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Effects of exogenous elevation of corticosterone on immunity and the skin microbiome of eastern newts (*Notophthalmus viridescens*)

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The amphibian chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*) threatens salamander biodiversity. The factors underlying *Bsal* susceptibility may include glucocorticoid hormones (GCs). The effects of GCs on immunity and disease susceptibility are well studied in mammals, but less is known in other groups, including salamanders. We used *Notophthalmus viridescens* (eastern newts) to test the hypothesis that GCs modulate salamander immunity. We first determined the dose required to elevate corticosterone (CORT; primary GC in amphibians) to physiologically relevant levels. We then measured immunity (neutrophil lymphocyte ratios, plasma bacterial killing ability (BKA), skin microbiome, splenocytes, melanomacrophage centres (MMCs)) and overall health in newts following treatment with CORT or an oil vehicle control. Treatments were repeated for a short (two treatments over 5 days) or long (18 treatments over 26 days) time period. Contrary to our predictions, most immune and health parameters were similar for CORT and oil-treated newts. Surprisingly, differences in BKA, skin microbiome and MMCs were observed between newts subjected to short- and long-term treatments, regardless of treatment type (CORT, oil vehicle). Taken together, CORT does not appear to be a major factor contributing to immunity in eastern newts, although more studies examining additional immune factors are necessary.

This article is part of the theme issue 'Amphibian immunity: stress, disease and ecoimmunology'.

1. Introduction

Emerging diseases are a significant threat to biodiversity and a growing conservation concern [1]. One disease of particular concern is chytridiomycosis. Chytridiomycosis is a skin disease of amphibians caused by amphibian chytrid fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) [2,3]. Both pathogens infect a broad range of amphibian hosts, but *Bd* causes the highest rates of mortality in anurans (frogs and toads) while *Bsal* seems to be most lethal in caudates (salamanders) [4,5]. *Bsal*'s recent emergence and spread throughout Europe has resulted in population crashes of both free-living and captive salamanders [6,7]. Despite *Bsal*'s presumed absence in North America, *Bsal* is predicted to soon arrive. A North American *Bsal* invasion could have devastating consequences for native salamanders, thereby, underscoring the importance of developing proactive disease mitigation strategies [8].

Salamander susceptibility to infection and chytridiomycosis caused by *Bsal* varies between and within species. For example, salamandrid salamanders (newts) are highly susceptible in that individuals accrue high infection loads and experience high rates of mortality following experimental exposure to *Bsal* [3,9]. By contrast, some species of ambystomatid (mole) and plethodontid (lungless)

salamander resist *Bsal* infection entirely [9,10]. Furthermore, rates of *Bsal*-induced mortality can vary greatly among conspecific salamander populations [11]. Disentangling the mechanisms underlying variation in *Bsal* susceptibility is critical to allocating conservation resources to high-risk populations and generating science-based disease management plans.

While *Bsal* susceptibility is probably driven by myriad factors, one hypothesis is that susceptibility is influenced by variation in host immunological responses [12,13]. Salamanders, like other jawed vertebrates have well-developed innate and adaptive immune systems [14]. Innate immunity rapidly responds to a broad range of pathogens in a non-specific manner. While innate defences include inflammatory responses, the complement system, and leucocytes, they also include constitutive defences of the skin [15]. For example, antimicrobial peptides (host defence peptides) of many amphibians including salamanders have bioactivity against numerous microbes and are considered critical defences against amphibian chytrid fungal pathogens [16–18]. The skin microbiome is also recognized for its role in protecting amphibians against amphibian chytrid fungi [19,20]. In fact, *in vitro* studies find that several bacterial symbionts commonly found on the skin of plethodontid salamanders inhibit the growth of *Bd* through the secretion of secondary metabolites [21]. In contrast with innate responses, adaptive immunity is slower to respond, targets specific pathogens and offers immune memory [22]. Pathogen specificity is mediated primarily through the action of T and B lymphocytes, which in salamanders, are produced in the spleen, liver and kidneys [12,23,24].

One key mediator of host immunity is glucocorticoid hormones (GCs). Baseline levels of GCs serve myriad roles in metabolism, development, reproduction, behaviour and immunity [25]. However, GCs also become elevated as part of the vertebrate stress response. Stress-induced elevations in GCs help an animal avoid or cope with a stressor by altering metabolic processes to divert resources to tissues necessary for immediate survival [26]. While GCs typically act by affecting gene expression, which can take hours for effects to be evident, non-genomic responses can be fast-acting [27]. Through negative feedback mechanisms, GC levels drop when an animal is removed from the stressor [28].

Compared to other vertebrates, little is known about the effects of GCs on salamander immunity [29–32]. In fact, fundamental GC-immune relationships that have been well established in humans and domesticated rodent models have seldom been tested in non-traditional model systems such as salamanders [33–35]. One example is the hypothesis that GCs enhance immune function when elevated for short periods of time and suppress immune function when elevated for long periods of time [36,37]. While elevations in corticosterone (CORT, the primary GC in amphibians) have been observed in salamanders following exposure to *Bsal*, the association between CORT, immunity and disease outcome remains unclear [10]. Further, results in plethodontid salamanders demonstrate that repeated elevations in CORT can result in higher *Bd* infection loads and delayed wound healing responses [33,38]. Although specific immune parameters were not measured, these results suggest that CORT elevations negatively impact salamander immunity [33,38].

Here, we used eastern newts, *Notophthalmus viridescens*, to test the hypothesis that CORT alters immune function. We predicted that while short-term CORT elevations would enhance immune function, long-term elevations would suppress immune function. This species is a suitable model for this study because, like other salamandrid salamanders, *N. viridescens* is highly susceptible to *Bsal* [9]. Further, *N. viridescens* has a widespread geographical distribution which overlaps with regions predicted to be greatly impacted by a *Bsal* emergence [39]. For these reasons, *N. viridescens* has served as one of the primary models for *Bsal* research [8].

In order to test our predictions in *N. viridescens*, we first needed to determine the concentrations of exogenous CORT required to produce short-term and long-term elevations in plasma CORT that were physiologically appropriate. To do so, we first measured endogenous CORT in free-living *N. viridescens* to establish the natural range of CORT at baseline and when elevated by a stressor (experiment 1). Next, we exposed captive *N. viridescens* to a range of exogenous CORT doses to determine the dose that elevates endogenous levels within the natural range for this species (experiment 2).

Using results from experiments 1 and 2, we tested whether periodic elevations in endogenous CORT influence salamander immune function (experiment 3). We exposed captive *N. viridescens* to repeated CORT doses over a short (two doses over 5 days) or long (18 doses over 26 days) period of time. We then compared measures of immunity (leucocyte profiles, plasma bacterial killing ability (BKA), splenic lymphocytes and hepatic melanomacrophages). We also assessed general measures of overall health (body mass, fat bodies and haematocrit) as these variables are influenced by hormones and stressors. In addition, we measured the skin microbiome to test whether CORT elevations altered the structure of skin bacterial communities. We also measured *Bd* infection loads to account for potential effects of *Bd* infection on immune parameters. Lastly, we measured plasma CORT to see if periodic CORT applications (achieved via dermal applications of exogenous CORT) elevated baseline CORT levels. Results from this study will help elucidate the relationship between CORT and salamander immune responses, as well as the potential for these interactions to influence salamander responses to emerging pathogens such as *Bd* and *Bsal*.

2. Material and methods

We performed one field experiment (experiment 1) and two laboratory experiments (experiments 2 and 3). For each experiment, fully aquatic adult *N. viridescens* were captured from Clarion County, Pennsylvania with appropriate permits from the Pennsylvania Fish and Boat Commission (permit nos. 2019-01-0082 and 2020-01-0028) and Duquesne University's Institutional Animal Care and Use Committee (protocol no. 1905-07). Dip nets were used to capture newts from a single breeding pond in October 2019 (experiment 2) and October 2020 (experiments 1 and 3), when ambient air temperatures were 13.3°C and 12.8°C, respectively.

For the laboratory experiments, newts were allowed time to acclimate to captive conditions and a terrestrial lifestyle indicated by changes in the skin, reduced tail size and loss of secondary sexual characteristics. Captive newts were individually maintained in 18.4 × 16.3 × 5.7 cm plastic home boxes lined with moistened paper towels and polyvinyl chloride (PVC) pipe cover items previously washed and bleached, in Biological Oxygen Demand incubators set at 12 : 12 light: dark cycle at 15°C. We chose to house newts at 15°C because it is the standard temperature for conducting *Bsal* susceptibility trials [9,11]. Newts were fed wax worms or maggots weekly.

(a) Experiment 1: natural range of endogenous corticosterone in free-living newts

To assess the natural variation in endogenous CORT levels, we measured plasma CORT from free-living male ($n = 17$) and female ($n = 5$) newts immediately after being captured (baseline CORT) or following a handling stressor (elevated CORT). To measure baseline CORT levels, newts were sacrificed by rapid decapitation and sampled for trunk blood. Because endogenous CORT can increase within minutes following a stressor (e.g. capture), newts were not anaesthetized prior to decapitation and trunk blood collection was completed within 3 min of capture [40]. To measure elevated CORT levels, newts were placed individually in a plastic bag containing 3–5 ml of sterilized nanopure water, vigorously handled for 15 min and allowed an additional 15 min to mount a physiological stress response (total time after capture = 30 min) before collecting trunk blood. Vigorous handling involved a combination of firmly massaging the body of a single newt and shaking the plastic bag continuously, as described by Pereira, Cava [41]. Blood samples were collected for all newts between 11.00 and 16.00 h in heparinized capillary tubes (Thermo Fisher Scientific, Waltham, MA, USA) and stored on ice. Within a few hours of collection (mean ± s.e.m., 5.8 ± 0.77 h), capillary tubes containing blood samples were centrifuged for 15 min (IEC Micro-MB bench top centrifuge, Thermo IEC, Needham Heights, MA) to isolate plasma. Plasma was stored in heparinized microcentrifuge tubes at −80°C until assayed for CORT (see below). Newts were dissected to validate sex. The sample size for baseline (not handled) and elevated (handled) CORT groups was 11 newts for each group. Body masses were similar for newts in both groups (mean ± s.e.m., 3.0 ± 0.11 g: $t_{20} = 0.20$, $p = 0.87$). Radioactive trace recovery was 99.6% and intra-assay variation was 5.6% for the CORT assay.

