HEMATOLOGY, PLASMA BIOCHEMISTRY, PROTEIN ELECTROPHORESIS, AND PATHOGEN SURVEILLANCE IN HEADSTARTED AND WILD-REARED POPULATIONS OF BLANDING'S TURTLES (EMYDOIDEA BLANDINGII) IN THREE NORTHERN ILLINOIS, USA, COUNTIES

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Hematology, Plasma Biochemistry, Protein Electrophoresis, and Pathogen Surveillance in Headstarted and Wild-Reared Populations of Blanding's Turtles (*Emydoidea blandingii*) in Three Northern Illinois, USA, Counties

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ABSTRACT: Blanding's turtles (*Emydoidea blandingii*) are a species of conservation concern throughout their natural range. Headstarting is a common chelonian conservation technique in which neonates are reared in managed-care settings before release, but health assessments are rarely incorporated. From 2020 to 2021 we assessed headstarted turtle health pre-release and 1 mo, 1 yr, and 2 yr after release using physical examination, hematology, plasma biochemistry, protein electrophoresis, and pathogen detection in three Illinois counties. Results were compared to wild-reared juveniles in the same habitats. Overall, 767 assessments from 561 turtles were included. Wild-reared and 2 yr post-release headstarts had higher incidence of hemoparasites, asymmetrical nares, and increased creatine kinase and aspartate aminotransferase activities ($P<0.05$) compared to all other groups. Erythrocyte sedimentation rate and heterophil:lymphocyte ratio were greater, while total leukocyte and lymphocyte counts were lower ($P<0.05$) in pre-release headstarts compared to wild-reared juveniles. Total solids, albumin, and beta globulins were higher, while the calcium:phosphorous ratio was lower ($P<0.05$) in pre-release headstarts and wild-reared juveniles vs. other groups. Bile acid levels were highest in pre-release headstarts ($P<0.05$). Body condition and gamma globulins increased following release, while alpha globulins and the albumin:globulin ratio decreased following release ($P<0.05$). Two pre-release and one post-release headstart tested positive for *Emydomyces testavorans*, one post-release headstart was positive for *Mycoplasmopsis* sp., and nine post-release turtles were positive for adenoviruses. Overall, rearing conditions have a profound and temporally dynamic impact on Blanding's health assessment parameters. Future studies should evaluate long-term impacts on morbidity and mortality to support positive health status and conservation outcomes.

Key words: Biochemistry, Blanding's turtles, *Emydoidea blandingii*, headstart, hematology, pathogen surveillance, protein electrophoresis.

INTRODUCTION

With more than half of chelonian species threatened with extinction (Rhodin et al. 2018), effective conservation efforts are urgently needed for this taxon. Chelonians are long-lived with delayed sexual maturity and high egg and juvenile mortality; thus conservation efforts need to consider years to decades in the future (Gibbons 1987; Congdon et al. 1993; Spencer 2002). Supplementing declining populations by translocation or release of captive-reared animals is a common intervention in several taxa, including chelonians (Burke 2015). Headstarting, an approach in which neonates are reared in managed-care settings before release, is the most common method of supplementing chelonian populations because of their oviparous reproductive strategy, negligible parental care, predictable and identifiable nesting sites, and straightforward neonatal husbandry (IUCN-SSC 2013; Burke 2015). This technique has been used to successfully bolster the populations of many turtle species, including green sea turtles (*Chelonia mydas*; Bell et al. 2005), Kemp's Ridley sea turtles (*Lepidochelys kempii*; Shaver 1996), hawksbill sea turtles (*Eretomchelys imbricata*; Okuyama et al. 2010), redbelly turtles (*Pseudemys rubriventris*; Haskell et al. 1996), northern diamondback terrapins (*Malaclemys terrapin terrapin*; Wood and Herlands 1997), wood turtles (*Glyptemys insculpta*; Mullin et al. 2020), and western pond turtles (*Actinemys marmorata*; Vander Haegen et al. 2009).

Complete health assessments on headstarts of any species following release are very rare; published studies include limited assessments of Blanding's turtles (*Emydoidea blandingii*) in Illinois, US (Cann et al. 2021) and common loons (*Gavia immer*) in Massachusetts, US (Kneeland 2020). Neither of these studies investigated the presence of pathogens prior to or after release in relation to changes in physiological parameters. One of the most frequently published concerns with headstarting is that release of previously captive individuals into the wild has the potential for concurrent release of dangerous pathogens (Dodd and Seigel 1991; Jacobson 1993; Smith 2015); while disease surveillance efforts are recommended to mitigate this risk, they are underemployed.

