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Physiological plasticity and acclimatory responses to salinity stress are ion-specific in the mayfly, *Neocloeon triangulifer*☆

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ABSTRACT

Freshwater salinization is a rapidly emerging ecological issue and is correlated with significant declines in aquatic biodiversity. It remains unclear how changing salinity regimes affect the physiology of sensitive aquatic insects. We used the parthenogenetic mayfly, *Neocloeon triangulifer,* to ask how ionic exposure history alters physiological processes and responses to subsequent major ion exposures. Using radiotracers (^{22}Na, $^{35}SO_4$, and ^{45}Ca), we observed that mayflies chronically reared in elevated sodium or sulfate (157 mg L^{-1} Na or 667 mg L^{-1} SO_4) had 2-fold ($p < 0.0001$) and 8-fold ($p < 0.0001$) lower ion uptake rates than mayflies reared in dilute control water (16 mg L^{-1} Na and 23 mg L^{-1} SO_4) and subsequently transferred to elevated salinities, respectively. These acclimatory ion transport changes provided protection in 96-h toxicity bioassays for sodium, but not sulfate. Interestingly, calcium uptake was uniformly much lower and minimally influenced by exposure history, but was poorly tolerated in the toxicity bioassays. With qRT-PCR, we observed that the expression of many ion transporter genes in mayflies was influenced by elevated salinity in an ion-specific manner (general upregulation in response to sulfate, downregulation in response to calcium). Elevated sodium exposure had minimal influence on the same genes. Finally, we provide novel light microscopic evidence of histomorphological changes within the epithelium of the Malpighian tubules (insect primary excretory system) that undergoes cellular degeneration and necrosis secondary to calcium toxicity. We conclude that physiological plasticity to salinity stress is ion-specific and provide evidence for ion-specific toxicity mechanisms in *N. triangulifer*.

1. Introduction

Many freshwater ecosystems are becoming increasingly salty throughout the world (Canedo-Arguelles et al., 2016; Kaushal et al., 2018, 2005). Various human activities such as road deicing, resource extraction, and irrigation of arid landscapes are some of the activities that contribute to elevated major ion concentrations in freshwaters (Entrekin et al., 2011; Pond et al., 2008a). Changes in precipitation patterns, drought, and seawater intrusion are additional climate driven changes to freshwater salinity regimes (Barlow and Reichard, 2010; Kinzelbach et al., 2003; Mosley, 2017). Because different environmental scenarios can lead to the elevation of specific major ions (e.g., NaCl in road-deicing, SO_4 and Ca in mountain-top coal mining), it is critical to understand how various ion matrices lead to different toxic effects in aquatic organisms (Griffith, 2017a; Kunz et al., 2013).

Ecologists report that sensitive aquatic organisms such as mayflies, are disproportionately affected by freshwater salinization (Beermann et al., 2018; Kefford, 2018; Pond et al., 2008a). Mayflies and other aquatic insects play an essential role in freshwater ecosystem function and serve as biological indicators (Hawkins, 2006; Kenney et al., 2009). Scientists have only recently begun to study the physiological mechanisms behind salinity stress in aquatic insects to better understand this ecological dilemma. Previous work demonstrates that ion influx rates increase with increasing concentration (Scheibener et al., 2017) and temperature (Orr and Buchwalter, 2020) in aquatic insects. However, our limited evidence suggests that these increased flux rates do not result in systemic dysregulation of the elements at the whole body level (Buchwalter et al., 2019; Scheibener et al., 2017) or alter hemolymph osmolality (Kefford, 2018; Dowse et al., 2017; Verberk et al., 2020). Thus, our limited evidence to date points towards mayflies as strict osmoregulators and we hypothesize, at least in the case of sulfate toxicity, that bioenergetic cost of this regulation (the ATP expense of

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excessive ion turnover) may explain delayed development, decreases in population fitness observed in the lab (Buchwalter et al., 2019; Hassell et al., 2006; Johnson et al., 2015; Soucek and Dickinson, 2015; Verberk et al., 2020), and ultimately the loss of species that have been observed in the field (Cormier et al., 2013b, 2013a; Pond et al., 2008b).

Extremely elevated salinities can be detrimental (Griffith, 2017a; Johnson et al., 2015), but are sensitive aquatic organisms able to acclimate to changing salinity regimes? This question has been approached in some organisms such as daphnids (Chen and Stillman, 2012; Coldsnow et al., 2017) and a species of mayfly (Dowse et al., 2017). However, mechanisms of acclimation (e.g., flux rates of specific major ions) remain unknown in aquatic insects. We hypothesize that mayflies can acclimate by making subtle physiological changes and better survive in a stressfully salty environment over time.

Here, we capitalize on recent advances in the establishment of the baetid mayfly, *Neocloeon triangulifer* as a laboratory model (Chou et al., 2018, 2017; Funk et al., 2006; Kim et al., 2017). We ask how major ion exposure history affects subsequent physiological responses to different ionic conditions. To specifically ask if and how *N. triangulifer* can acclimate to different salinity regimes, we used radiotracers to assess how exposure history affects ion transport rates for sodium, sulfate, and calcium. We also developed a suite of RT-qPCR probes for several genes related to ion transport to assess how exposure history affects gene expression at the mRNA level. Additionally, we ask if and how these physiological changes associated with exposure history are manifested at the whole organism level by comparing the survival of larvae with different exposure histories to acute salinity challenge. Finally, we performed routine histological evaluation on mayfly larvae to assess tissue morphological changes in the important ion regulatory organ, the Malpighian tubules.

2. Methods

The overarching strategy for these studies was to compare how *N. triangulifer* larvae reared under different ionic conditions respond to changing salinity regimes (See Supplementary Fig. 1). Artificial soft water (ASW) (Table 1) serves as our routine culture media, and was the control treatment for these experiments, in addition to being the base water to which all ions of interest were added. Pre-exposure concentrations (Supplementary Table 2) were intended to be challenging, but not acutely lethal to increase potential acclimatory effects. The elevated sulfate concentrations consisted of a blend of Ca- and Mg–SO_4 and were chosen based on previously published full life cycle studies for this ion (Buchwalter et al., 2019). The elevated sodium pre-exposure concentrations were chosen based on previously published studies of NaCl toxicity in this species (Jackson and Funk, 2019; Soucek and Dickinson, 2015). Finally, calcium pre-exposure concentrations were raised in similar magnitudes, due to lack of previous toxicity data.

