabolism	Energy production, DNA synthesis, protein synthesis↑, alanine ↓ aspartate ↓, α-ketoglutamine ↑ (Vastag et al., 2011)	Aquatic conditions, including chemical pollution, pathogens, predation (Kiesecker, 2011)	Whole-organism metabolites (Vastag et al., 2011)	Embryo mortality; cessation of development
Metamorphosis	Metabolism	Increasing energy needs, anabolic activity, tail apoptosis; greater dehydration from fasting (Rowland et al., 2023); purine, arginine and pyrimidine, urea cycle metabolites, arginine and purine/pyrimidine, cysteine/methionine, sphingolipid, and eicosanoid metabolism (Ichu et al., 2014)	↑ galactose metabolism and lactose degradation with xenobiotic exposure (Glinski et al., 2021) or reduced resources; ↓ glutathione (Ichu et al., 2014); galactose predictive of chytrid (Wang et al., 2021); predation and pesticide exposure alter aminoacyl-tRNA biosynthesis, galactose and glutathione metabolism, arginine biosynthesis (Snyder et al., 2022); pesticide exposure impacts serine and threonine, histadine, linoleic acid, and sphingolipid metabolism	Whole-organism or tissue metabolomics (Ichu et al., 2014)	Delayed development, reduced transition to juvenile stage
	Redox signalling	Lipid peroxidation ↑, glutathione ↓, catalase ↓, SOD, CAT, MDA expression altered during intestinal development and tail resorption, ascorbic acid ↑ for collagen synthesis (Menon and Rozman, 2007; Guo et al., 2022); glutathione peroxidase ↓, GST ↓, sulfhydryl groups ↓ (Petrović et al., 2021)	Lower antioxidant activity and increased lipid peroxidation to xenobiotics or environmental conditions (abiotic or density effects; Burraco et al., 2017; Petrović et al., 2021); ↑ thiol and CAT in pesticide and nematode infection (Marcogliese et al., 2021)	ROS production in tissues; antioxidant enzymatic responses of SOD, CAT, MDA, GST (Menon and Rozman, 2007; Chen et al., 2017; Guo et al., 2022); decreased expression of GSH; increased TBARS	Delayed development; reduced transition to juvenile stage
	Endocrine response	CS in response to ↑ TH, regulate development via diodination, glucuronidation, sulfation, affecting HPT, HPA, HPG axes (Denver, 2009; Duarte-Guterman et al., 2014; Thambirajah et al., 2019)	GC ↑ to some xenobiotics (Burraco and Gomez-Mestre, 2016; Trudeau et al., 2020), environmental conditions (Sachs and Buchholz, 2019; Thambirajah et al., 2019), predators (Narayan et al., 2013) ; neurogenerative, oxidative, mitochondrial, teratological effects (Di Lorenzo et al., 2020)	CS and TH levels in tissue or immersed water (Gabor et al., 2013a); tissue/organism enzyme activity or DGE in AR, TR, tra, trb, dio2, dio3 (Thambirajah et al., 2022); ambient water assay; size at metamorphosis (Rowland et al., 2023); vitellogenin indicative of feminization (Venturino and de D'Angelo, 2005)	Time to metamorphosis; cohort sex ratio; carryover to juvenile immunity, survival, fecundity (Kiesecker, 2002, Denver, 2009; Kohli et al., 2019; Ruthsatz et al., 2020; Le Sage et al., 2022)
	Immunity	Endocrine-driven development of immunity; immunosuppression at metamorphosis (Rollins-Smith, 2017)	Viral loads, resistance, and parasite prevalance affected by pesticides and abiotic factors (Kerby et al., 2011; Kiesecker, 2011; Knutie et al., 2017; Pochini and Hoverman, 2017); Microbiome in tadpoles impacted by xenobiotic exposure	Gut microbiome diversity; at advanced developmental stages – blood leukocytes, white cell lymphocytes and granulocytes (basophils, neutrophils, eosinophils); DGE (Row et al., 2016)	Reduced survival due to pathogens and parasites (Kiesecker, 2011)
Juvenile maturation to adult	Endocrine	TRH influences TSH (Paul et al., 2022)	Food constraints ↓ CORT (Prokić et al., 2021); variance in CORT along latitudinal cline (Le Sage et al., 2022)	Dermal swab, fecal content, tissue or ambient water assay of CS (Gabor et al., 2013a)	Behavioral responses to stressors, reduced dispersal
	Immunity	Gut microbiome linked to resistance of parasites (Knutie et al., 2017), skin microbiome linked to resistance of pathogens (Krynak et al., 2017; McCoy and Peralta, 2018; Jiménez et al., 2021); possibly compromised by shortened developmental hydroperiod (Brannelly et al.,	Dermal microbiome and pathogen vulnerability impacted by xenobiotic exposure (Krynak et al., 2017; McCoy and Peralta, 2018; Jiménez et al., 2021); habitat degradation affects vulnerability to pathogens (Stevens and Baguette, 2008; Costa et al., 2021; Becker et al., 2023)	Microbiome diversity in skin mucosa (Neely et al., 2022); antimicrobial peptides (Huang et al., 2016); white cell lymphocytes and granulocytes (basophils, neutrophils, eosinophils); B and T cells in organs,	Susceptibility to pathogens, reduced juvenile survival or limited dispersal due to disease or deformities (Kiesecker, 2002, Rohr et al., 2006; Krynak et al., 2017; Kohli et al., 2019)

(Continued)