(b) Corticosterone preparation and application

We dissolved crystalline CORT (cat. no. Q1550-000, Steraloids Inc.) in 500 µl 100% ethanol and then added 10 ml sesame oil (oil vehicle) to generate a stock CORT concentration of 0.5 mg ml^{-1}. As a control, we combined 500 µl 100% ethanol with 10 ml sesame oil (oil vehicle control). Stock and control solutions were left open overnight on separate stir plates to allow ethanol to evaporate. Final CORT doses were made from the stock solution in January 2020. Final CORT doses and oil vehicle control vials contained a stir bar and were stored on separate stir plates until needed. Final CORT and oil doses were used for experiment 2 in January 2020 and for experiment 3 in November 2020. Our previous work in *Desmognathus* salamanders found that CORT applications elicited changes in body mass and wound healing after CORT preparations were stored at room temperature for a minimum of eight months, thus, demonstrating the long-term stability of CORT in sesame oil [33,42]. Therefore, it is unlikely that CORT degradation occurred over the course of experiments 2 and 3.

In amphibians, plasma CORT can be elevated by applying exogenous CORT to the skin. CORT passes through the skin and enters circulation (transdermal delivery method) [43]. To apply CORT and oil vehicle doses to newts, we first transferred newts (in their home boxes) from the 15°C incubator to a bench at room temperature. CORT or oil vehicle control doses (2.5 µl) were pipetted directly onto the newt's dorsal skin surface between the forelimbs. Efforts were made to avoid disturbing the newts. To minimize risk of cross-contamination, CORT and oil vehicle doses were applied on separate benches lined with clean bench paper, changing gloves between dose-types. In addition, separate pipets and pipet tip boxes previously cleaned with 70% ethanol were used to apply CORT and oil vehicle doses, changing tips between newts. After each application, home boxes were immediately closed and left at room temperature for 45 min to allow doses to be absorbed before sampling trunk blood. We chose the 45 min time point based on previous work, in which plasma CORT remained elevated in plethodontid salamanders for 30 min to 4 h following the application of dermal CORT [43]. We have observed comparable results in *N. viridescens* (K. E. Pereira 2018, unpublished data).

(c) Experiment 2: elevation of endogenous corticosterone in captive newts

The dose of exogenous CORT (µg) that elevates plasma CORT within the natural range can vary between amphibian species [43]. To determine the exogenous CORT dose that elevates plasma CORT within the natural range for *N. viridescens* (see results for experiment 1), trunk blood was collected from captive male ($n = 9$) and female ($n = 16$) newts approximately 45 min after receiving a dermal application of an oil vehicle (control), 0.1 µg CORT, 0.2 µg CORT, 0.3 µg CORT or 0.5 µg CORT. Blood samples were collected for all newts between 14.00 and 15.30 h in heparinized capillary tubes (Thermo Fisher Scientific) and stored on ice. Plasma was isolated from blood samples and stored as in experiment 1 and assayed for CORT (see below). Newts were dissected to validate sex. The sample size for each treatment dose was five newts. The total time in captivity for all newts was 71 days. Body masses were similar for newts in all treatments (mean ± s.e.m., 2.8 ± 0.06 g; main effect treatment: $F_{4,24} = 0.89$, $p = 0.49$). Radioactive trace recovery was 96% and intra-assay variation was 6.9% for the CORT assay.

(d) Experiment 3: corticosterone, immunity and the skin microbiome

To investigate whether periodic elevations in endogenous CORT affect immunity or skin microbial communities, captive male newts were tested within a two factor experimental framework. We only used male newts to control for potential sexual differences in immunity, which have been well documented for numerous vertebrate taxa [44]. The first factor was treatment in which newts received a dermal application of oil vehicle (control) or 0.3 µg CORT. We used 0.3 µg CORT because experiment 2 showed that this concentration elevated plasma CORT to handling-induced CORT levels (see results for experiment 1). We used the same preparation and application procedures for CORT and the oil vehicle that were used in experiment 2. Dermal applications began in November 2020 after newts had been in captivity for 23 days and were administered between 8.00 and 16.00 h.

The second factor was treatment time in which newts received dermal applications for a short-term or long-term duration. For the short-term duration, newts received two dermal applications (control or CORT) over 5 days (electronic supplementary material, table S1). Our timing for applying short-term doses was based on previous studies in *N. viridescens* and ambystomatid salamanders that showed while changes in some parameters of immunity (e.g. leucocyte populations and plasma BKA) were apparent within 48 h following a single injection of hydrocortisone (a synthetic GC) changes in other parameters (e.g. splenic lymphocytes) were not observed until much later (4–16 days after injection) [31,45]. By applying short-term doses at two time points, we hoped to capture potential immediate and delayed immune responses. For the long-term duration, newts received 18 dermal applications (control or CORT) over 26 days (electronic supplementary material, table S1). We chose to apply long-term doses consistently throughout the experimental period based on previous studies in plethodontid salamanders, in which 9- and 29-day consecutive dermal applications of CORT resulted in higher *Bd* infection loads and delayed wound healing, suggesting that repeated CORT applications negatively impact salamander immunity [33,38].

The sample size for each treatment type × treatment time (duration) group was 12 newts. We conducted the experiment in a staggered fashion over a 42 day period so that the total time in captivity was similar within and between each group (mean ± s.e.m.; 57.5 ± 2.6 days in captivity; effect of treatment: $F_{1,47} = 0.003$, $p = 0.96$; effect of time: $F_{1,47} = 3.6$, $p = 0.07$; interaction treatment × time: $F_{1,47} = 0.96$, $p = 0.33$; electronic supplementary material, table S1). We also rotated newts weekly among incubator shelves to minimize potential effects of a newt's placement within the incubator. Newts were randomly assigned to treatment groups. Body masses measured at the start of the experiment were similar among groups (mean ± s.e.m., 2.1 ± 0.05 g; effect of treatment: $F_{1,47} = 0.61$, $p = 0.44$; effect of time: $F_{1,47} = 0.50$, $p = 0.48$; interaction treatment × time: $F_{1,47} = 0.011$, $p = 0.92$).

Approximately 24 h before the start of the experiment, newts were weighed for initial body mass and a skin swab was collected, stored at −80°C, and later used for *Bd* and *Bsal* detection using standard methods [46]. Approximately 24 h after administering the final CORT and oil vehicle doses, newts were weighed for final body mass, and swabbed a second time with two swabs simultaneously for *Bd* and *Bsal* detection and skin microbiome sampling using standard methods [46]. The next day (approx. 48 h after final doses were applied), newts were sacrificed by rapid decapitation and trunk blood was collected within 3 min (electronic supplementary material, table S1). We used 2 µl of blood to prepare blood smears. Blood smears were immediately fixed in 100% methanol, and later stained (CAMCO Stain Pak, Cambridge Diagnostic Products, Inc., Fort Lauderdale, FL, USA) and coverslipped. The remaining blood was collected for all newts between 10.05 and 12.08 h in heparinized capillary tubes (Thermo Fisher Scientific) stored on ice, centrifuged, and used to measure haematocrit (% haematocrit = haematocrit (mm)/total length of sample (mm) × 100; a common measure of overall health) and isolate plasma [47]. Plasma was isolated, stored as in experiment 1, and assayed for CORT (see below). Radioactive trace recovery was 90.8% and the intra-assay coefficient of variation was 2.6% for the CORT assay. Plasma samples (stored at −80°C for mean ± s.e.m., 291.5 ± 2.6 days) were also used for measures of immunity as described below.

Following sacrifice, newts were stored on ice for less than 2 h and dissected to validate sex. We also collected fat bodies to assess overall health, and spleen and liver tissues for immunological measurements (electronic supplementary material, table S1). Fat bodies were blotted dry and weighed to the nearest 0.1 mg. Spleens were blotted dry, weighed to the nearest 0.1 mg, placed in a 1.5 ml microcentrifuge tube with 100 µl phosphate-buffered saline (PBS), immediately placed on ice, and then stored at 4°C for less than 24 h. Livers were stored in 5 ml microcentrifuge tubes containing 1 ml 10% formalin at room temperature.

(e) Measures of immunity

(i) Leucocyte counts

Total leucocytes were enumerated from stained blood smears (CAMCO) to calculate neutrophil to lymphocyte ratios (NLR), a bioindicator of physiological stress and GC-induced immunomodulation [48]. Stained blood smears were observed under an optical microscope at a total magnification of 400×. For each smear (one per newt), a minimum of 50 leucocytes were counted and classified as neutrophils, lymphocytes, monocytes, eosinophils, basophils or thrombocytes based on morphology. NLR was calculated by dividing the number of neutrophils by the number of lymphocytes.