2.1. Mayfly husbandry

The parthenogenetic baetid mayfly, *Neocloeon triangulifer*, was originally collected from White Clay Creek in Pennsylvania (WCC-2 clone) and gifted to us by the Stroud Water Research Center (SWRC; Avondale, PA) (Sweeney and Vannote, 1984). *N. triangulifer* larvae were reared in laboratory conditions in 4 L glass Pyrex dishes at room temperature (21–23 °C) and a 14:10 h light:dark photoperiod. Collaborators at the SWRC also gifted us natural periphyton cultured on plastic plates as nutrition for the mayfly larvae.

2.2. Ion flux experiments

To assess the plasticity of ion transport, mayfly hatchlings were seeded into either control (ASW) or one of the three elevated salinity treatments (157 mg L^{-1} Na, 735 mg L^{-1} SO_4, or 122 mg L^{-1} Ca) within 24 h of hatching. After rearing for approximately 21 days, mature larvae were placed in experimental waters to perform the transplant ion flux measurements (Fig. 1).

Radioactive experimental waters were made in ASW with $^{45}CaCl_2$ or dual-labeled with $^{22}NaCl$ and $Na_2^{35}SO_4$ (PerkinElmer, Billerica, MA, USA) with exposure activities ranging from 156 to 260 Bq mL^{-1}. Exposure waters were measured with the Beckman LS6500 Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA) directly before the experiments. Experiments were performed in 100 mL high-density polyethylene beakers with 15 mL of exposure water, gentle aeration, and sealed with a ParaFilm™ covering. All experiments had 8 replicates ($n = 8$) for each of the 3 time points used to calculate mass-specific, unidirectional uptake rates. Details of this calculation method can be found in previous publications (Orr and Buchwalter, 2020) and Supplementary Fig. 2.

After each timepoint, mayflies were removed from the radioactive exposure waters, rinsed in two consecutive baths of ASW to remove any loosely adsorbed ions from the exoskeleton and weighed. In the ^{45}Ca experiments, mayflies were additionally rinsed with 0.05 M EDTA and 0.1 M L-ascorbic acid sodium salt due to the adsorptive nature of Ca on insect exoskeletons (Poteat and Buchwalter, 2014). After rinsing,

Table 1
Water chemistry for all experimental exposure waters. Cations (Na, Ca, Mg, K) and anions (SO_4, Cl, CO_3) are all listed in mg L^{-1}. All waters were sampled, filtered, and verified by North Carolina State University's Environmental and Agriculture Testing Services Lab (ICP-EATS) and concentrations were within 10% of nominal values except for 743 Na, which had a 21% error. Measured values are listed in parentheses beside nominal values.

	Na	Ca	Mg	K	S	SO_4	Cl	CO_3
Control (ASW)	15.3 (16.5)	12.7 (11.6)	3.4	1.4	7.8 (7.9)	23.3	14.1	42.6
121 Ca	15.3	121 (113)	3.4	1.4	7.8	23.3	97	42.6
480 Ca	15.3	480 (471)	3.4	1.4	7.8	23.3	158	42.6
686 Ca	15.3	686 (646)	3.4	1.4	7.8	23.3	208	42.6
980 Ca	15.3	980 (933)	3.4	1.4	7.8	23.3	280	42.6
1400 Ca	15.3	1400 (1338)	3.4	1.4	7.8	23.3	380	42.6
2000 Ca	15.3	2000 (1896)	3.4	1.4	7.8	23.3	525	42.6
157 Na	157 (152)	12.7	3.4	1.4	7.8	23.3	255	42.6
205 Na	205 (228)	12.7	3.4	1.4	7.8	23.3	334	42.6
280 Na	280 (298)	12.7	3.4	1.4	7.8	23.3	452	42.6
387 Na	387 (414)	12.7	3.4	1.4	7.8	23.3	615	42.6
535 Na	535 (559)	12.7	3.4	1.4	7.8	23.3	844	42.6
743 Na	743 (915)	12.7	3.4	1.4	7.8	23.3	1165	42.6
360 SO_4	15.3	101	40.4	1.4	120 (129)	360	14.1	42.6
515 SO_4	15.3	139.2	56.4	1.4	172 (173)	515	14.1	42.6
735 SO_4	15.3	193.7	79.1	1.4	245 (256)	735	14.1	42.6
1050 SO_4	15.3	271.8	111.5	1.4	351 (383)	1050	14.1	42.6
1500 SO_4	15.3	383	157.9	1.4	501 (532)	1500	14.1	42.6

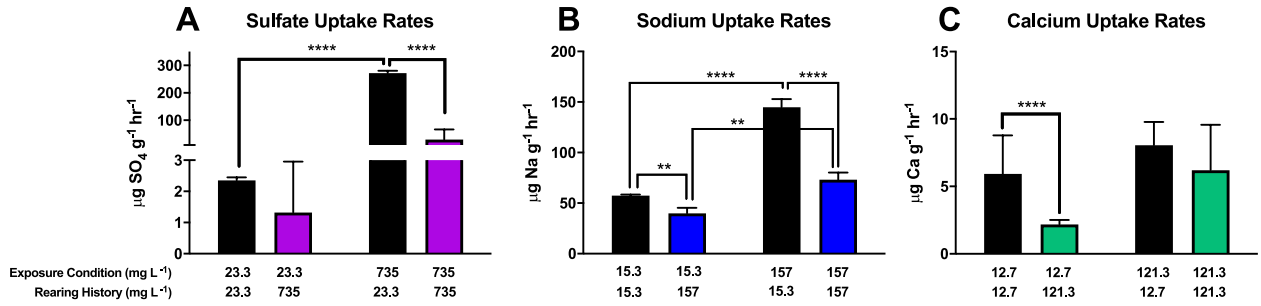

Fig. 1. Ion uptake rates in *N. triangulifer* for sulfate (A), sodium (B), and calcium (C). The y-axis indicates both rearing history and exposure condition for each major ion of interest. Each column represents a time-course study demonstrated in Supplementary Fig. 2. Errors bars represent standard error from the mean ($n = 8$). A one-way ANOVA with Tukey's multiple comparisons was performed and asterisks indicate statistically significant differences between groups (* indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$).