TABLE 1 Continued

Stage	Systemic response	Endogenous activity	Exogenous stressors	Assessment methods	Demographic endpoint
		2019); Lower juvenile immunity relative to mature adults		MHC-II; antibodies - IgA/X, IgD, IgF, IgM, IgY	
	Metabolism	Related to endocrine activity; longer hydroperiod ↑ lipid stores (Scott et al., 2007)	Influenced by temperature, water loss, xenobiotics; food deprivation ↓ CORT; pesticides altered sucrose and starch pathway regulation (Zaya et al., 2011, Dornelles and Oliveria, 2016, Van Meter et al., 2018)	Body condition; energy metabolism in tissue	Reduced juvenile survival
	Redox signalling	Increased antioxidants during estivation in preparation for oxidative stress; lower oxidative metabolism enzyme activity during estivation (Rowland et al., 2023)	Food constraints ↑ lipid peroxide; ↓ SOD, glutathione peroxidase, GST, glutathione and sulfhydryl groups (Prokić et al., 2021); pesticides and pathogens ↑ thiol; nematode infections ↑ thiol, ↑ catalase (Marcogliese et al., 2021)	ROS production; anti-oxidant enzymatic responses of SOD, glutathione peroxidase, glutathione, GST, thiol, catalase (Marcogliese et al., 2021; Prokić et al., 2021)	Survival to following breeding season, potentially a function of size/ condition at end of season
Adult fecundity	Endocrine activity	Gonadotropins released by pituitary; estrogen, androgen, progestogen regulate reproduction (Duarte-Guterman et al., 2014)	Endocrine disrupting compounds can disrupt gonadal development, sexual differentiation (Lambert et al., 2015; Marlatt et al., 2022); temperature impact on sex determination (Lambert et al., 2015; Ruiz-García et al., 2021); density-dependent resource availability (Kissel et al., 2020)	ER/AR binding; Aromatase inhibition; impairment of steroidogenesis; vitellogenin expression in response to xenoestrogen exposure; zona radiata, zona pellucida, DGE in er, bteb, tra, trb, thbzip,	Altered population sex ratio (Roco et al., 2021; Baranek et al., 2022); reduced fecundity

Endogenous activity associated with transitional phases of the anuran lifecycle, exogenous stressors that alter biological processes, methods to assess organismal effects, and potential demographic impacts. ↑ upregulation or increased expression; ↓ downregulation or decreased expression.

stages (F, T_e, T_l, T_j in Figure 1) but present potential effects on survival and development as well.

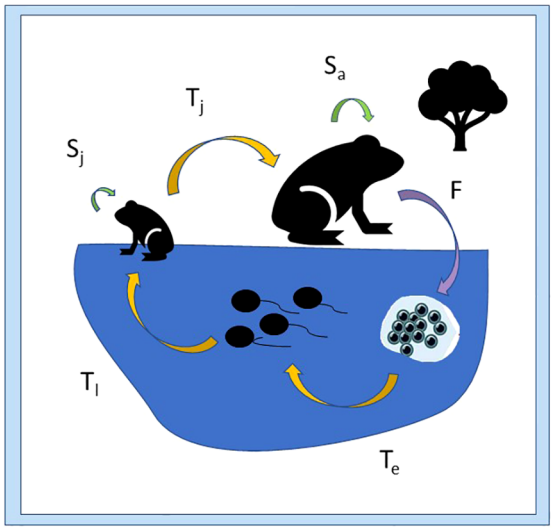

FIGURE 1
Vital rates (indicated by arrows) for the anuran life cycle, including survival rates (green arrows; S) for terrestrial juvenile (Sj) and adult stages (Sa), transition rates (yellow arrows; T) from embryo to larval stage (Te), from larval to juvenile stage (Tl), and from juvenile to adult stage (Tj); and fecundity (purple arrow; F).

2.1 Development (embryo transition to tadpole stage; T_e)

Survival during the relatively brief stage of embryo development is largely dependent on a suitable environment to avoid predators, pathogens, or pollution, and the costs associated with such defenses differ among amphibian species and developmental mode (Brooks and Kindsvater, 2022). Amphibian clutches can experience high mortality from pathogens or predation, depending on the geographic location and ecological community composition (Kiesecker, 2011), such that habitat characteristics and regional observations are most informative in identifying these threats (Wake and Vredenburg, 2008). Pesticides aggregated as runoff in wetlands provide another potential stressor for developing embryos (Smalling et al., 2015) with lethal or sublethal impacts on individuals. Ultimately, a systematic review revealed that the time to hatching for embryos was influenced more by taxonomy and exposure to pollution, rather than experimental setting (lab vs. field; Egea-Serrano et al., 2012). Singly or in combinations, stressors during embryogenesis can lead to delayed, wide-ranging effects, resulting in a diverse array of phenotypic outcomes associated with aspects of developmental plasticity that are not observed until later life history stages (Jonsson et al., 2022).

Given the relatively brief duration of this stage in most anuran species, and rapidly changing metabolism, identifying potential stressors based on organismal condition or response could be a challenging diagnostic approach, compared to assessment of anomalous response during later life stages. Endogenous variation

in embryonic metabolite levels is suggestive of energy production primarily, presumably for DNA synthesis (Vastag et al., 2011). Contaminant levels in egg masses that are linked to deformities and reduced offspring viability can result from maternal transfer of contaminants rather than indicating direct environmental exposure alone (Todd et al., 2011; Metts et al., 2013). Determining physiological response to a variety of stressors (e.g., contaminant mixtures and abiotic factors), is a complex challenge that might be approached by evaluating exposure-based epigenetic changes (e.g., DNA methylation, histone acetylation) in developing embryos (Fogliano et al., 2023) or simply assessing differential responses in later life stages.