(ii) Bacterial killing ability

BKA of plasma, which includes complement proteins, lysozyme, antibodies and other antimicrobial constituents, was assayed by adapting methods outlined in French & Neuman-Lee [49]. Briefly, 18 µl of plasma (diluted with PBS to a 1 : 3.6 ratio) was combined with 6 µl *Escherichia coli* at an estimated concentration of 10^6 cfu ml^{-1} within the wells of a 96-well microtitre plate. The dilution ratio for plasma samples and estimated bacterial concentrations were previously optimized in separate BKA assays (see the electronic supplementary material). Plasma dilutions were plated in duplicate, when possible, but most were plated in singlicate because plasma volumes were limited. Controls consisted of 18 µl PBS only (five replicate wells representing no BKA), 18 µl 50 µg ml^{-1} colistin antibiotic combined with 6 µl *E. coli* (duplicate wells) and 18 µl PBS combined with 6 µl *E. coli* (six replicate wells representing maximum bacterial growth). Plates were incubated at 37°C for 1 h, briefly removed from the incubator to add 125 µl tryptone soy broth to all sample and control wells and returned to 37°C for a second incubation of an additional 6 h. Optical density (OD) at 490 nm was measured hourly using a SpectraMax iD3 plate reader (Molecular Devices, San Jose, CA, USA).

BKA values were calculated by first determining the change in OD_{490} (ΔOD_{490}) for all sample and control wells by subtracting OD_{490} at the start of the second incubation from the OD_{490} after 6 h. The 6 h time point was chosen because it is within the exponential growth phase for *E. coli* (electronic supplementary material, figure S1). The ΔOD_{490} of plasma samples and controls was then divided by the ΔOD_{490} of the control containing *E. coli* and PBS (average of well replicates) and subtracted by one. For ease of interpretation, the absolute value of these calculations was multiplied by 100 (to generate a percentage) and is presented in the results. Increasing percentages were interpreted as greater BKA and immune function.

(iii) Splenocyte count

Splenic lymphocytes (splenocytes) were enumerated from spleen tissues, the primary site for T and B lymphocyte proliferation in amphibians [23,50]. Spleens in 100 µl PBS were removed from 4°C, placed on ice and macerated for 2 min using a clean dissection needle to suspend splenocytes. Fifty microlitres of the splenocyte-PBS mixture was then combined with 50 µl 0.4% Trypan blue stain (Life Technologies Corporation, Grand Island, NY, USA) within a new 600 µl microcentrifuge tube to differentiate viable and non-viable cells (electronic supplementary material, figure S2). Six microlitres of the stained splenocyte mixture was loaded into the chamber of a Bulldog Bio 4-chip disposable haemocytometer (cat. no. DHC-N420). Chambers were observed under an optical microscope at a total magnification of 400×. For each chamber, a minimum of 100 viable splenocytes were counted from multiple squares and averaged. Mean splenocyte counts were corrected for dilution factor and used to extrapolate total splenocytes in 100 µl PBS, hereafter total splenocytes. Total splenocytes were used for statistical analysis.

(iv) Melanomacrophage centres

Melanomacrophage centres (MMCs) are pigmented cell aggregations found in the livers of fishes and amphibians (electronic supplementary material, figure S3) [51]. In amphibians, available data suggest that MMC function is multifaceted and involved in immune processes [52,53]. Livers previously stored in 10% buffered formalin were rinsed in double-distilled water, placed in a 30% sucrose solution (in PBS at room temperature) overnight with agitation, embedded in frozen section compound (Leica Biosystems Richmond, Inc., Richmond, IL, USA), and cryosectioned into 20 µm thick sections using an IEC Minotome (International Equipment Company, Chattanooga, TN, USA). Each 20th cryosection was collected, mounted on a gelatin subbed slide, placed on a slide warmer set at 37°C for 1 h, and left at room temperature overnight before coverslipping. The

number of cryosections collected per liver varied (3–17 sections). This was because livers varied in size and preliminary analysis indicated that measures of MMC variables were similar regardless of the number of sections examined (K. E. Pereira 2021, unpublished data). Each cryosection was observed and photographed at a total magnification of 40× using an Olympus BX51 System Microscope (Olympus Corporation, Shinjuku City, Tokyo, Japan) equipped with a Moticam BTU8 Tablet Camera (Motic Instruments Inc., Richmond, British Columbia, Canada).

Photographed cryosections (micrographs) were then analysed using the imaging software, ImageJ to quantify MMC count and MMC size [54]. For MMC count, the total number of MMCs in a single micrograph was counted and divided by the area of the liver section examined. This was completed for at least three micrographs per liver. Values were averaged to generate MMC count (MMCs μm^{-2} liver section). For MMC size, the areas of MMCs within a single micrograph were measured and averaged across micrographs. This was completed for at least three micrographs per liver. Values were averaged to generate MMC size (μm^2).

(v) Skin microbiome

Skin swabs collected at the end of experiment 3 were used for detecting and characterizing the skin bacteria community. As a treatment control, sterile swabs were dipped into the oil vehicle control or CORT (+ oil vehicle) vial after all experiments were completed, stored separately at −80°C, and analysed in a similar manner. We extracted total genomic DNA from skin and treatment control swabs using a gMAX mini Genomic DNA kit (IBI Scientific, Dubuqu, IA, USA) following a modified version of the animal tissue protocol. Briefly, the initial cell lysis steps were modified to include a lysozyme step to facilitate lysis of Gram-positive bacteria. This included a 1 h incubation at 37°C with an enzymatic lysis buffer (20 mM Tris-CL, 2 mM sodium EDTA, 1.2% Triton X-100, 20 mg ml^{-1} lysozyme), followed by a 30 min incubation at 70°C with proteinase K and GSB buffer (IBI Scientific). The manufacturer's protocol was followed for the remaining steps.

The V4 region of the 16S rRNA gene was polymerase chain reaction (PCR)-amplified for bacterial communities in duplicate with single index barcoded primers (bacterial: 515F–806R, EMP 2018) with the addition of bovine serum albumin (BSA) to increase PCR efficiency. PCR-reactions contained: 10 µl 5 Prime HotStart MasterMix, 0.3 µl BSA (20 µg ml^{-1}), 0.5 µl of each primer (10 µM), 11.7 µl of DNA-free water and 2.0 µl of sample DNA. The PCR cycle conditions included a denaturation step of 94°C for 2 min, followed by 28 cycles at 94°C for 20 s, 55°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min [55]. Oil vehicle control and CORT (+ oil vehicle) treatment control samples did not yield detectable DNA and were excluded from subsequent sequencing. Duplicate PCR products were pooled, purified and equalized to the same concentration using the Omega Biotek MagBead Normalization Kit (Omega Biotek, Norcross, GA, USA). Samples were sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) at the University of Massachusetts Boston using 300 cycle paired-end v2 chemistry kit, sequenced in one direction for 300 cycles.

Raw sequences were initially processed in QIIME2 (v2021.8) [56]. Following demultiplexing, sequences with a minimum quality score of 20 were then trimmed to 150 bp, chimaeras were removed, and amplicon sequence variants (ASVs, also referred as sub-operational-taxonomic-units or sOTUs) were generated by denoising with Deblur [57]. Contaminants were identified in R with the package decontam, using the 'prevalence' method and a threshold of 0.5, with experimental (water), extraction and PCR controls used to identify contamination. A table of ASVs with contaminants removed was reimported into the QIIME2 environment, where it was further filtered to remove chloroplasts and mitochondria sequences. Samples were then rarefied at a depth of 6600 (electronic supplementary material, figure S4).

Metrics for alpha diversity (diversity within a community; Faith's phylogenetic diversity, richness, evenness, Shannon) and beta diversity (diversity relative to other communities; Bray–Curtis and weighted UniFrac distances) were calculated using the core-metrics-phylogenetic pipeline in the q2-diversity plugin [56]. Phylogeny was constructed using the align-to-tree-mafft-fasttree pipeline (q2-phylogeny), and taxonomy assigned with the q2-feature-classifier using classify-sklearn and Greengenes 13_8 99% reference sequences [58–60].

(vi) *Bd* and *Bsal* detection

Skin swabs collected before the start and at the end of experiment 3 were used for the detection and quantification of *Bd* and *Bsal* from each newt. Total genomic DNA was extracted from swabs using the 'animal tissue' protocol for the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) with a final elution volume of 200 µl. A quantitative PCR (qPCR) assay using a QuantoStudio 3 Real Time PCR system (Applied Biosystems, Waltham, MA, USA) was run at the University of Pittsburgh to detect and quantify the presence of *Bd* and *Bsal* DNA [61]. Twenty-five µl reactions containing 12.5 µl of 2x SensiFast probe Lo-Row Mix (Meridian Bioscience, Memphis, TN, USA), PCR primers at a concentration of 900 nM, minor groove binder probes at 240 nM, 400 ng μl^{-1} BSA, 1/3x TaqMan exogenous internal positive control (IPC) reagents (Applied Biosystems, Waltham, MA, USA), and 5 µl of template DNA. Negative and positive control samples and a seven-fold dilution series of *Bd* and *Bsal* plasmid standards (Pisces Molecular, Boulder, CO, USA) were included in each qPCR run. Negative controls were generated by running the DNA extraction protocol on a clean swab. As an additional negative control, wells including 20 ul of qPCR reagents and 5 ul buffer AE (Qiagen, Hilden, Germany) were included in each qPCR run. Positive controls were generated by running the DNA extraction protocol on a swab that had been dipped in a broth culture of *Bd*. DNA was amplified using 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. All samples were run in singlicate. Whole-swab *Bd* load (genetic equivalents, GE) was calculated by multiplying detected *Bd* DNA copies by 40 to account for the proportion of the full extraction that was aliquoted for qPCR. *Bsal* DNA was not detected on any of the experimental swabs. Wells where the IPC did not amplify were excluded from the analysis.