mayflies were blotted dry, weighed, and digested in 500 μL of Soluene 350 (PerkinElmer) in a 20 mL glass vial for 48 h. Digestates were neutralized with 500 μL of glacial acetic acid and 12 mL of scintillation cocktail (PerkinElmer Ultima Gold uLLT) was added to measure radioactivity by liquid scintillation counting. We applied appropriate corrections for spill-over and quench, and only measurements with lumex values < 5% and error rates <10% were used in analyses.

2.3. Gene expression

We examined the expression of eight unique genes in *N. triangulifer* that we hypothesized may be affected by salinity (e.g., ion transporters). The gene names and primer information are listed in Supplementary Table 2. Primers lacking accession numbers have been verified through NCBI BLAST to ensure high similarity (>90%) between other more established species (e.g., *Aedes aegypti, Aedes gambiae,* and/or *Drosophila melanogaster*). *N. triangulifer* hatchlings were seeded into either control (ASW) or one of three elevated salinity (735 mg L^{-1} SO_4, 157 mg L^{-1} Na, or 121 mg L^{-1} Ca) waters within 24 h of hatching. After rearing for approximately 21 days, mature larvae were placed in experimental waters to perform a full transplant assessment (i.e., all four combinations of exposures for each major ion of interest) for an acute 8-h exposure (Supplementary Fig. 1). To assess the relative gene expression of important transporters, we randomly sampled mayfly larvae at the end of each exposure and flash froze 2 composited larvae per replicate in liquid nitrogen ($n = 5$). Samples were stored in a −80 °C freezer until RNA extraction was performed using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions and quantified using a NanoDrop™ 1000 (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using the MultiScribe™ MuLV reverse transcriptase and random primers (Applied Biosystems, Carlsbad, CA) in 20 μL reaction tubes using a Bio-Rad iCycler (Bio-Rad, Hercules, CA).

Quantitative real-time PCR (qRT-PCR) was performed using diluted cDNA, diluted primers, and SYBR Green Master Mix (Bio-Rad, Hercules, CA) on a QuantStudio™ 3 machine (Thermo Fisher Scientific, Waltham, MA) in 10 μL reactions with technical triplicates. Custom primers were designed through Integrated DNA Technologies (IDT, Coralville, Iowa) and are listed in Supplementary Table 2. Standard PCR parameters were used and followed by a melt curve to ensure high quality of all samples.

2.4. 96 hour toxicity bioassay

To assess survival across treatment groups with differential exposure histories, we seeded *N. triangulifer* hatchlings into either control (ASW) or elevated salinity (205 mg L^{-1} Na, 360 mg L^{-1} SO_4, or 480 mg L^{-1} Ca) waters within 24 h of hatching. After approximately 10–12 days of rearing, middle-aged larvae were seeded into 6-well plates across a series of concentrations for each ion of interest: 15.3 (ASW), 205, 280, 387, 535, or 743 mg L^{-1} Na, or 23.3 (ASW), 360, 515, 735, 1050, 1500 mg L^{-1} SO_4, or 12.7 (ASW), 480, 686, 980, 1400, 2000 mg L^{-1} Ca. All waters were sampled and verified by North Carolina State University's Environmental and Agriculture Testing Services Lab (ICP-EATS) and concentrations were within 10% of nominal values, except 743 mg L^{-1} Na, which had a 21% error.

Each replicate well contained 10 larvae and each treatment was represented by 3 replicates ($n = 3$) or 6 ($n = 6$, for control groups only). Sterile 6-well plates were filled with 8 mL (approximately 75% full) of each water and aerated fully. Wells were given 2 drops (~200 μL) of food slurry (0.1 g periphyton per mL of corresponding water) to prevent alterations of salinity concentrations within the wells. All plates were aerated (60 s/well) and inspected daily with a Leica MZ 16 F stereoscope and any mortalities were recorded and removed. At 48 h, a 50% water change was performed to remove excess debris/feces and replace it with new test solution. Additional food was added from corresponding food slurries as needed. Previous 96-h bioassays in our lab have indicated that these methods prevent substantial changes to water conductivities and pH (Orr and Buchwalter, 2020).

2.5. Microscopic pathology

Mature *N. triangulifer* larvae were treated with various salinity treatments for 96 h: control (ASW, $n = 5$), sodium (674 mg L^{-1} Na as NaCl; $n = 7$), sulfate (735 mg L^{-1} SO_4 as $CaSO_4$ and $MgSO_4$; $n = 5$), or calcium (980 mg L^{-1} Ca as $CaCl_2$; $n = 7$). After the exposure, mayflies were immediately rinsed, fixed in 10% neutral buffered formalin for 24 h, transferred to ethanol 70%, embedded in paraffin in sagittal orientation, sectioned into 4-5 μm-thick, and stained with hematoxylin and eosin (HE) at the histology laboratory at the North Carolina State University Histopathology core facility. Multiple serial sagittal HE sections of mature *N. triangulifer* larvae from each group were blindly examined by one American College of Veterinary Pathologists (ACVP) board-certified anatomic pathologist to determine and characterize any histological alterations including inflammatory and/or degenerative processes on targeted organs and lesion severity with light microscopy.

2.6. Data analysis

Flux rates were determined by linear regression analysis using GraphPad Prism (v6, GraphPad Software, La Jolla, CA, USA). Uptake rates were calculated based on the slopes of linear regression analysis of each time course based on wet weights. Comparisons of flux rates among groups were performed via one-way ANOVA with Tukey's multiple comparisons test for all experiments. All data were also analyzed for normality.