2.2 Metamorphosis (transition from larval to juvenile stage; T_l)

The morphological restructuring for transition from larval stage to juvenile stage is dependent on endocrine drivers, specifically surges in thyroid hormones (TH), regulated by thyroid hormone receptors and retinoic acid receptors (TR and RXR, respectively; reviewed in Paul et al., 2022). Endocrine regulation and body morphogenesis during the larval stage are controlled by the hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-adrenal/interrenal axes (HPA/HPI) as well as the hypothalamus-pituitary-gonadal (HPG) axis (Duarte-Guterman et al., 2014). Development and metamorphosis are regulated largely by the release of the thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) and modulated by the corticosteroids (CS) corticosterone (CORT) and aldosterone (ALDO; Denver, 2009). Regulation of TH signal involves cellular processes of deiodination, glucuronidation, and sulfation (Thambirajah et al., 2019). Metabolism and cardiac functions associated with development and metamorphosis are also regulated by CS. Ruthsatz et al. (2020) showed that certain metamorphic stages were significantly more susceptible to changes in growth and development due to increased TH levels, with high TH levels associated with reduced weight and size in tadpole and froglet stages as well as increased heart rate and reduced energy stores across all stages.

TH inhibition or impairment can delay development, while CS production is often associated with accelerated metamorphosis in response to pond drying or other stressors (Sachs and Buchholz, 2019; Thambirajah et al., 2019), although the role of CORT as a homeostatic response to stress is complex. The corticotropic releasing hormone (CRH) regulates the HPA axis as well as the HPT axis, thereby contributing to additional crosstalk between these pathways and circulating hormone levels (Thambirajah et al., 2022). CORT levels in southern leopard frogs increased with exposure to multiple aquatic stressors, specifically a nitrogenous fertilizer, a pesticide, and salt (Adelizzi et al., 2019). However, relatively elevated CORT levels were associated with populations less tolerant to contaminant exposure, such that differences in stress response could be indicative of exposure history (Shidemantle et al., 2022). Predator detection can also elicit an increased CORT response (Narayan et al., 2013). Signals of agrochemical disruption of endocrine function among interactions of the thyroid, gonadal, and metabolic axes in amphibians was reviewed in detail by Trudeau et al. (2020). Early life stage stressors that elevate CS production can alter endocrine response throughout the lifecycle of the individual (Denver, 2009).

Stressor perturbations in endocrine functions are particularly impactful in metamorphosing amphibians and can influence

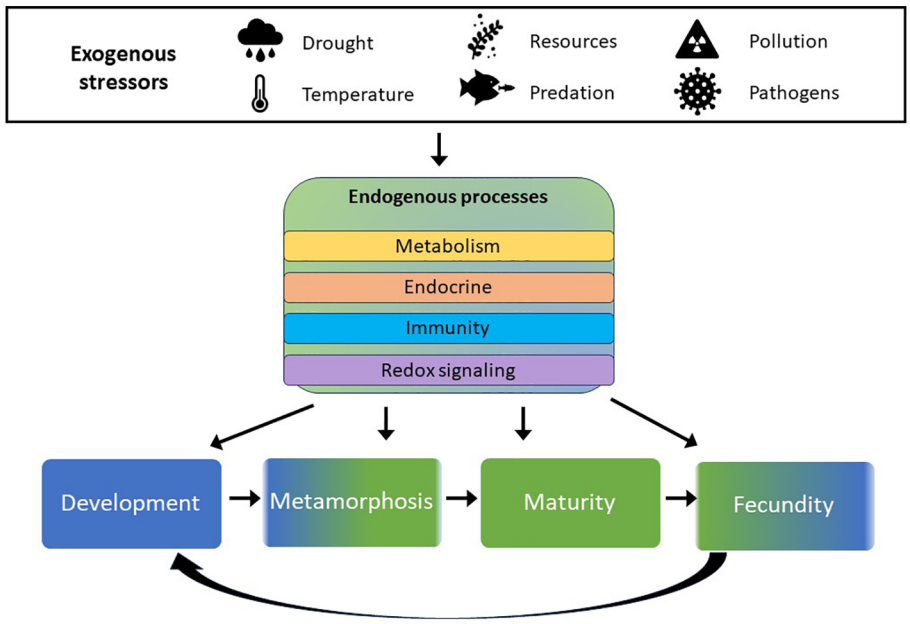

FIGURE 2
Symbols represent influential abiotic factors and extrinsic sources of population regulation. These potential stressors impact endogenous processes (shown in center block) in similar ways physiologically, and can affect individuals differently, depending on the life stage at which the stress occurs.

immunity, survival, and fecundity in subsequent terrestrial life stages (Kiesecker, 2002; Kohli et al., 2019). A meta-analysis determining effects on time to metamorphosis found taxonomy, pollution, and timing of exposure to be more influential than the experimental setting (Egea-Serrano et al., 2012). Pond drying constraints influencing larval development are expected to be impacted in various ways by climate change, depending on regional location (Walls et al., 2013). The duration of larval stage and developmental mode, combined with community dynamics between larval competitors and predators, can distinguish species resilience and response to such unpredictable environmental stressors (Belden et al., 2010; Moss et al., 2021; Brooks and Kindsvater, 2022). Interannual variability in hydrologic regime at temporary wetlands determines the length of the developmental period and the density of developing anuran larvae (e.g., Pechmann et al., 1989). Developmental plasticity in metamorphic climax allows variable phenotypic response to interannual conditions and is driven by the neuroendocrine processes responsible for the development of the immune system, highlighting a potential tradeoff between accelerated development and resistance to disease and parasites (Kohli et al., 2019). Likewise, tradeoffs between development and microbiota diversity or immunology are demonstrated later in life with increased susceptibility to pathogens (Le Sage et al., 2022). As northern leopard frog tadpoles approach metamorphic climax, tail tissue decreases expression of mitochondrial energy genes and upregulates expression of immunity genes (Row et al., 2016). Post-metamorphic immune function may be compromised in amphibians experiencing shorter hydrologic regimes (Brannelly et al., 2019), which may further exacerbate disease susceptibility. Therefore, interannual variance in aquatic habitat suitability can have lasting impacts on cohort fitness.