(vii) Corticosterone assays

Plasma samples were used to measure plasma CORT levels at the time of sacrifice. CORT was assayed by the Endocrine Technologies CORE (ETC) at the Oregon National Primate Research Center (ONPRC) following standard radioimmunoassay (RIA) procedures. Briefly, plasma samples were combined with 0.1% gel-PBS and CORT was extracted with 5 ml diethyl ether by vigorous inversion for 3 min. Samples were centrifuged for 5 min (2000*g* at 4°C), and the aqueous phase was frozen using a dry ice bath. The organic phase was decanted into a new 13 × 100 mm tube, dried under forced air in a 37°C water bath, redissolved in 0.1% gel-PBS and assayed for CORT using an in-house RIA. Each sample was assayed in singlicate because of limited sample volume. A standard curve (5 pg per tube to 1000 pg per tube) was created using 3H-corticosterone (American Radiolabeled Chemicals, St Louis, MO, USA). All samples were within the standard curve. A commercially available anti-corticosterone antibody (cat. no. ab77798; Abcam, Cambridge, MA, USA) was used to complete RIAs. RIA sensitivity was 5 pg per tube. CORT values were corrected for extraction losses determined by radioactive trace recovery at the same time each sample was extracted.

(viii) Statistical analysis

All statistical analyses, except for bacterial data, were performed using IBM SPSS Statistics software version 27 (IBM Corp.,

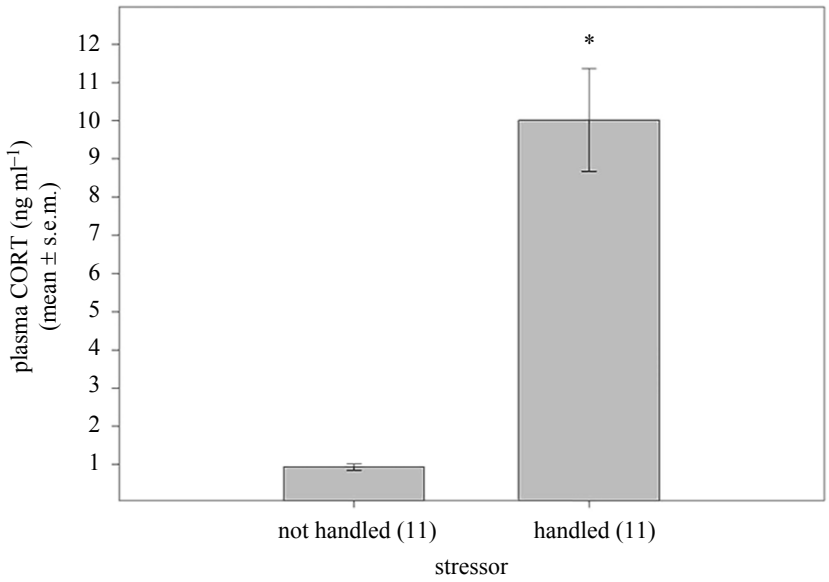

Figure 1. Natural range of endogenous CORT in free-living male and female *Notophthalmus viridescens* (experiment 1). Plasma CORT was measured from newts that were not handled (baseline CORT) and newts subjected to a 15 min handling stressor (elevated CORT). Sample sizes are indicated in parentheses on the *x*-axis (*$p < 0.001$).

Armonk, NY, USA), SigmaPlot software version 11 (Systat Software Inc., Richmond, CA, USA), and R version 4.2.2 [62]. For experiments 1 and 2, we used general linear models (GLMs) to compare differences in plasma CORT ($\log_{10}$ transformed) between treatment groups. For experiment 3, we used GLMs to test the effects of CORT treatment and treatment duration on variables for immunity (NLR, BKA, total splenocytes, MMC count and MMC size) and overall health (body mass lost, fat body mass, % haematocrit). Covariables were included in experiment 3 GLMs when relevant (electronic supplementary material, table S2). Significant effects ($p \leq 0.05$) were further analysed using post hoc paired comparisons (Holm-Sidak; electronic supplementary material, table S2). When necessary, data used in experiment 3 GLMs were transformed to better satisfy the assumptions of parametric statistics (electronic supplementary material, table S2). For experiment 3, we also performed a separate series of linear regressions to test for relationships between amphibian *Bd* infection load (initial and change in infection load) or plasma CORT and immune variables. Because many newts cleared *Bd* infections by the end of experiment 3 (final infection load = 0 GE), we performed a series of zero-inflated gamma regression models to test for relationships between final *Bd* infection load and immune variables or plasma CORT.

In experiment 3, statistical analyses of bacterial data were performed using R version 4.0.2 [62]. We used linear models to compare differences in alpha diversity metrics between treatments (oil vehicle, CORT) and treatment times (short-term, long-term) (ANOVA, car v3.0.13), and transformations were done when necessary, using the bestNormalize package (v1.8.3) [63–65]. We used PERMANOVAs (vegan v2.6.2, Adonis2) [62] to compare differences in beta diversity metrics between treatments (oil vehicle, CORT) and treatment times (short-term, long-term) which were assessed using the Bray–Curtis and weighted UniFrac distances [66,67]. Dispersion of communities grouped by either treatment (oil vehicle, CORT) or treatment time (short-term, long-term) was assessed with the PERMDISP2 procedure (betadispr, vegan v2.6.2)[68].

3. Results

(a) Experiment 1: natural range of endogenous corticosterone in free-living newts

A 15 min handling stressor elevated endogenous (plasma) CORT in male and female newts (range: 2.1–19.5 ng ml^{-1}) compared to newts that were not handled (baseline controls, range: 0.5–1.5 ng ml^{-1}; effect of stressor treatment: $F_{1,21} = 52.36$, $p < 0.001$; figure 1). Plasma CORT was similar for male and female newts (effect of sex: $F_{1,21} = 0.89$, $p = 0.36$; interaction treatment * sex: $F_{1,21} = 0.083$, $p = 0.78$).

(b) Experiment 2: elevation of endogenous corticosterone in captive newts

In total, 45 min after dermal application of exogenous CORT, plasma CORT was elevated relative to oil vehicle controls in a dose-dependent manner in male and female newts (effect of CORT treatment: $F_{4,24} = 13.65$, $p < 0.001$; figure 2). Plasma CORT elevations in newts that received 0.3 µg CORT (range: 7.5–35.6 ng ml^{-1}) were most similar to the natural elevated range (see experiment 1 results; figure 1) for *N. viridescens*. Plasma CORT was similar for male and female newts (effect of sex: $F_{1,24} = 0.24$, $p = 0.63$; interaction treatment × sex: $F_{4,24} = 0.097$, $p = 0.98$).

(c) Experiment 3: corticosterone, immunity and skin microbial communities

(i) Immunity

Most immune parameters were similar for CORT and oil vehicle-treated newts, regardless of the number of treatment applications. NLR was less in CORT-treated newts compared with oil vehicle-treated newts (effect of treatment: $F_{1,47} = 4.03$, $p = 0.05$). While NLR seemed highest for short-term newts treated with oil, this trend was not significant (interaction treatment × time: $F_{1,47} = 3.4$, $p = 0.07$). NLR was similar for short- and long-term-treated newts (effect of time: $F_{1,47} = 0.98$, $p = 0.33$; figure 3*a*). While plasma BKA was similar for CORT and oil vehicle-treated newts, (effect of treatment: $F_{1,43} = 2.4$, $p = 0.13$), plasma BKA was less in long-term-treated newts compared with short-term-treated newts regardless of whether treatments consisted of CORT or oil vehicle (effect of time: $F_{1,43} = 4.01$; $p = 0.05$; interaction treatment × time: $F_{1,43} = 0.46$, $p = 0.50$; figure 3*b*). BKA of the colistin control was 100% for both replicate wells. No bacterial growth was observed for

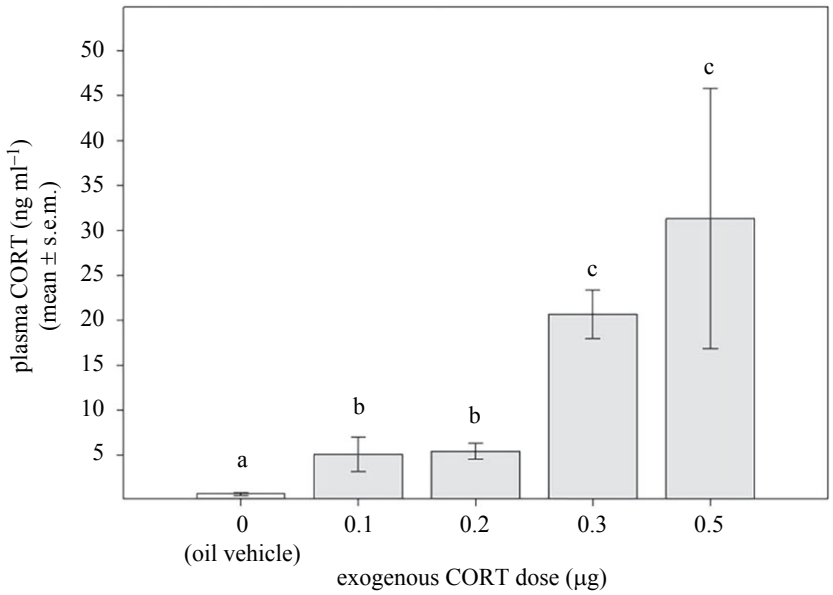

Figure 2. Elevation of plasma CORT in captive male and female *Notophthalmus viridescens* using transdermal delivery method (experiment 2). Plasma CORT was measured from newts 45 min after administering a dermal application of an oil vehicle (control) or exogenous CORT dose. Sample size for each bar was five newts. Bars sharing the same letter are statistically similar.

controls containing colistin or PBS only (see the electronic supplementary material, figure S1 for representative growth curve). Total splenocytes were also similar for all newts (effect of treatment: $F_{1,45} = 0.22$, $p = 0.64$; effect of time: $F_{1,45} = 0.01$, $p = 0.93$; interaction treatment × time: $F_{1,45} = 3.33$, $p = 0.08$; figure 3*c*).