For gene expression experiments, the delta-delta CT method (Pfaffl, 2001) was used to calculate relative expression and normalized to the housekeeping gene, β-actin. Data were normalized again to the control

samples and relative fold change was analyzed with a one-way ANOVA and Tukey's multiple comparisons. Finally, the toxicity bioassays were also analyzed using a one-way ANOVA with Tukey's multiple comparisons and LC_{50} values were calculated using probit analysis. For all plots, error bars represent mean ± SEM and a p-value of 0.05 was chosen *a priori*.

3. Results

3.1. Ion flux experiments

Flux rates in control reared larvae were concentration dependent for sodium and sulfate, but not calcium (Fig. 1). For example, control mayflies (23.3 mg L^{-1} SO_4) had a basal sulfate uptake rate of 2.4 ± 0.1

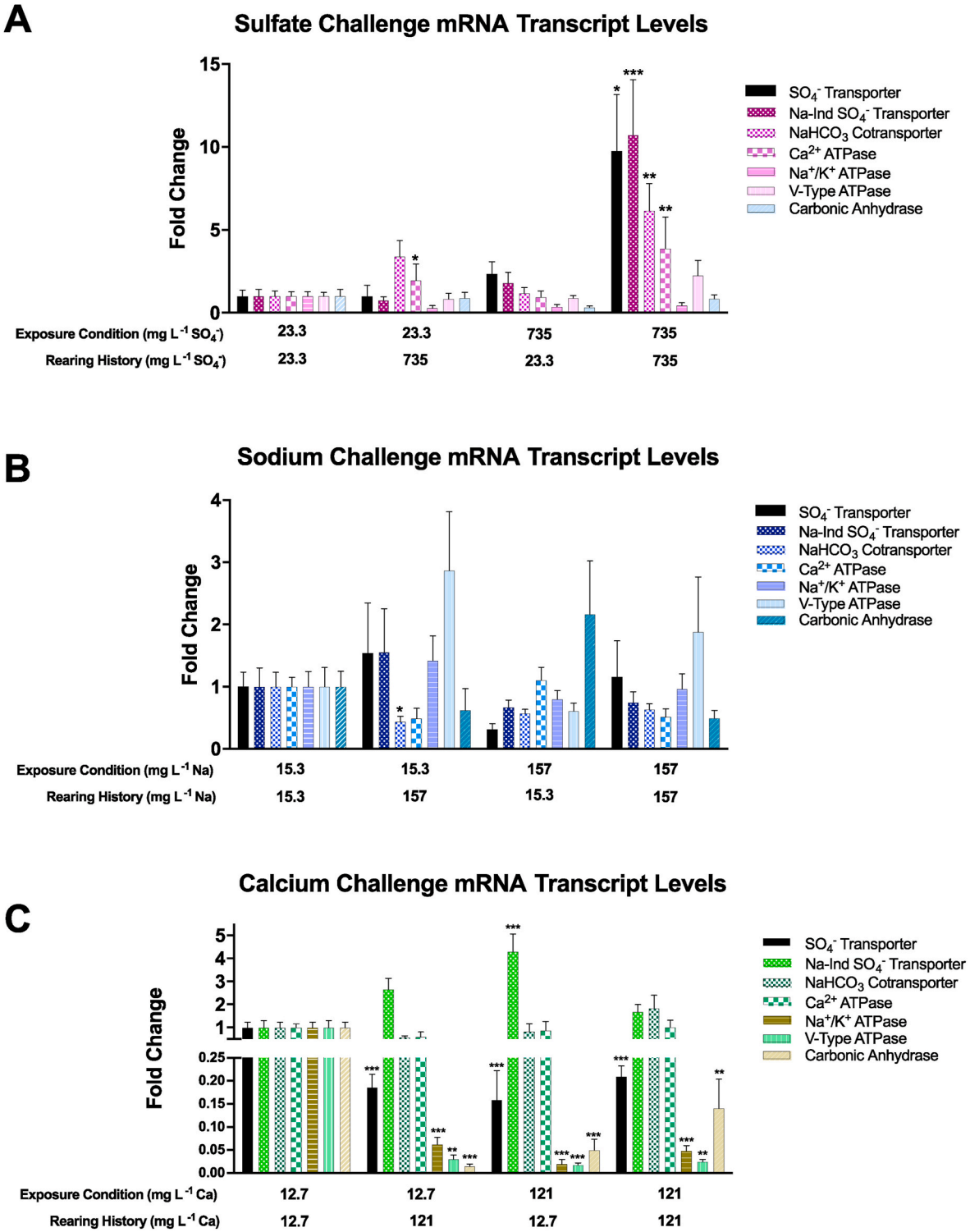

Fig. 2. mRNA transcript levels in *N. triangulifer* exposed to sulfate (A), sodium (B), or calcium (C). Errors bars represent standard error from the mean ($n = 5$). A one-way ANOVA with Tukey's multiple comparisons was performed and asterisks indicate statistically significant differences from the control group (* indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$).

μg SO_4 g^{-1} hr^{-1}, but increased sulfate uptake to 272.1 ± 8.1 μg SO_4 g^{-1} hr^{-1} when placed in elevated sulfate (735 mg L^{-1} SO_4) ($p < 0.0001$) (Fig. 1A). Similarly, control mayflies (16 mg L^{-1} Na) had a sodium uptake rate of 57.4 ± 1.0 μg Na g^{-1} hr^{-1}, but increased sodium uptake to 144.9 ± 7.9 μg Na g^{-1} hr^{-1} in elevated sodium (157 mg L^{-1} Na) ($p < 0.0001$) (Fig. 1B). Interestingly, calcium uptake rates changed minimally between treatment waters. Control mayflies (12.7 mg L^{-1} Ca) had mean calcium uptake rates of 5.934 ± 2.8 μg Ca g^{-1} hr^{-1} in control water and 8.1 ± 1.7 μg Ca g^{-1} hr^{-1} in elevated calcium (121 mg L^{-1} Ca) waters ($p = 0.21$) (Fig. 1C).