During metamorphic climax, metabolic activity changes, reflecting increasing energy needs, anabolic requirements, and tail apoptosis; these energetic requirements and fasting effects create a vulnerable transition from larva to juvenile in which contaminant body burdens can amplify (Rowland et al., 2023). The aquatic phase of the amphibian life cycle is also susceptible to reduced growth in response to pathogens that have been introduced to waterbodies, although survival is rarely impacted at this stage (Nolan et al., 2023). Although phylogeny and abiotic environmental variables determine the initial likelihood of *Bd* or ranavirus occurrence in areas of viral compatibility, other stressors such as pesticides can further influence viral loads and the resistance of the host population as well as the prevalence of parasite-induced deformities (e.g., Kerby et al., 2011; Kiesecker, 2011; Pochini and Hoverman, 2017).

2.3 Maturity (juvenile transition to reproductive adult; T_j)

The literature on this critical amphibian life stage is scarce, due in part to the complexity of rearing and maintaining juvenile amphibian populations in a laboratory setting through maturation, as well as the challenge of monitoring individual juvenile amphibians from metamorphosis through reproduction in a field setting (see Petrovan and Schmidt, 2019). Furthermore, a systematic analysis of factors affecting survival found pollutants and the experimental setting (lab vs. field) to be more influential than taxonomic group or developmental stage in the study (Egea-Serrano et al., 2012). Although pre-metamorphic environmental conditions directly influence post-metamorphic life stages, there may also be distinctly different age or stage-specific stress responses in amphibians, as evidenced by variations in sucrose and starch pathway regulation following pesticide exposure in larval and juvenile amphibians (Zaya et al., 2011; Dornelles and Oliveria, 2016; Van Meter et al., 2018). Survival to reproduction was positively correlated with lipid stores at metamorphosis among two *Ambystoma* salamander species, and lipid stores were greater among individuals emerging from longer hydroperiod wetlands. Furthermore, total rainfall during years of juvenile development was also positively associated with survival to reproduction (Scott et al., 2007).

Potential carry-over effects from compromised development can exist (Kiesecker, 2002; Rohr et al., 2006; Kohli et al., 2019) with additional risk from stressors in the terrestrial environment. Terrestrial habitat degradation and habitat fragmentation influences the connectivity of both amphibian populations and their potential pathogens (Stevens and Baguette, 2008; Costa et al., 2021; Becker et al., 2023). Reduced skin and gut microbiota in the larval stage can also reduce parasite resistance in adults (Knutie et al., 2017). Amphibian skin contains antimicrobial peptides linked to immunity and defense functions as well as to biosynthesis and metabolism (Huang et al., 2016). Skin secretions have demonstrated antimicrobial antioxidant properties and can be beneficial to healing (Wang et al., 2020). Bacterial and fungal taxonomy in skin secretions is associated with disease resistance (Bates et al., 2022), with the skin microbiome affected by the same abiotic factors that influence *Bd* occurrence (Ruthsatz et al., 2020). Some species' secretions contain sufficient toxins to be lethal to predators, thereby reducing mortality via predation (Liu et al., 2022). Compromised skin microbiome diversity is implicated in important physiological functions such as electrolyte and hydration loss, disease susceptibility, and increased pesticide effects. Pesticide exposure has been linked to disruption of the skin microbiome and antimicrobial peptides of amphibians, thereby affecting vulnerability to pathogens (Krynak et al., 2017; McCoy and Peralta, 2018; Jiménez et al., 2021). Habitat disturbance has also been associated with skin microbiome diversity, primarily via pathogen dispersal (Neely et al., 2022), underscoring the ecological complexity of proximate mechanisms of multiple stressors and their potential interactions. Enhanced data collection efforts on juvenile amphibians are essential to improve risk assessment and inform management decisions at the local scale.

2.4 Fecundity (adult production of embryos; F)

Reproductive failure associated with insufficient hydroperiod is a determinant of lifetime reproductive success in species dependent on ephemeral wetlands for breeding (Taylor et al., 2006; Stevens and

Baguette, 2008). In years with suitable hydroregime, terrestrial density dependence and sex ratio can affect fecundity within a population (Kissel et al., 2020; Baranek et al., 2022). Effects of endocrine disruption in developmental phases as well as during gamete production could also reduce fecundity via altered gonadal development, or a high incidence of intersex individuals in the population (Lambert et al., 2015; Marlatt et al., 2022). Sex-specific age at maturation could further restrict operational sex ratio in amphibian populations (Baranek et al., 2022). In addition to the endocrine disruption associated with xenobiotic exposure, temperature can affect sex determination, with potential impact on operational sex ratio following extended periods of anomalous temperatures (Lambert et al., 2015; Ruiz-García et al., 2021). The lasting impact of such shifts will vary depending on the species life history and genetic sex determination (Bókony et al., 2017).