Regarding MMC, MMC count and size were similar for CORT and oil-treated newts (MMC count, effect of treatment: $F_{1,46} = 0.00$, $p = 1.00$; MMC size, effect of treatment: $F_{1,46} = 0.07$, $p = 0.79$). However, MMC count was greater in newts that received two CORT or oil vehicle applications over 5 days (short-term treatment time) compared with newts that received 18 applications over 26 days (long-term treatment time; effect of time: $F_{1,46} = 4.8$, $p = 0.03$; interaction treatment × time: $F_{1,46} = 0.328$, $p = 0.57$; figure 3*d*). MMC size was similar for all newts, regardless of treatment time (effect of time: $F_{1,46} = 1.07$, $p = 0.31$; interaction treatment × time: $F_{1,46} = 0.004$, $p = 0.95$; figure 3*e*).

(ii) Overall health and final corticosterone

Final body mass was similar for all newts (mean ± s.e.m., 1.9 ± 0.04 g). However, newts that received long-term CORT treatments lost less body mass compared to newts in the remaining groups (effect of treatment: $F_{1,47} = 1.04$, $p = 0.32$; effect of time: $F_{1,47} = 0.58$, $p = 0.45$; interaction treatment × time: $F_{1,47} = 4.8$, $p = 0.034$, figure 4*a*). Per cent haematocrit was similar for CORT and oil vehicle-treated newts regardless of the number of treatment applications (effect of treatment: $F_{1,46} = 2.1$, $p = 0.16$; effect of time: $F_{1,46} = 0.14$, $p = 0.71$; interaction treatment × time: $F_{1,46} = 0.072$, $p = 0.79$; figure 4*b*). Likewise, fat body mass (with body mass as a covariate) was similar for all newts (effect of treatment: $F_{1,46} = 0.95$, $p = 0.34$; effect of time: $F_{1,45} = 1.1$, $p = 0.31$; interaction treatment × time: $F_{1,46} = 1.2$, $p = 0.29$; figure 4*c*). Lastly, CORT measured approximately 48 h after applying the last CORT or oil vehicle treatment (final CORT) was similar for all newts (effect of treatment: $F_{1,46} = 2.8$, $p = 0.101$; effect of time: $F_{1,46} = 0.22$, $p = 0.65$; interaction treatment × time: $F_{1,46} = 0.45$, $p = 0.504$; figure 4*d*). Final CORT was positively correlated with NLR ($R^2 = 0.30$, $F_{1,43} = 19.6$, $p \leq 0.001$) and BKA ($R^2 = 0.08$, $F_{1,43} = 4.54$, $p = 0.04$; electronic supplementary material, table S3). Final CORT was not correlated with total splenocytes or MMC variables ($p > 0.05$; electronic supplementary material, table S3).

(iii) Bacterial communities

Alpha diversity was similar for CORT and oil vehicle-treated newts (Faith's phylogenetic diversity: $F_{1,43} = 0.29$, $p = 0.59$; richness: $F_{1,43} = 0.005$, $p = 0.94$; evenness: $F_{1,43} = 0.77$, $p = 0.38$; Shannon: $F_{1,43} = 0.56$, $p = 0.47$; electronic supplementary material, tables S6 and S7). However, alpha diversity was greater in short-term-treated newts compared with long-term-treated newts regardless of whether treatments consisted of CORT or oil vehicle (Faith's phylogenetic diversity: $F_{1,43} = 11.11$, $p < 0.01$; richness: $F_{1,43} = 14.29$, $p < 0.01$; Shannon: $F_{1,43} = 6.87$, $p = 0.01$; evenness: $F_{1,43} = 4.28$, $p = 0.05$; figure 5; electronic supplementary material, tables S6 and S7). Newts that received short-term applications had more detectible members in their communities compared with long-term newts, indicating higher species richness and the composition of these communities also had higher phylogenetic diversity. Additionally, overall species distribution (evenness) was also greater in the short-term application group, suggesting that community membership was less balanced with greater treatment application times. Thus, different patterns of ASV representation were observed in the communities of both short- and long-term applications. Overall composition of skin bacterial communities was also similar for all newts. Specifically, no differences were observed for dispersion (PERMDISP2, $p > 0.1$; electronic supplementary material, tables S8 and S9) or beta diversity, as indicated by Bray–Curtis and weighted UniFrac dissimilarities (PERMANOVA, $p > 0.1$; figure 6; electronic supplementary material, tables S10 and S11).

(iv) *Bd* and *Bsal* infection (experiment 3 only)

Bd was detected from all skin swabs collected from newts at the start of experiment 3. Initial *Bd* infection load ranged from

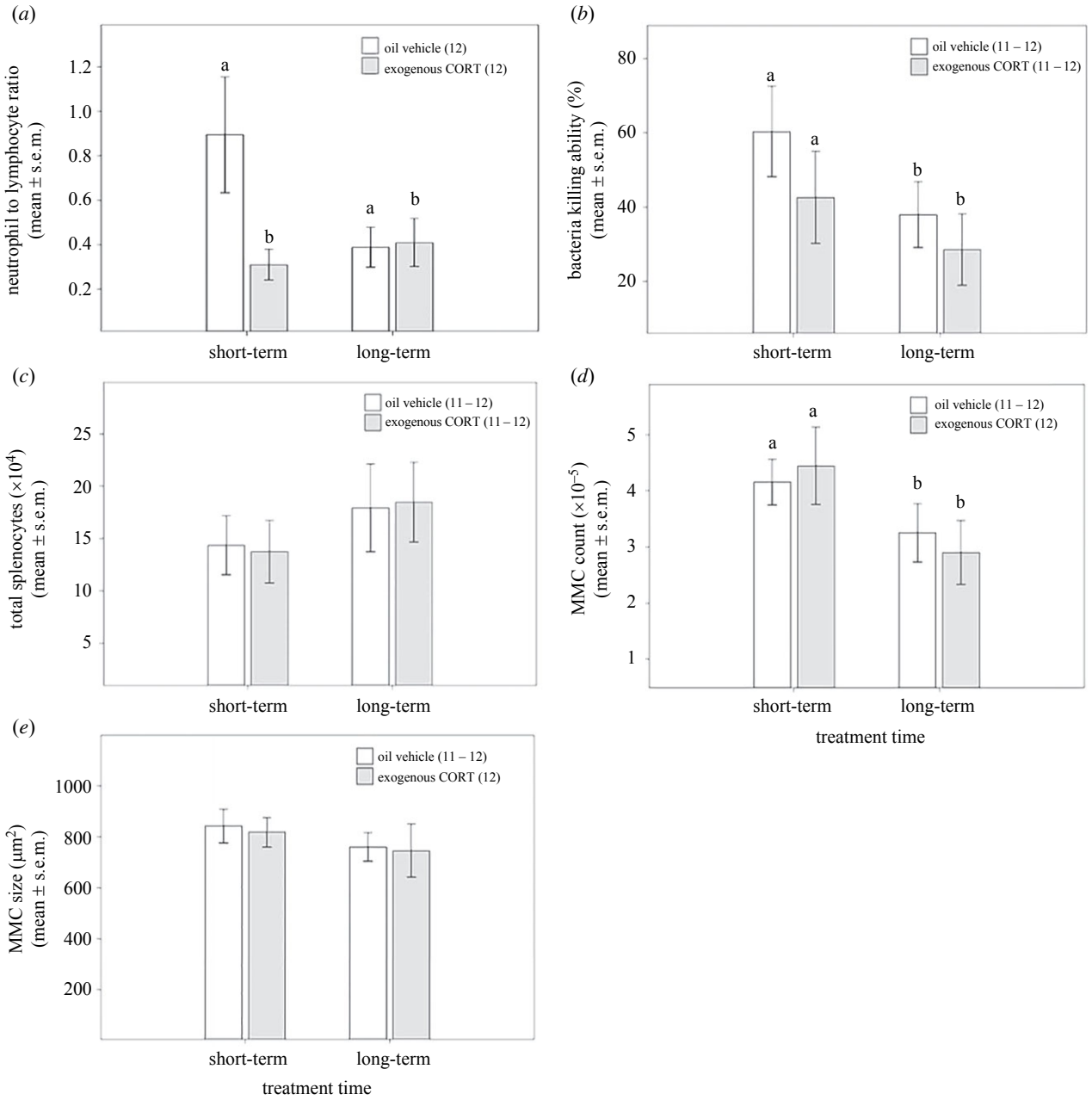

Figure 3. Effects of elevated plasma CORT on parameters of immunity in captive male *Notophthalmus viridescens* (experiment 3). Dermal treatment with an oil vehicle control or CORT was administered for a short-term (two treatment applications over 5 days) or long-term period (18 treatments applications over 26 days). Immune parameters consisted of NLR (*a*), plasma BKA (*b*), total splenocytes (*c*), melanomacrophage centre (MMC) size (*d*), and MMC count (*e*). Sample sizes are indicated in parentheses. Bars sharing or lacking letters are statistically similar.

403.3 to 2.5×10^7 GE. *Bd* infection load measured at the end of the experimental period ranged from 56.8 to 75 000 GE and was sometimes cleared entirely (final infection load = 0 GE). Measures of *Bd* infection (initial, final, and change in infection load) were not correlated with any measures of immunity or final CORT (all $p > 0.05$; electronic supplementary material, tables S4 and S5). All animals tested negative for *Bsal*.