Previous exposure history generally decreased ion uptake rates within each exposure water (control or elevated concentration). Control mayflies exposed to elevated sulfate (735 mg L^{-1} SO_4) waters had a sulfate uptake rate of 272.1 ± 8.1 μg SO_4 g^{-1} hr^{-1}, but mayflies that were reared in that elevated sulfate concentration had 8-fold slower sulfate uptake rate at 30.1 ± 36.8 μg SO_4 g^{-1} hr^{-1} ($p < 0.0001$). Similarly, pre-exposed mayflies reduced their sulfate uptake rate by 1.7-fold from 2.4 ± 0.1 μg SO_4 g^{-1} hr^{-1} to 1.3 ± 1.3 μg SO_4 g^{-1} hr^{-1}, but this change was not statistically significant ($p = 0.54$) (Fig. 1A).

We observed similar patterns in larvae reared under elevated sodium conditions (157 mg L^{-1} Na). Mayflies that were naïve to this salinity challenge had a sodium uptake rate of 144.9 ± 7.9 μg Na g^{-1} hr^{-1}, but pre-exposed mayflies reduced their sodium uptake rate 2-fold to 73.0 ± 7.0 μg Na g^{-1} hr^{-1} ($p < 0.0001$). Pre-exposed mayflies also reduced their sodium uptake rate 1.5-fold in control waters to 39.7 ± 5.5 μg Na g^{-1} hr^{-1} compared to control counterparts with a sodium uptake rate of 57.4 ± 1.0 μg Na g^{-1} hr^{-1} ($p < 0.01$) (Fig. 1B). Mayflies presented with an elevated calcium challenge had little differences in calcium uptake rate between rearing histories ($p = 0.10$). However, pre-exposed mayflies were able to reduce their calcium uptake rate 2.7-fold in control water to 2.2 ± 0.3 μg Ca g^{-1} hr^{-1} compared to 5.9 ± 2.8 μg Ca g^{-1} hr^{-1} of naïve mayflies ($p < 0.0001$) (Fig. 1C).

3.2. Gene expression

We assessed changes in relative mRNA expression of 6 genes of interest across all four treatment combinations for each major ion of interest (Fig. 2). Control-reared mayflies that were placed into elevated sulfate (735 mg L^{-1}) water had no statistically significant changes in expression in any of the selected genes relative to controls. However, mayflies that had been reared in elevated sulfate and were subsequently exposed to control water for 8 h, experienced a 1.9-fold upregulation in the Ca^{2+} ATPase gene ($p < 0.05$). Mayflies that had been reared in and exposed to elevated sulfate had the largest response with significant upregulation in SO_4^- Transporter (9.8-fold, $p < 0.05$), Na-independent SO_4^- Transporter (10.7-fold, $p < 0.001$), $NaHCO_3$ Cotransporter (6.1-fold, $p < 0.01$), and Ca^{2+} ATPase (3.9-fold, $p < 0.01$) (Fig. 2A).

Sodium challenge had seemingly little effect on mRNA transcript levels; only one gene in a single treatment group was significantly changed. Mayflies that had elevated sodium (157 mg L^{-1} Na) rearing histories and were subsequently exposed to control water significantly reduced the expression of $NaHCO_3$ Cotransporter to 0.4-fold ($p < 0.05$) (Fig. 2B).

Finally, we examined gene expression changes in mayflies that had experienced calcium challenge. Control-reared mayflies that were exposed to elevated calcium (121 mg L^{-1} Ca) had a significant 4.3-fold upregulation in the Na-independent SO_4 Transporter gene ($p < 0.001$). Similarly, pre-exposed mayflies that were exposed to control water also had a 2.7-fold upregulation in this gene ($p < 0.05$). Most interestingly, all treatment groups with any amount of elevated calcium exposure had a significant downregulation in SO_4 Transporter ($p < 0.001$), Na/K ATPase ($p < 0.001$), V-Type ATPase ($p < 0.01$), and Carbonic Anhydrase ($p < 0.01$) (Fig. 2C).

3.3. Toxicity bioassays

To evaluate whether any of the physiological or molecular acclimatory responses described above could influence survival we performed 96-h toxicity bioassays for each major ion of interest (sulfate, sodium, and calcium) (Fig. 3). Mayfly larvae that had been acclimated to elevated sulfate, performed worse than their control counterparts (Fig. 3A). Control mayflies had no significant changes in survival at any concentration of sulfate, however, pre-exposed mayflies experienced mortalities at the three highest concentrations tested (735, 1050, and 1500 mg L^{-1} SO_4). The most challenging concentration, 1500 mg L^{-1}, only had a 33% survival rate in acclimated mayfly group, compared to the near perfect survival of control mayflies in the same treatment group ($p < 0.01$). Because of these results, we calculated an acute LC_{50} of 1939 mg L^{-1} SO_4 for only the pre-treated group.

In contrast, larvae with previous exposure history to elevated Na unaffected by even extremely high Na exposures (up to 1500 mg L^{-1}). Control larvae experienced significant mortalities in the three highest concentrations of sodium: 73% survival in 387 mg L^{-1} Na ($p < 0.05$), 63% survival in 535 mg L^{-1} Na ($p < 0.001$), and 67% survival in 743 mg L^{-1} Na ($p < 0.001$) (Fig. 3B). Control-reared mayflies had an acute LC_{50} of 1169 mg L^{-1} Na.

Finally, pre-exposure to elevated calcium resulted in poor performance at all concentrations tested excluding the control (15 mg L^{-1}) (Fig. 3C). The control reared mayflies performed better than the Ca-pre-exposed mayflies, at the two mid-range concentrations (480 and 696 mg L^{-1} Ca). Larvae with a history of previous exposure only had a 17% and 27% survival at these two concentrations, respectively. For 980 and 1400 mg L^{-1} Ca treatment groups, both acclimated and control larvae survived poorly and in the highest concentrations (2000 mg L^{-1} Ca), not a single larva from either group survived. An acute LC_{50} value for the control-reared group was calculated to be 901 mg L^{-1} Ca.