3 Field-based measures of stressor response

Assessing the status of a wildlife population or relative condition of an individual within its habitat is a challenge complicated by the heterogeneity of both organismal response and stressor distribution. Acquiring an adequate sample size for a meaningful detection of environmental or stressor effects could limit the practical scope of most field-based efforts, while standardization of conditions can bias the interpretation of stressor response in most laboratory or mesocosm settings. Stage-specific physiology, along with ecological or life history vulnerabilities, provides additional context for interpretation of potential stressor effects (Venturino et al., 2003). For example, intestinal development and tail resorption in larvae are coincident with signs of oxidative stress (Menon and Rozman, 2007). Establishing baseline physiology with common biomarkers provides context of endogenous variability during the amphibian life cycle. Identifying these informative endpoints and sensitive stages can preclude the need for extensive accounting of stressor-specific effects and interactions.

3.1 Physiological processes

Systematic responses to stress include endocrine disruption, oxidative stress, metabolic perturbation, and compromised immunity (Venturino et al., 2003). Specifically, elevated CORT and standard metabolic rate as well as decreased antioxidant enzymes are common signals of abiotic and xenobiotic physiological stress (Burraco and Gomez-Mestre, 2016). Endocrine disruption in the interconnected hormonal axes can also trigger responses in other systems, such as neurodegenerative effects, oxidative damage, impairment of mitochondrial function, and teratological effects (Di Lorenzo et al., 2020). Taxonomic family and pollutant exposure were significant determinants of developmental abnormalities in a systematic review of ecotoxicological studies (Egea-Serrano et al., 2012), and specific phenotypical abnormalities can be ascribed to different classes of chemicals (Venturino et al., 2003).

Endocrine-driven developmental processes are highly conserved in vertebrates (Paul et al., 2022), as are many physiological endpoints associated with both homeostatic and lethal responses to pesticides and contaminant exposure. Larval amphibians are especially susceptible to endocrine disruption due to their reliance on hormonal cues for initiation and timing of metamorphosis and sex determination (Duarte-Guterman et al., 2014). Crosstalk between hormonal axes includes an evolutionary history of HPT and HPG signaling (Thambirajah et al., 2022). Genetic sex determination during developmental stages varies between and within amphibian species due to rapid turnover of genes such that either males or females can be heterozygotic, with some species having three sex chromosomes (Miura, 2017). Sex reversal in response to external temperature or steroid hormones can also affect the sex ratio of a cohort (Roco et al., 2021). Although estrogenic and androgenic effects have been studied much more extensively in mammals, intersex amphibian larvae resulting from reproductive steroid hormone exposure have been associated with effects on the androgen receptor (AR) and thyroid receptor (TR) and altered expression of dio1, dio2, dio3, and thrb (Thambirajah et al., 2022). Increased vitellogenin production is a common indication of feminization (Venturino and de D'Angelo, 2005), and increased formic acid has been suggested as an indicator of androgen receptor binding and anti-androgenic effects in larvae (Melvin et al., 2018).

Endogenous changes in metabolism are also associated with lifecycle-dependent physiological processes (Ichu et al., 2014). During metamorphosis, metabolic pathways are dramatically altered in the liver and the tail as a result of lipid and carbohydrate metabolism (Zhu et al., 2020). Metabolomic changes during metamorphosis demonstrate physiological processes associated with morphological restructuring in metabolic pathways, including the urea cycle as well as arginine and purine/pyrimidine, cysteine/methionine, sphingolipid, and eicosanoid metabolism; however, similar metabolite expression in humans is associated with disease (Ichu et al., 2014). As the tadpole tail regresses and intestines restructure, lipid peroxidation is increased; depleted catalase (CAT) and glutathione contribute to oxidative stress, as demonstrated by CAT, superoxide dismutase (SOD), and malondialdehyde (MDA) expression; and the antioxidant ascorbic acid increases as organs develop (Menon and Rozman, 2007; Guo et al., 2022). Epidermal galactose levels, and specifically 25 uniquely expressed genes, are predictive of chytrid outbreaks and are life stage dependent, with higher expression at metamorphosis (Wang et al., 2021). Food constraints in the juvenile stage were associated with higher lipid peroxidase and lower SOD, glutathione S-transferase (GST), glutathione peroxidase, glutathione and sulfhydryl groups (Prokić et al., 2021).

Antioxidant system response (AOS) and oxidative stress is highest at metamorphic peak, and associated with lower glutathione, CAT, glutathione peroxidase, GST, and sulfhydryl groups, and oxidative stress is exacerbated by decreasing water levels (Petrović et al., 2021). Hepatic GST activity has been proposed as a biomarker indicative of TH signaling imbalance and developmental effects (Chen et al., 2017). Upregulated

pathways include transamination and the urea cycle because of hepatic catabolism, TCA cycle and oxidative phosphorylation resulting from energy metabolism (although these are downregulated in the tail), and hepatic glycogen phosphorylation and gluconeogenesis (Zhu et al., 2020). Decreased activity occurred in β-oxidation and the pentose phosphate pathway, and downregulation of glycolysis, β-oxidation, and transamination in the tail accompanied reduced protein synthesis and lower energy production and consumption, although glycogenesis, fatty acid elongation and desaturation, and lipid synthesis were maintained (Zhu et al., 2020).

Indication of oxidative stress is a common detoxification response to many chemical classes and is characterized by altered expression of mixed function oxidases (MFO; e.g., CYP1A, EROD, demethylase), GSH, lipid peroxides, and antioxidant enzymes (CAT, SOD; Venturino and de D'Angelo, 2005). Oxidative stress and lipid peroxidation, as demonstrated by increased SOD and CAT activity were also associated with hepatotoxicity resulting from increasing organophosphate exposure, although GST activity was unchanged, and MDA decreased (Li et al., 2017). Common indications of oxidative stress as a detoxification response include glutathione deficits and production of GST (Venturino and de D'Angelo, 2005). Interactive oxidative stress effects of pesticide concentration and parasite abundance were observed in thiol levels of recent metamorphs, with nematode infection related to elevated thiol and catalase expression (Marcogliese et al., 2021).