4. Discussion

GCs are widely regarded to modulate vertebrate immune function, but we found no evidence that physiologically appropriate concentrations of CORT altered immunity in eastern newts (*N. viridescens*). Following repeated presumed elevations of plasma CORT (via dermal treatments of exogenous CORT), most parameters of immunity (NLR, plasma BKA, splenocytes, hepatic MMC), and measures of overall health (body mass, fat body mass, haematocrit) were similar between newts treated with CORT and newts treated with an oil vehicle control, regardless of whether treatments were short-term (two treatments over 5 days) or long-term (18 treatments over 26 days). Thus, contrary to our predictions, with the exception of NLR, we found no changes in immunity after CORT treatments. Similarly, we found no differences in the skin microbiome between CORT and oil vehicle-treated newts. However, we did find an effect of time (short-term, long-term) on plasma BKA, MMCs and the skin microbiome. We discuss our results in more detail below.

(a) Treatment with corticosterone-elevated plasma corticosterone to physiologically relevant levels

It is important to highlight that our CORT treatments produced physiologically relevant concentrations of plasma

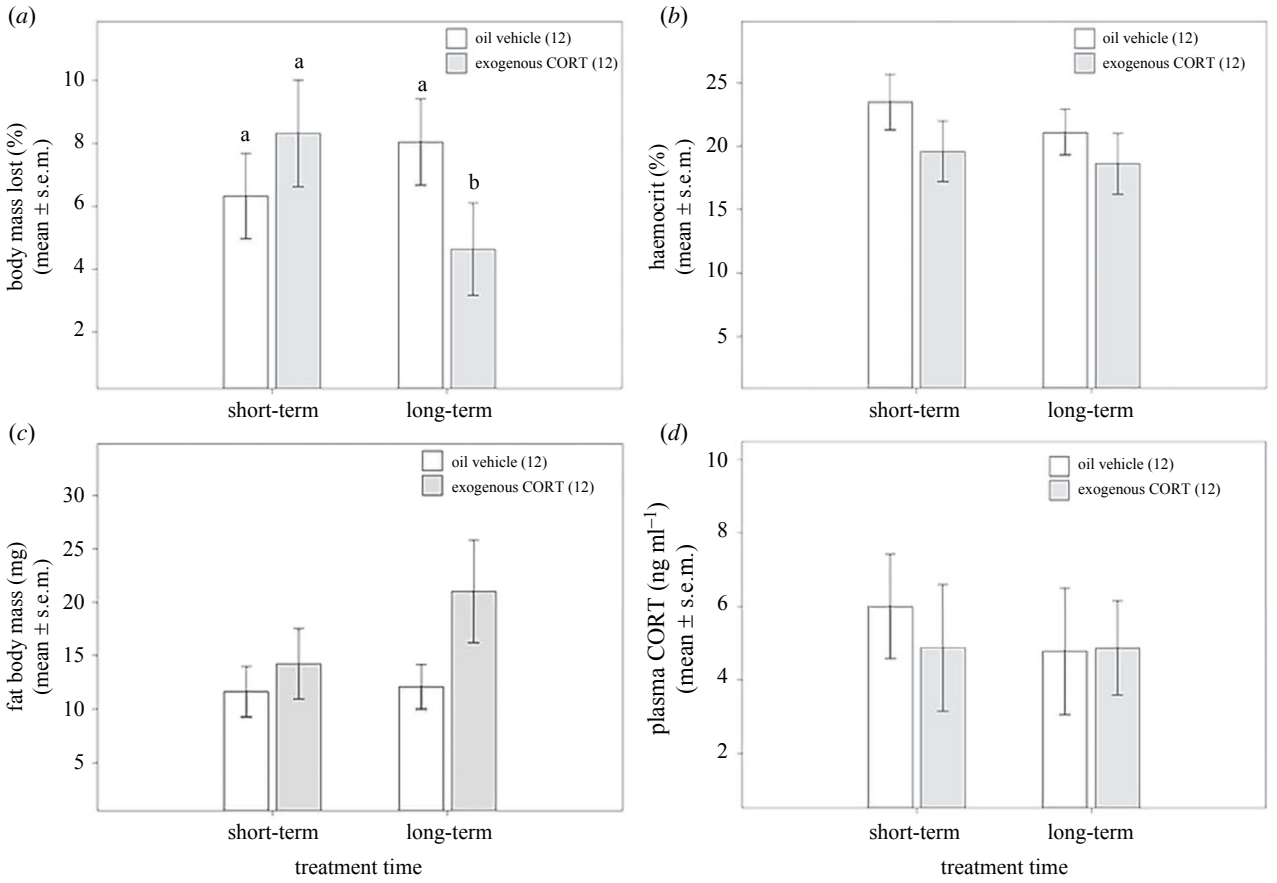

Figure 4. Effects of elevated plasma CORT on parameters of overall health (*a–c*) and baseline plasma CORT (*d*) in captive male *Notophthalmus viridescens* (experiment 3). Dermal treatments of an oil vehicle control or CORT were administered for a short-term (two treatment applications over 5 days) or long-term period (18 treatment applications over 26 days). Sample sizes are indicated in parentheses. Bars sharing or lacking letters are statistically similar.

CORT, unlike previous studies where newts were treated with pharmacological doses of GCs [31]. To ensure that we were using physiologically relevant CORT concentrations, we examined free-living newts to determine baseline and handling-induced levels of CORT. We found that plasma CORT was significantly elevated in free-living *N. viridescens* following a 15 min handling stressor relative to non-handled control newts and peaked at about 20 ng ml^{-1}, consistent with a previous study [69]. Next, we carefully determined that a dermal dose of 0.3 µg CORT achieved a plasma CORT level closely resembling stressor (handling)-induced levels observed in free-living newts. Finally, plasma CORT in control newts that received dermal treatment with an oil vehicle was similar to that of free-living newts not subjected to a handling stressor ($t_{14} = 1.49$, $p = 0.16$). This result suggests that neither dermal applications of treatments nor long-term captive conditions, in themselves, caused an increase in plasma CORT in *N. viridescens*.

(b) No effect of corticosterone on neutrophil to lymphocyte ratios and splenocytes

After receiving short- or long-term treatments, NLR values were mostly similar among CORT and oil-treated newts. However, NLR was the greatest in newts subjected to short-term oil treatments. This result was unexpected because CORT-induced changes in leucocyte distributions are well documented across a broad range of vertebrate taxa, including amphibians [34,70,71]. In fact, NLR is commonly used as an index for physiological stress in wild vertebrates [48]. We expected that CORT treatments, especially when repeated for an extended period of time (i.e. 26 days), would result in increased circulating neutrophils and decreased lymphocytes, consequently, increasing NLR values [70]. However, NLR for CORT and control newts were generally similar and mirrored baseline values previously reported in free-living *N. viridescens* [72,73]. Lymphocytes enumerated from the spleen (splenocytes) were also similar for CORT and control newts, further supporting that CORT had little to no effect on leucocyte proliferation or distribution. Together, these results suggest that neither CORT elevations nor the captive condition altered leucocyte distributions in *N. viridescens*.

Our results do not align with a previous study in free-living plethodontid salamanders (*Desmognathus ochrophaeus*) in which seasonal elevations in plasma CORT were associated with decreases in lymphocyte populations [74]. However, our results are consistent with results in captive *D. ochrophaeus* where physiological elevations in CORT (via dermal CORT treatment or handling stressor) did not affect leucocyte ratios, thereby, suggesting that the relationship between CORT and leucocytes may be context-dependent [42].

(c) Effect of time but not corticosterone on bacterial killing ability

In studies of amphibian disease, the BKA of plasma is often used to assess the strength of complement-mediated innate immunity but may also reflect bioactivity of lysozyme and antibody constituents [75]. Indeed, results in anurans show that