3.4. Microscopic pathology

The histological results were similar in the different groups: control, sodium, and sulfate treated groups. Microscopic lesions were not detected in any of the assessed tissues including the cephalic, or organs within the thoracic and the abdominal cavities. A comparison of the control and treated mayfly larvae did not provide any evidence of toxic lesions by light microscopy. Interestingly, within all samples from the calcium treated group, the epithelial cells of the Malpighian tubules had multifocally acute cellular degeneration and/or necrosis (Fig. 4). Acute cellular degeneration (hydropic degeneration) was characterized by the evidence of cellular swelling, rounded edges, and feathery to vacuolated cytoplasm. Necrotic cells showed fragmented cytoplasm with associated fade basophilic nuclei (karyolysis) or nuclear fragmentation (karyorrhexis).

4. Discussion

To our knowledge, the question of how ionic exposure history affects physiological processes and/or toxicological outcomes has been unexplored in aquatic insects. This question is important because these organisms are used routinely to evaluate ecological conditions in nature (Cormier et al., 2013a; Hawkins, 2006; Hawkins et al., 2010; Johnson et al., 1993) where they are typically exposed over generations. In contrast, toxicity evaluations are almost always performed with naïve animals, and data for relevant insect surrogate species remain relatively scarce (Buchwalter et al., 2019; Hassell et al., 2006; Johnson et al., 2015; Scheibener et al., 2017, 2016; Soucek and Dickinson, 2015; Soucek et al., 2018). Here, we take a first step in assessing how exposure history influences physiological and organismal performance in a relevant insect model, *N. triangulifer* (Buchwalter et al., 2019; Chou et al., 2020, 2018; 2017; Funk et al., 2006; Kim et al., 2017; Orr and Buchwalter, 2020; Soucek et al., 2018; Soucek and Dickinson, 2015; Sweeney

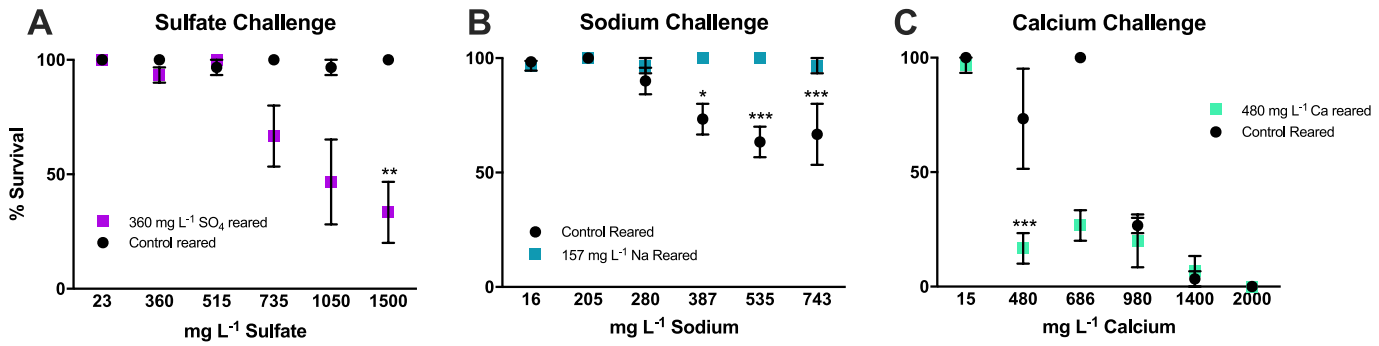

Fig. 3. Survival of *N. triangulifer* in 96-h toxicity bioassays to examine the effects of previous exposure to sulfate (A), sodium (B), or calcium (C). Errors bars represent standard error from the mean ($n = 3$). A one-way ANOVA with Tukey's multiple comparisons was performed and asterisks indicate statistically significant differences of % survival between the control reared or pre-exposed mayfly larvae (* indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$).

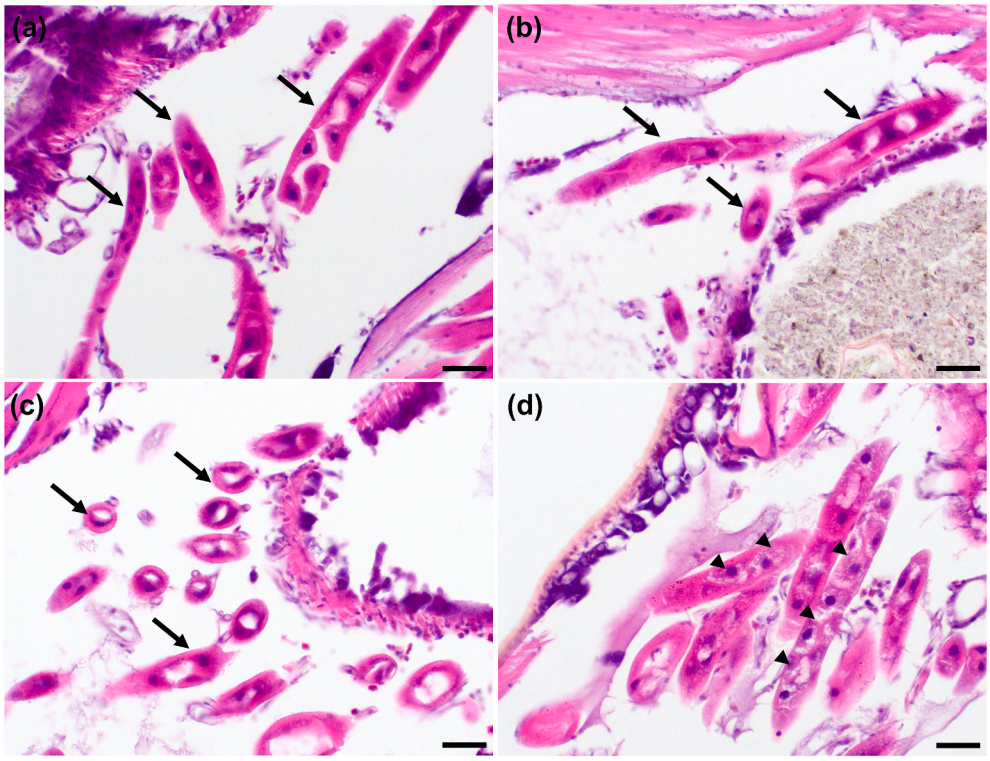

Fig. 4. Sagittal sections of *N. triangulifer* larvae. Hematoxylin and eosin (HE), bar = 20 μm. The cellular epithelium of the Malpighian tubules from the control (a), sodium-treated (b), and sulfate-treated (c) groups is within normal limits (arrow). The small and luminal tubules are lined by a simple cuboidal epithelial on a thin basement membrane with lack of muscular layer. The Malpighian tubules (arrowhead) in the calcium-treated group (d) are multifocally undergoing cellular degeneration and necrosis.