Amphibian physiological responses to environmental stressors have been well documented (Park and Do, 2023), and the various threats that impact amphibian populations can elicit similar physical effects. For example, xenobiotic exposure or threatening environmental conditions (e.g., pond drying or predator presence) is commonly associated with oxidative stress and production of reactive oxygen species (ROS; Burraco et al., 2017). Habitat fragmentation and degradation, coincident with anthropogenic infringement and climate change, contribute to invasive species introduction, disease outbreak, and increased pollution, multiplying threats to immunocompetency (Kiesecker, 2011). Immune functions impacted by common amphibian stressors and their interactions are indicated in various matrices and measurements. For example, glucocorticoids (GC) are CS hormones influencing the immune system, tissue inflammation, and cardiovascular response (Rollins-Smith, 2017), and frequently indicate physiological stress. However, some stressors, e.g., food deprivation, can yield differential endocrine responses, with reduced CORT in juveniles conserving energy resources as opposed to increased CORT levels in food-deprived tadpoles (Crespi and Denver, 2005).

3.2 Omics technologies

Stressor-specific measurements of organismal response introduce complexity to both laboratory and field-based assessment approaches, as well as to the interpretation of multi-stressor scenarios. Evaluating biomarker expression can help identify biochemical perturbations indicative of systemic stress to environmental conditions. The suite of 'omics technologies, including genomics, transcriptomics, proteomics, and metabolomics, can shed light on the underlying biological processes and provide a means to identify specific genes, metabolites, and pathways that are affected in an amphibian ecological risk assessment.

Comparative genomics is increasingly recognized as a valuable tool for conservation purposes (Kosch et al., 2023). This includes the use of reference genomes in eDNA approaches for monitoring populations (Breton et al., 2022; Saeed et al., 2022), informing genetic rescue efforts for threatened amphibians (Kosch et al., 2023), and using sequence information to predict protein similarity and infer ecotoxicological implications across species (LaLone et al., 2023). However, compared to other vertebrate classes, genomic coverage for amphibians is currently recognized as lacking (Hotaling et al., 2021). Kosch et al. (2023) provided a review of the 32 available amphibian reference genomes and found variable annotation quality for the available genomes and uneven coverage across amphibian families, with genomic comparison further complicated by the presence of large, repetitive genomes. This limited availability of amphibian reference genomes presents challenges for generalizing ecotoxicological results to higher taxonomic levels within the class Amphibia. Despite these challenges, genetic approaches can provide conservation insights. This is particularly true for amphibian species with cryptic habits and biphasic life cycles, which complicate traditional field-based measurements of survival, fecundity, and migration (Mazerolle et al., 2007; Funk et al., 2021). Landscape genetics, for instance, can reveal connectivity within a population as well as isolated subpopulations (Watts et al., 2015). This information can then be used to prioritize conservation targets for threatened amphibians (e.g., Forester et al., 2022). The pressing need for increasing knowledge of amphibian genomes to assist in conservation efforts was highlighted by Calboli et al. (2011). It is hypothesized that only a relative few, simple mechanisms of gene alterations are indicated in amphibians' response to numerous environmental stressors. Functional genomics has been used to probe the molecular underpinnings of field observations concerning the sexual differentiation in amphibians (Bögi et al., 2002), fragmentation of populations (McCartney-Melstad et al., 2018), and pathogen-host interactions (Zamudio et al., 2020).

In the laboratory, transcriptomics approaches leverage differential gene expression (DGE) approaches by contrasting the expression level of transcripts in stressed individuals versus control individuals. Changes in gene expression can reveal which genes are upregulated or downregulated, thereby identifying perturbations in specific biochemical pathways regardless of the origin of the stressor. The magnitude of the response could indicate functional points of departure (e.g., Ewald et al., 2022; Mittal et al., 2022). Transcriptomics data, generated from controlled laboratory exposures, provide a comprehensive view of gene expression changes comparable to traditional apical endpoints. The large volume of data, coupled with the fact that the expression responses are specifically associated with the mechanism of the stressor, suggests the possibility of developing expression-based "fingerprints" or signatures resulting from single and multi-stressor exposures. These can be used to determine if the

magnitude of an exposure to a toxicant or stressor of a particular mode of action is likely to elicit biological perturbations that can be linked to or predictive of apical effects. Furthermore, high-throughput transcriptomics (HTTr) methods have been developed to observe changes in gene expression in cells, rather than in test species, after exposure to chemicals (Schirmer et al., 2010). These methods are less resource-intensive than traditional toxicity testing and can be used to determine at what concentration chemicals impact cellular biology and to develop adverse outcome pathways (AOPs). For vertebrates, such regulatory testing programs aim to evaluate the potential endocrine-disrupting effects of chemicals, utilizing the conservation of certain endocrine pathways among vertebrate classes to evaluate the feasibility of extrapolating data across taxa. These approaches integrate functional genomics with transcriptomics to establish the confidence levels in pathway conservation while identifying the specific needs for additional data to advance read-across methods for estrogen, androgen, thyroid, and steroidogenesis pathways in vertebrate ecological receptors (McArdle et al., 2020).