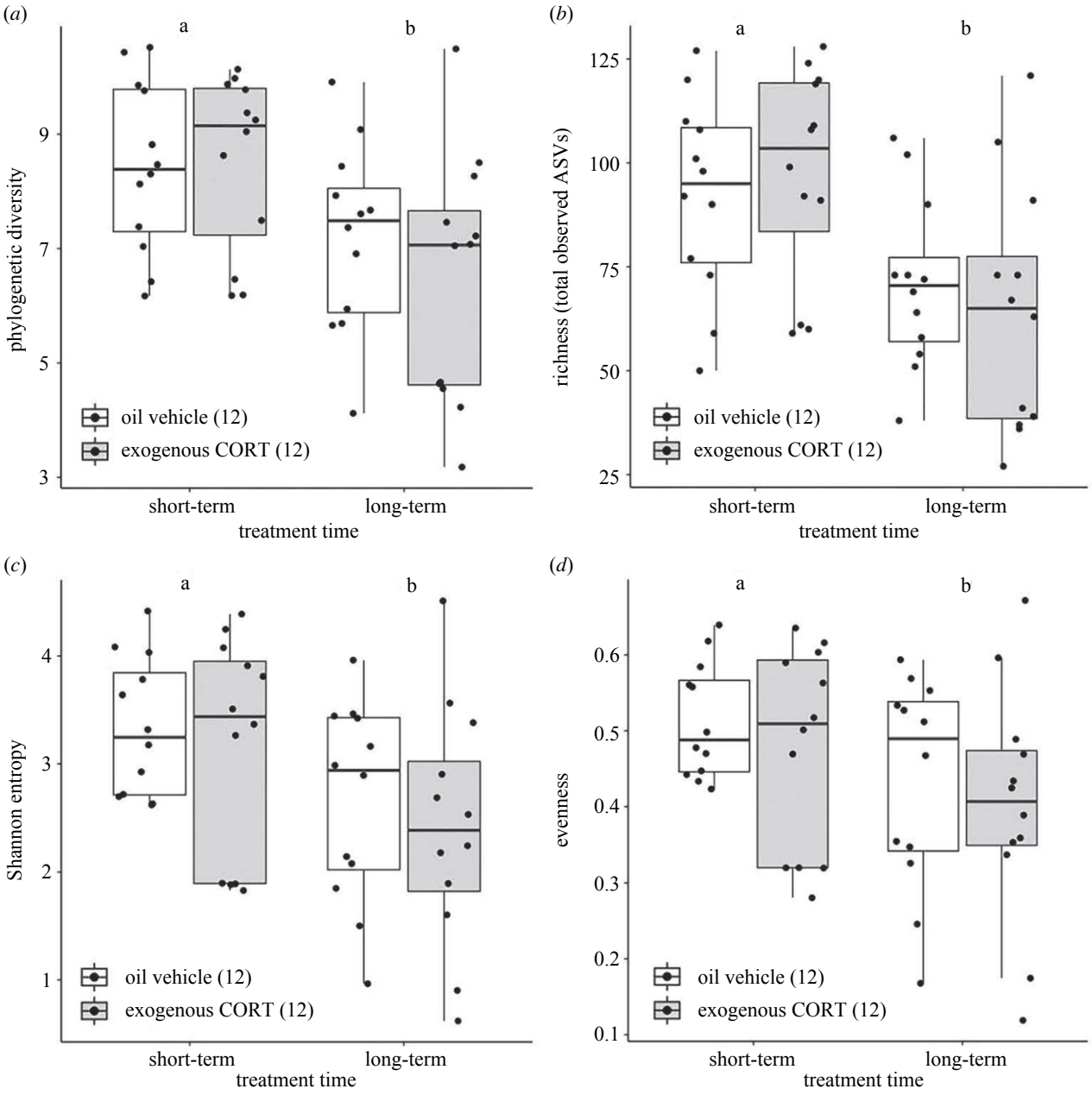

Figure 5. Effects of elevated plasma CORT on alpha diversity of skin bacterial communities in captive male *Notophthalmus viridescens* (experiment 3). Rarefied ASV tables were used to calculate phylogenetic diversity (*a*), overall community richness (*b*), Shannon entropy (*c*), and evenness (*d*) from skin sampled following dermal treatments of an oil vehicle control or CORT administered for a short-term (two treatment applications over 5 days) or long-term period (18 treatment applications over 26 days). Sample sizes are indicated in parentheses.

higher plasma BKA is associated with reduced susceptibility to *Bd* infections, chytridiomycosis and mortality [76]. In *N. viridescens*, we observed a minute relationship between CORT concentrations and plasma BKA (electronic supplementary material, table S3). This result is similar to results in cryptobranchid salamanders (*Cryptobranchus alleganiensis*) in which plasma CORT and BKA were not related [35]. However, plasma BKA was significantly less in newts subjected to long-term treatments compared with newts subjected to short-term treatments, regardless of whether treatments consisted of CORT or oil vehicle.

The differences in plasma BKA between short-term and long-term-treated newts are not owing to time in captivity, as both groups were in captivity for the same amount of time. Instead, the differences could have been owing to differences in environmental temperature. For each application (CORT or oil vehicle), newts were removed from 15°C and placed at room temperature (mean ± s.e.m.; 23.9 ± 0.16°C) for 45 min. Consequently, newts that received long-term treatments were exposed more frequently to higher ambient temperatures compared with newts that received short-term treatments. While results in cryptobranchid salamanders (*C. alleganiensis*) provide some evidence that natural fluctuations in temperature increase plasma BKA, results in anurans (*Lithobates catesbeianus*) suggest that temperatures above 20°C negatively impact plasma BKA [77,78].

(d) Effect of time but not corticosterone on melanomacrophage centres

We included measures of MMCs to assess potential CORT-induced changes in immunity in *N. viridescens*. In fishes, MMC parameters generally increase in response to exposure to environmental contamination and pathogens [79]. Studies in anurans indicate that MMCs are also influenced by pesticide and parasite exposure [80]. To the best of our knowledge, this is one of the first studies to investigate MMCs in *N. viridescens*

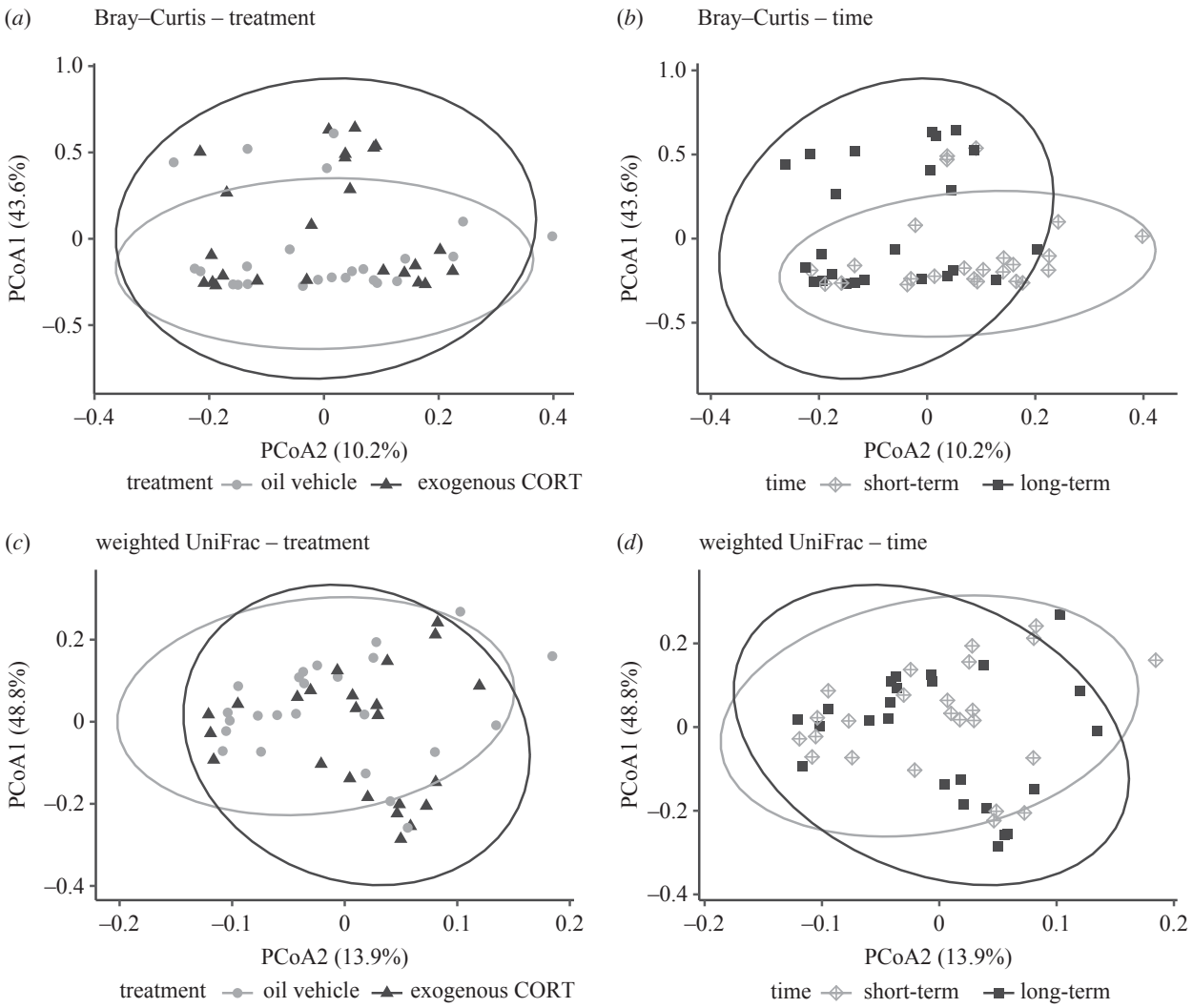

Figure 6. Effects of elevated plasma CORT on beta diversity of skin bacterial communities in captive male *Notophthalmus viridescens* (experiment 3). Distance matrices were calculated from rarefied ASV tables from skin sampled following dermal treatments of an oil vehicle control or CORT using QIIME2. Ordination of Bray–Curtis (*a*,*b*) and weighted UniFrac (*c*,*d*) dissimilarities are shown, where each point represents the bacterial skin community of an individual newt. The sample size for each treatment and time combination was 12 newts.

[81]. While our results suggest that MMCs are not sensitive to CORT, newts subjected to long-term treatments had fewer MMCs compared with newts subjected to short-term treatments regardless of whether treatments consisted of CORT or oil vehicle.

As explained above for BKA, we suspect that the effect of time (long-term versus short-term) on MMCs was because of placing the newts at room temperature for 45 min for each dermal application. There is evidence that MMCs, like plasma BKA, are influenced by environmental temperature. In anurans (*Eupemphix nattereri*), extreme changes in environmental temperature are associated with decreased pigmentation of hepatic MMCs [82]. Likewise, anuran MMCs also vary seasonally. For instance, results in *Rana esculenta* found that MMC numbers were significantly higher during the winter compared with the summer [83]. While short-term newts were more consistently held at lower temperatures relative to long-term newts, it is possible that differences in environmental temperature also contributed to differences in MMC numbers.