and Vannote, 1984) in the context of three different major ions: sodium, sulfate, and calcium. For the first time, we demonstrate that exposure history to salinity can significantly influence ion transport rates in a mayfly.

4.1. Ion flux experiments

Our data show that mayfly larvae that had been reared in an elevated major ion concentration generally decreased their uptake rates compared to naïvely exposed counterparts. The marked reduction we observed in both sulfate and sodium transport rates seems to be an appropriate acclimatory response. We previously observed that exposure to elevated sulfate delayed development (Buchwalter et al., 2019), and we speculated that the energetic cost of maintaining hemolymph homeostasis was responsible for developmental delays and toxicity. Other researchers have also observed similar developmental delays (Johnson et al., 2015; Soucek and Dickinson, 2015). Thus, a reduction in flux rates would serve to minimize the energy costs of maintenance. Interestingly, calcium uptake rates are remarkably slow in this species (Orr and Buchwalter, 2020) as they are for other aquatic insects (Poteat et al., 2012; Poteat and Buchwalter, 2014), relative to other ions. Even when concentrations are raised considerably, *N. triangulifer* maintains very slow uptake of calcium. Under control exposure conditions, the elevated calcium reared larvae clearly down-regulated uptake, but a high degree of interindividual variability under that higher concentrations precluded the finding of statistical significance in this comparison. Nevertheless, the apparent reduction of calcium influx rates also appears to be a response to calcium challenge.

While literature is relatively sparse for salinity acclimation ion flux studies, our findings are consistent with other aquatic taxa. One study found that freshwater mussels (*Toxolasma texasensis*) acclimated to 10% seawater significantly reduced renal sulfate reabsorption compared to controls that conserved sulfate (Dietz et al., 2000). Similarly, other researchers found that calcium uptake was reduced significantly in rainbow trout that had been acclimated to waters amended by Ca $(NO_3)_2$) for 50 days both *in vivo* and *in vitro* (Perry and Wood, 1985). Shorter acclimation periods (3 d) to elevated calcium in tilapia larvae also reduced calcium uptake rates compared to larvae reared in low

calcium (Hwang et al., 1996; Lin et al., 2016). Although our results are in support of these studies, it is important to note that calcium may play different roles in freshwater vertebrates that rely heavily on the ion for maintaining healthy bony skeletons.

4.2. Gene expression

At the gene expression level, we observed some stark differences in between pre-exposed and naïve larvae. For example, we observed that the pre-exposed group left in elevated sulfate dramatically upregulated the mRNA expression of two sulfate transporting genes, likely in tissues associated with efflux, such as the Malpighian tubules. We also observed unexpected crosstalk with other ion transporters, $NaHCO_3$ cotransporter and Ca ATPase. In contrast, larvae reared under elevated sodium appeared to have a stimulation of V-Type ATPase expression but a high degree of variability in those data impedes us from making definitive conclusions. Finally, calcium pre-exposure generally decreased expression of several genes. This could be due to an attempt to reduce uptake, or alternatively, could result from a forfeiture of gene regulation because of the severe toxicity of calcium observed in our toxicity bioassays. We were somewhat handicapped in this line of inquiry by the very small size of *N. triangulifer* larvae, and our lack of understanding of the tissue specific distributions of these genes of interest. Thus, this "whole organism" level of expression represents an exploratory effort that we can hopefully refine in the future.

Few studies have analyzed gene expression changes of mayflies in response to salinity stress. Our lab has previously demonstrated a slight decrease in the mRNA expression of a sulfate transporter and a Na-independent sulfate transporter in *N. triangulifer* in response to 24-h of sulfate stress (Buchwalter et al., 2019). However, the majority of literature on aquatic insect salinity-induced gene expression focused on mosquitoes due to their importance as a human disease vector. Several studies report changes in *A. aegypti* mRNA expression in response to salinity stress including genes such as P-type Na/K ATPase and V-type H-ATPase (Patrick et al., 2006), sodium-hydrogen antiporter 3 (Durant and Donini, 2019), aquaporins (Akhter et al., 2017; Misyura et al., 2020), and snakeskin and mesh homeostasis-related genes (Jonusaite et al., 2016). In addition, RNA-Seq experiments in *Anopheles coluzzii* and *Anopheles merus* larvae have indicated significant changes in the mRNA expression of AgJNKa (MAPK gene) and AgAE2 (ion transport candidate gene) in response to seawater exposure (Uyhelji et al., 2016). Studies for osmoregulatory gene expression in response to elevated salinity have been conducted in other aquatic taxa such as Palaemonid prawns (Rahi et al., 2020), Hawaiian Anchialine shrimp (Havird et al., 2014), Chinese mitten crab (Yang et al., 2019), alewife fish (Velotta et al., 2017), among others (Havird et al., 2013; Rahi et al., 2018). Unfortunately, most of the aforementioned studies were only performed using seawater and thus, ion-specific conclusions cannot be drawn.