Metabolomics technology may also provide a means to address the uncertainties surrounding chemical risk assessment of single and multiple stressors. Available technology measures the changes in hundreds (if not thousands) of metabolites simultaneously, effectively capturing a metabolomic fingerprint as a snapshot of an organism's altered physiology. This metabolomic fingerprint of subcellular biological responses often represents immediate or early response within the organism to stresses and can be associated with signaling networks that are linked to adverse outcomes at higher levels of biological organization. Successful application of metabolomics to differentiate multi-stressor response was achieved by Snyder et al. (2022). Similarly, the use of transcriptomics and proteomics for advancing amphibian toxicogenomic studies was reviewed in Helbing (2012). Relying on 'omics technologies to identify meaningful suites of stressor response and target demographic vulnerabilities for sample collection could offer a comparative physiology approach for detecting impacted individuals and populations.

Exogenous impacts of xenobiotic exposure can exacerbate stressors that accompany particular life stages. Reduction in body size during metamorphosis and fasting during hibernation result in increased metabolic demands and greater body burdens of contaminants due to biomagnification (Rowland et al., 2023). Aquatic exposures of various pesticides were associated with increased galactose metabolism and lactose degradation, indicating effects on energy metabolism (Glinski et al., 2021). Pathways associated with glucogenesis and glycolysis were also indicators of energy metabolism impacts in terrestrial juvenile frog exposures (Van Meter et al., 2022). The urea cycle was frequently impacted by various pesticides, and the purine metabolism pathway was also enriched, indicating increased energy production as a response to toxicity. Reduction in glutathione levels is another common result of pesticide exposure indicative of oxidative stress in both larval and juvenile amphibians (Ichu et al., 2014). Reduced citrate, α-ketaglutarate, and fumarate were also proposed as oxidative stress biomarkers, as intermediates of the tricarboxylic acid cycle (Melvin et al., 2018).

The magnitude of altered metabolite regulation during later life stage terrestrial exposures was not indicative of bioaccumulation, and exposure to combinations of pesticide did not always have a synergistic effect on juvenile toads (Van Meter et al., 2018). In fact, extrinsic sources of urea as fertilizer at low concentrations can counteract combined pesticide effects, presumably by facilitating excretion and detoxification, although excessive doses can be detrimental (Van Meter et al., 2022). Hepatic metabolome analyses revealed altered pathways indicating stress caused by both predation and pesticide exposure; these include aminoacyl-tRNA biosynthesis, galactose metabolism, glutathione metabolism, and arginine biosynthesis (Snyder et al., 2022). Transgenerational fertility effects of endocrine disruption due to pesticide exposure were associated with greater mass, increased palmitoleic:palmitic acid ratio, and decreased glucose (Karlsson et al., 2021). As studies of multistressor deviations from normal metabolite activity continue, identification of meaningful pathway perturbations could provide a systematic method of identifying locations of environmental impacts without prerequisite knowledge of specific land use changes or fate and exposure of particular pollutants.

3.3 Sampling strategies

Traditional measures of contaminant impacts on amphibians focus on body burden concentrations and somatic indices of body condition (e.g., Băncilă et al., 2010) as well as general indicators of genotoxic and mutagenic impacts (i.e., comet and micronucleus assays) and more targeted analyses of cellular-level endpoints. For larger taxa (e.g., birds and mammals), marking individuals and collecting tissue samples can be a routine, noninvasive procedure conducted in the field to inform physiological condition. However, the small body size of amphibians hinders repeated sampling of blood or plasma for various analyses. For smaller amphibians, sampling techniques might involve toe-clipping for both individual identification as well as tissue collection, or sample collection might require sacrifice of individuals, particularly at earlier life stages.

Many common assays measure hematological enzymatic responses typical of exposure to specific xenobiotic contaminants (Ohmer et al., 2021). For example, esterase activity (acetylcholinesterase, butyryl cholinesterase, and carboxyl esterase) can indicate potential developmental effects, but response varies greatly within and between species (Venturino et al., 2003; Venturino and de D'Angelo, 2005). Hematological measures representative of immune response include leukocytes, neutrophil/lymphocyte ratio, bacterial killing assays, and delayed hypersensitivity assays (Ohmer et al., 2021). Changes in neutrophils and lymphocytes are often proportional with increased glucocorticoid levels, indicating physiological stress; however, neonicotinoid exposure altered leukocyte profiles relative to neutrophils or eosinophils but did not affect CORT levels in northern leopard frogs (Gavel et al., 2021). Neutrophil to lymphocyte ratios were also a good indication of environmental stressors and were associated with total dissolved solid levels in aquatic habitats that impacted growth and development (Ruso et al.,

2021). Combinations of physiological indices are also informative to link endpoints with individual condition (e.g., Park et al., 2021).

Several minimally or non-invasive techniques that may be more effective for amphibians are now routinely used including urinalysis, fecal sampling, waterborne sampling, salivary swabs, and dermal swabs (Narayan et al., 2019). Among these techniques, saliva has only been validated in adults and not juveniles. Janin et al. (2012) concluded that CORT concentrations in saliva were highly correlated with urine measurements in toads. Urinalysis has been previously used to track endocrine response such as the reproductive hormones estradiol, progesterone, and testosterone within both captive and wild caught amphibians (Narayan, 2013). Additionally, CORT levels can be quantified from urine to identify differences in stress response due to predation risk or pathogen prevalence (Kindermann et al., 2012; Narayan et al., 2019). While urine samples have the distinct advantage of being highly concentrated for measuring endocrine functions along with physiological stress, the method is not always ideal for smaller amphibians producing lower volumes (Narayan, 2013; Baugh et al., 2018).