(e) Health measures

We measured body mass (final and amount lost), fat body mass and haematocrit as measures of overall health. In vertebrate species, including plethodontid salamanders, elevations in plasma CORT were associated with significant losses in body mass and depletion of fat stores [42,84]. Haematocrit percentage is broadly used as an indicator of general health although it is influenced by numerous factors including hydration level, reproductive status, sex and parasite load [47]. We found that body mass, fat body mass and haematocrit percentages were similar among all newts regardless of treatment type (CORT or oil vehicle) or time (short-term, long-term). One unexpected result was that newts subjected to long-term treatments with CORT lost less body mass relative to newts in the other treatment groups. While this result is puzzling because we would expect CORT to cause more, not less, body mass loss, previous work found that birds exposed to prolonged CORT treatments also had greater body mass relative to controls [29,84,85].

(f) Effect of time but not corticosterone on the skin microbiome

Although CORT altered gut microbial communities in birds and lizards, to our knowledge, no studies have investigated whether CORT alters the skin microbiota of amphibians [86,87]. Here, alpha and beta diversity metrics were similar

for CORT and oil-treated newts. However, the alpha diversity of the skin microbiome was less in long-term-treated newts compared with short-term-treated newts, regardless of whether treatments consisted of CORT or oil vehicle control. The effect of time on the skin microbiome is not owing to differences in the time in captivity, as all groups were in captivity for the same amount of time.

It was unexpected that treatment time had a significant impact on the skin microbiome of *N. viridescens*. Because both CORT and control treatments were prepared using a sesame oil vehicle, one potential explanation is that differences in the total volume of oil added to long-term (2.5 µl × 18 treatments) and short-term (2.5 µl × 2 treatments) newts drove changes in bacterial communities by acting as an additional source of carbon. Vegetable oil (EVO) has been proposed as a method of increasing microbial activity for environmental bioremediation and impacting environmental microbial community composition [88–90]. However, we feel that it is unlikely that the oil itself affected the skin microbiome because treatments were only applied to a small patch of skin, while the entire body was swabbed to assess the skin microbiome. It is important to highlight that the CORT and oil treatments used in the current study did not contain DNA, further suggesting that the treatments themselves do not explain differences in the skin microbiome.

Alternatively, other factors, such as the handling frequency (i.e. opening and closing of home boxes), and changes in temperature during treatment applications may have contributed to observed differences in the skin microbiome. It is unlikely that handling frequency contributed to differences in alpha diversity because short-term newts (less frequent opening and closing of home boxes) had greater bacterial diversity than long-term newts (more frequent opening and closing of home boxes). This is counterintuitive because we would expect that increased handling frequency would increase the potential of introducing contaminant DNA into home boxes [91]. Instead, differences in bacterial diversity could be owing to differences in environmental temperature. Our results align with a previous study of free-living *N. viridescens*, where bacterial phylogenetic diversity and species richness was greater on the skin of newts at 14°C compared with newts at 22°C [39].

The differences in the skin microbiome between short-term and long-term-treated newts may also be related to observed differences in plasma BKA and MMCs. Many complement proteins (as assessed by plasma BKA) circulate as inactive proteins until they are activated by antigenic stimulation [75]. Thus, the increased bacterial diversity observed in short-term newts could have activated complement activity as reflected by higher plasma BKA. On the other hand, in humans, reduction in complement activity was linked to changes in the overall structure of skin bacterial communities and a significant decrease in diversity [92]. Therefore, it is equally plausible that reduction in plasma BKA, as observed in long-term newts, drove reductions in skin bacterial diversity. The variables of amphibian MMCs are also responsive to antigenic stimulation, such as that elicited by ranavirus and *Bd*, but the relationship between skin microbiota and MMCs remains largely unexplored [93,94].

(g) Explanations for the lack of corticosterone effects

Collectively, our results indicate that CORT had no effect on our immune measures, including skin microbiome alpha diversity. While it would be premature to conclude a complete lack of CORT-immune relationship in *N. viridescens*, there are several potential explanations for the lack of CORT effect observed herein including: infection status or captivity might have desensitized the newts to CORT treatments, CORT might have impacted immune factors that we did not measure, or our timing was not optimized for detecting the influence of CORT.

First, *Bd* infections may have confounded CORT-immune interactions. In anurans (*Aletes obstetricans*, *Litoria wilcoxii*, *Litoria caerulea*), infection with *Bd* was strongly associated with elevated CORT, probably owing to the infection or disease activating a CORT response [95–97]. Similar CORT elevations have been observed in ambystomatid salamanders (*Ambystoma maculatum*) following experimental exposure to *Bsal* [10]. All newts harboured natural *Bd* infections at the beginning of experiment 3. Although no newts exhibited clinical signs of chytridiomycosis, it is possible that subclinical *Bd* infections triggered release of endogenous CORT, in turn, desensitizing newts to CORT treatments. Likewise, other infections that were not screened in the current study (e.g. ranavirus, endoparasites) may have also influenced CORT and immune responses [98,99]. Given that *Bd* can have profound impacts on amphibian immunity, it was surprising that we found no correlations between *Bd* infection, and the immune parameters included in the current study [12,100].

Second, it is possible that captivity triggered the release of endogenous CORT, in turn, desensitizing newts to our CORT treatments. We think this is unlikely because: (i) plasma CORT levels in control newts in experiment 2 (that were in captivity for roughly the same amount of time and housed in the same manner as newts in experiment 3) were the same as field-sampled newts; and (ii) baseline plasma CORT measured at the end of experiment 3 (final CORT) was similar for all newts and only slightly above baseline measures previously observed in free-living newts [101]. For these reasons, captivity probably had a negligible effect on baseline CORT levels. We were also very careful not to disturb newts during the process of administering treatments, and it takes persistent and intense handling before newts exhibit any sort of behavioural or CORT response [41] (K. E. Pereira 2018, unpublished data).

Third, despite measuring numerous parameters of immunity, another possibility is that we did not measure the right parameters. The small body size of adult *N. viridescens* (less than 5 g) greatly limited the number of immunological tests we could perform. For instance, the amount of plasma collected per newt ranged from around 8 to 39 µl and was used entirely for BKA and CORT assays. Because of this limitation, we chose parameters that had a high probability of CORT-sensitivity (e.g. NLR), while also exploring more novel parameters (MMCs) [48,51]. It is important to highlight that the vertebrate immune system is vast and comprised many interacting elements [102]. Further, GC receptors are found on most nucleated cells but the functional responses to GCs are cell-specific [103]. Thus, although repeated CORT elevations did not alter immune measures in the current study, we cannot conclude a lack of CORT-immune relationship in *N. viridescens* [104,105].

Fourth, another variable to consider is the timing of our experimental design (electronic supplementary material, table S1). GCs can have both rapid and delayed effects on immune processes, depending on whether effects are mediated via genomic or non-genomic pathways [27,103]. Here, we

collected plasma and tissue samples 48 h after the final CORT treatment. This decision was partly based on previous work in *N. viridescens* in which immune changes were not observed until several days following a pharmacological dose of hydrocortisone (a synthetic GC) [31]. While it is possible that immune responses to short-term CORT treatments were more transient, we feel that it is unlikely that immune changes resulting from 18 CORT treatments (long-term) would not overlap with our experimental timing.

Lastly, it is possible that cortisol, rather than CORT, modulates immune function in *N. viridescens*. A recent paper in cryptobranchid salamanders (*C. alleganiensis*) found that plasma cortisol levels were 5–10 times higher than plasma CORT, leading to the conclusion that cortisol is the predominant GC in this species [106]. To begin to address the possible role of cortisol in *N. viridescens*, we measured plasma cortisol and CORT in a set of eight newts and found that concentrations were similar (mean ± s.e.m.; CORT: 1.8 ± 0.45 ng ml^{-1}; cortisol: 1.1 ± 0.19 ng ml^{-1}) (K. E. Pereira 2021, unpublished data). Additional research is needed to compare stressor-induced CORT and cortisol concentrations, and to examine potential regulatory roles of cortisol on immune function in *N. viridescens*.

5. Conclusion

Understanding the mechanisms underlying variation in salamander responses to the emerging amphibian chytrid fungus, *Bsal* is critical for allocating conservation resources to high-risk populations and generating science-based disease management plans [8]. Collectively, our results shine light on the relationship between GCs and salamander immune responses, while also providing valuable physiological information for eastern newts (*N. viridescens*), a species predicted to be widely impacted by *Bsal* emergence [100]. We found little evidence that elevations in corticosterone (CORT) influence immunity, overall health or skin bacterial communities.

Although insensitivity of immune factors to CORT in *N. viridescens* was unexpected, we provide several possible explanations and highlight important areas for future study. For example, *Bd* infections may have influenced immunological responses to elevated CORT. Given that *Bd* infections are pervasive in free-living populations of *N. viridescens* and may increase mortality rates caused by *Bsal*, research is urgently needed to better understand the implications of *Bd* infection for salamander immunity, stress physiology and disease outcome [107–109]. Further, additional research is needed to better understand the implications of environmental temperature for the skin microbiome, immunity and disease susceptibility of salamanders.

Ethics. All animal use was conducted with appropriate permits from the Pennsylvania Fish and Boat Commission (Permit Nos. 2019-01-0082 and 2020-01-0028) and Duquesne University's Institutional Animal Care and Use Committee (Protocol No. 1905-07).

Data accessibility. Data is available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.qv9s4mwj5 [110].

The data are also provided in the electronic supplementary material [111].

Authors' contributions. K.E.P.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, writing—original draft, writing—review and editing; J.A.M.: data curation, formal analysis, funding acquisition, software, writing—original draft, writing—review and editing; M.C.B.: data curation, formal analysis, writing—original draft, writing—review and editing; D.C.W.: funding acquisition, resources, supervision, writing—review and editing; S.K.W.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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