4.3. Toxicity bioassays

To characterize the survival and acute toxicity of major ions, we performed toxicity bioassays with a range of relevant concentrations chosen based on both prior work and environmental values. Mayflies that were reared in moderately elevated salinities performed better in sodium challenge, but worse in sulfate challenge. This surprising finding may reveal why aquatic insects are disappearing in nature, especially in sites that have particularly high levels of sulfate, such as West Virginia streams (Cormier et al., 2013a; Pond et al., 2008a). Due to the extremely slow uptake of calcium, we chose a higher concentration for the acclimation period before the calcium bioassays. However, the calcium exposures were significantly more harmful than the other salt exposures and the larvae appeared visibly less healthy than the larvae acclimated to sodium or sulfate. Notably, the "acclimatory" conditions were moderately elevated salinities and it is possible that chronic exposure to lower concentrations may be more beneficial. We acknowledge that our choice of higher calcium concentrations may require future investigations to determine where thresholds occur.

We find our toxicity data to be relatively consistent with other labs. In the present study, acute LC_{50} values of control-reared mayflies were calculated for sodium (1169 mg L^{-1} Na) and calcium (901 mg L^{-1} Ca). For sulfate, however, only pre-exposed animals were used to calculate the LC_{50} value (1939 mg L^{-1} SO_4), which may be more reflective of natural exposures, but less comparable to other naïve laboratory exposures. Similarly, other groups have calculated LC_{50}s for Cl (1062 mg L^{-1}), SO_4 (1227 mg L^{-1}) (Soucek and Dickinson, 2015), and NaCl (2755 mg L^{-1}) (equivalent to 1084 mg L^{-1} Na) (Jackson and Funk, 2019). Toxicity data such as these are essential for establishing ion-specific environmental regulations and protecting freshwater ecosystems.

4.4. Microscopic pathology

There has been limited work to examine tissue morphological changes caused by increased salinity in aquatic insects and scarce publications regarding the normal anatomy and histology of mayfly larvae. We hypothesized that increases in salinity would alter the tissue morphology of important osmoregulatory organs and the primary excretory system (e.g., gills and/or Malpighian tubules). Insects rely on Malpighian tubules for osmoregulatory homeostasis and urine formation, which may be impacted by elevated major ion exposures (Larsen et al., 2014). Through a blind approach, we observed that only calcium-treated mayflies had significant cellular degeneration and necrosis of the Malpighian tubules. We recognize that our calcium concentration was relatively high compared to most natural waters (Cormier et al., 2013a), but our microscopic pathology concentration choices were chosen for mechanistic exploratory purposes. Due to the particularly low calcium uptake rates, we were surprised by the severity of histopathological effects detected. These observations led us to consider alternate mechanisms of toxicity for calcium, specifically. Salinity-induced histological changes have been demonstrated in other species of aquatic organisms including yellow perch (Nero et al., 2006), snow trout (Shahriari Moghadam et al., 2018), and sea bass (Hamedi et al., 2016), but none of these studies specifically examined the effects of calcium. Furthermore, histopathological studies have been performed in mayflies (Liarte et al., 2014), but did not directly correlate with salinity-induced changes as seen herein.

4.5. Ion-specific mechanisms of toxicity

Our study specifically examined three major ions: sodium, sulfate, and calcium, but ions cannot be studied in complete isolation. Rather, we used NaCl, $CaSO_4$/$MgSO_4$, or $CaCl_2$ to manipulate our water chemistries experimentally. Our sulfate exposures were balanced with two cations, calcium and magnesium, to minimize cation influence. Although our waters elevated with sodium and calcium had the same anion, chloride, our acute toxicity values were dramatically different. In fact, our sodium elevated waters with chloride concentrations ranging from 255 to 1165 mg L^{-1}, were significantly less toxic than our calcium elevated waters with 97–525 mg L^{-1} Cl (Table 1). These results may suggest that the cations of interest, rather than chloride, influenced our observed results in mRNA expression, ion flux rates, Malpighian tubules hydropic degeneration and necrosis, and acute toxicity.

There are many hypotheses regarding major ion toxicity in sensitive aquatic insects (Kefford, 2018), but data produced from our lab has largely supported the energy depletion theory (Buchwalter et al., 2019). The cost of active ion transport across epithelia makes up a large portion of an aquatic insect's energy budget, but is exacerbated by salinity stress (see Verberk et al., 2020). However, the uniformly low uptake of calcium and extreme toxicity provokes us to reconsider the mechanism of toxicity for this ion. Like most major ions, calcium is essential to numerous biological functions, and low concentrations are often reported to be especially dangerous for aquatic life (Dam et al., 2010). One

group found that calcium stimulates sodium uptake in *D. magna* (Glover and Wood, 2005). Could the presence of calcium increase the uptake and consequential toxicity of other ions present (e.g., Cl)? Although there is little evidence for dysregulation in mayflies (Dowse et al., 2017), other groups suggest that hemolymph acidosis may be the root of salt stress in mosquito larvae (Durant and Donini, 2019). Recently, Wood and colleagues found that changes in trans-epithelial potential across the gills correlate with major ion toxicity in several species of fishes (Po and Wood, 2021; Wood et al., 2020). These authors speculate that the mechanism of toxicity may be related to metabolic acidosis. We suggest the mechanisms of toxicity for salinity may differ between ions and taxa.

5. Conclusions

Historically, ecological assessments are often performed with conductivity as the surrogate descriptor of the salinity. However, it is now known that the elevations of major ions differ between environmental scenarios (Cormier et al., 2013c) and that these ions affect organisms differently (Clements and Kotalik, 2016; Griffith, 2017b; Kunz et al., 2013). Indeed, in the present study we show significant evidence for ion-specific effects. In summary, our data suggests that the history of previous exposure to elevated ions in mayflies reduces ion uptake rates. Further, we found ion-specific changes in gene expression, tissue morphology, and survival of more challenging concentrations of major ions. Our results support the idea of acclimation to elevated sodium, but not to sulfate or calcium in *N. triangulifer* larvae. We believe that elevated sulfate imposes steep energetic consequences on mayflies, while calcium may have a completely different mechanism of toxicity. Understanding the osmoregulatory physiology of important aquatic insects, such as mayflies, is an essential step to improve regulatory efforts and protect our freshwater ecosystems.

Author statement

SEO and DBB conceived the concepts of the work. SEO conducted the research and wrote the manuscript. TTNW provided histopathological expertise. DBB provided research oversight and editorial assistance of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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