Another minimally invasive technique for endocrine analysis for amphibians of any size is immersing the individual in a clean container of water for a designated length of time, after which the released hormones can be quantified from the water. Most studies have used this technique to measure CORT release rates, which have been validated with circulating plasma levels (Gabor et al., 2013a). Waterborne sampling has evaluated CORT release rates in the presence or absence of *Bd*, predation, or pesticide exposure (Gabor et al., 2013b; Van Meter et al., 2019; Snyder et al., 2022) and can also be indicative of nitrate stress in amphibians (Ruthsatz et al., 2023). CORT is a potentially useful biomarker for amphibians to indicate stress, and non-invasive sampling methods offer the potential of serially sampling the same individual (Narayan et al., 2019; Tornabene et al., 2021). Therefore, within a short timeframe baseline values and acute elevation in CORT are quantifiable (Hammond et al., 2018). Szymanski et al. (2006) collected feces of adult anurans to quantify reproductive hormones, enabling sex identification. In addition to CORT, two other reproductive hormones, progesterone and estradiol, have also been quantified in water from amphibians (Baugh et al., 2018).

While dermal swabbing is most notable for detecting the presence of pathogens in amphibians (e.g., Standish et al., 2018), more recent studies have expanded on what can be tested in amphibian mucus, such as DNA collection and glucocorticoids (Poorten et al., 2017; Narayan et al., 2019; Santymire et al., 2021). A lab-based salamander study examined the difference between liver metabolomics and dermal swab metabolomics, to determine if similar pathways are impacted in the presence of pesticides, measuring for presence/absence of pathogens and glutathione as well (Van Meter et al., in press). Dermal swabs enable *in situ* field sampling with minimal handing time, which is particularly advantageous for threatened and endangered species and allows serial sampling of the same individual or environment (Santymire et al., 2018, 2021; Scheun et al., 2019; Tornabene et al., 2021).

Sampling techniques that are well-established at the individual level can also provide a comparison, through DGE or hepatic metabolites, of localized stressor response indicative of differential population-level effects. Such response-based metrics could preclude the necessity to anticipate synergies, compensation, and interactions between ubiquitous stressors that might be heterogeneously distributed. Rather than spatially explicit analysis of stressors within the species distribution, identifying variance in relative response within the population could target conservation concerns more rapidly within a diverse landscape while accounting for baseline fluxes in physiology. For example, the complex physiological changes during metamorphosis comprise endocrine interactions and changes in energy allocation as tail resorption and leg growth occurs, such that tissues sampled could vary in the cellular-level activity. Stage-specific fluxes in endocrine activity also affect response observed in individuals, such that standardizing measurements within consistent developmental stages is advisable when sampling larvae. Field-based observations could also be influenced by the environment of the individuals, making observations about water quality, larval density, and community composition relevant to evaluating stress response. As measures of individual response are considered within appropriate life stage time points, comparable evaluation of location-specific perturbations in baseline physiological functions can guide more targeted conservation actions.

4 Discussion

As amphibians are impacted by multiple stressors and their interactions, the capability to assess cumulative impacts on biochemical pathways within an organism's native habitat facilitates quantification of exposure risk and possible additive or synergistic effects. However, even at the organismal level, amphibians often lack sufficient toxicology data for evaluation of cellular-level responses (Marlatt and Martyniuk, 2017), and variance in individual response and chronic exposure obscure definitive metrics of detrimental effects on the population. Additional research is needed to identify reliable biomarkers that are consistently indicative of points of departure from normal cellular function in response to environmental stressors. Standardized indices of biomarker perturbations in response to stressors enables identification of reliable predictors of long-term impacts (Pham and Sokolova, 2023); however, caution must be taken to verify cellular responses are linked to demographic effects, rather than a homeostatic response to stressors (Forbes et al., 2006). Evaluating potential threats linked to synergistic exposure effects (e.g., reduced dermal microbiota) or multiple exposure routes (i.e., aquatic and terrestrial) requires situation-specific ecological context. Implementation of weight of evidence effects could further classify cumulative threat levels of variable biomarker responses (Cecchetto

et al., 2023). An initial step towards multi-stressor risk assessment is outlined here, namely by exploring stage-specific variance in biochemical pathways and identifying points of physiological vulnerability in the life cycle as a screening-level conservation approach.

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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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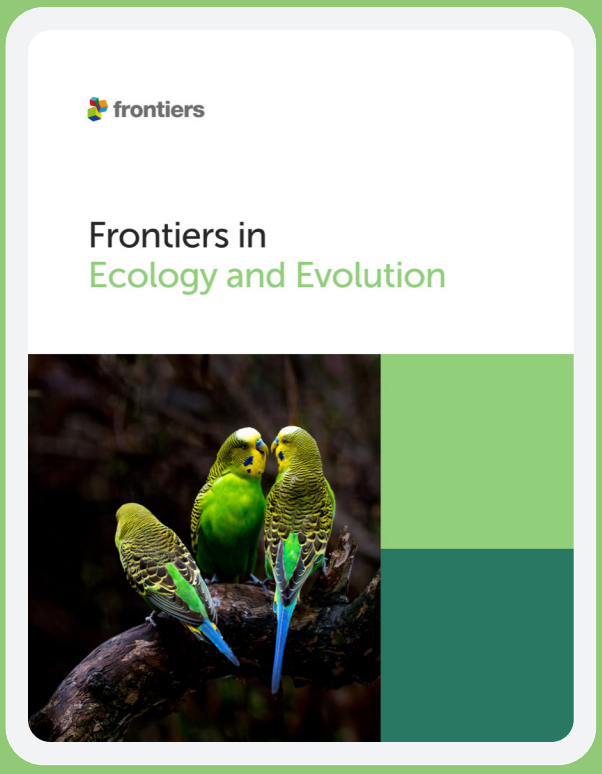

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