Blanding's turtles are medium-sized freshwater turtles native to the North American Great Lakes region and the northeastern US. These turtles are endangered or threatened throughout their natural range (van Dijk and Rhodin 2011) and are frequently targeted for conservation activities. This long-lived species has been reported to reach more than 75 yr old, with greater survivorship and reproductive frequency in older individuals (Congdon et al. 2001). In Illinois, Blanding's turtle conservation initiatives include mesopredator removal, habitat restoration, and headstarting programs initiated in 1996 by the Forest Preserve District of DuPage County (FPDDC), in 2006 by the Lake County Forest Preserve District (LCFPD), and in 2020 by the Forest Preserve District of Kane County (FPDKC; Glowacki and Kuhns 2010; Thompson et al. 2020). These programs have increased Blanding's turtle population size and viability and have incorporated health assessments and pathogen surveillance for wild populations, dramatically increasing knowledge about Blanding's turtle wellness (Lindemann et al. 2018, 2019; Newman et al. 2019; Mumm et al. 2019; Thompson et al. 2020; Winter et al. 2020; Andersson et al. 2021a, b; King et al. 2021; Sander et al. 2021; Golba et al. 2022). However, the health and disease status of the headstart population in Illinois is currently uncharacterized.

We aimed to fill knowledge gaps for Blanding's turtle conservation projects in Illinois by broadly assessing numerous components of health in headstarted turtles. Our objectives were to compare physical examination findings, hematology, plasma biochemistry, protein electrophoresis, and pathogen prevalence of pre-release headstarts, post-release headstarts, and wild juveniles. We hypothesized that health status would decline immediately post-release (Carstairs et al. 2019; Cann et al. 2021) but would rebound over time to equal or surpass the health status of wild juveniles.

MATERIALS AND METHODS

Fieldwork and animal sampling

Live animal use was approved through the University of Illinois IACUC (Protocols 18000 and 20258) and the Northern Illinois University IACUC (LA 16-0015). Scientific collecting permits were obtained from the Illinois Department of Natural Resources (HSCP 19-14, HSCP 19-46, 6828, 6939, 6941, 7281, 7421, 10906, 10955, 12098, and 13258).

Sampling was performed from June to August 2020 and May to July 2021. Pre-release headstarted turtles were sampled at an indoor rearing facility in Lake County, Illinois, and were subsequently released in their county of origin (Lake, Kane, or DuPage County, Illinois). Husbandry protocols, individual identification methods such as marginal scute notching and passive integrated transponder (PIT) tagging, and release criteria have been previously published (Thompson et al. 2020). Transmitters (SOPR 2038, 2070, and 2190, Wildlife Materials International Inc, Murphysboro, Illinois, USA) were glued to the carapace (using J-B Weld KwikWeld epoxy or J-B Weld

SteelStik epoxy putty, J-B Weld Company, Marietta, Georgia, USA) of a subset of turtles (n=175). The mass of the transmitters and epoxy was less than 8% of each turtle's mass. Headstarted turtles were released across the three counties as part of a separate biological, movement, and outcome study. Released and wild turtles were captured with the aid of radiotelemetry, baited hoop and minnow or crawfish traps, or incidentally by hand during fieldwork. All methods were used at all field sites.

Post-release turtles were sampled from two LCFPD sites, four FPDKC sites, and two FPDDC sites. Some sites have been previously described (Thompson et al. 2020; Golba et al. 2022); additional site descriptions are available upon request based on sensitivity of site information in this species. Turtles captured in the field were identified via a county-specific marginal scute notch code. Those with no marginal scute notches and no PIT tag were assumed to be wild-born and were assigned an individualized notch code and implanted with a PIT tag for permanent identification following established protocols (Cagle 1939; Buhlmann and Tuberville 1998). Post-release turtles were not sampled until at least 1 mo following release.

Physical examination and sample collection

Straight carapace length (SCL), shell height, carapace width, plastron length, and mass were recorded. Body condition index (BCI) was calculated using mass and SCL based on a previous study (Newman et al. 2019). Physical examinations were performed, noting visual appearance of the eyes, nares, oral cavity, tympanic membranes, appendages, digits, shell, integument, and cloaca. Each individual was characterized as juvenile (<250 g) or subadult (250–750 g) (Mumm et al. 2019). Sex of headstarted turtles was known for most individuals, as it was predetermined by the temperature set during incubation (Gutzke and Packard 1987). Sex of some subadults and juveniles could be determined based on position of cloacal opening, with cloacal openings caudal to the plastron indicative of males (Mosimann and Bider 1960). The sex was classified as unknown when a definitive determination could not be made.

Whole blood (maximum 0.8% body weight) was collected from the subcarapacial sinus via a 25-gauge needle, placed into lithium-heparinized (LH) collection tubes (BD microtainer, Becton, Dickinson and Co., Franklin Lakes, New Jersey, USA) and stored on ice in a cooler until analysis. Cotton tip applicators were used to swab the oropharynx, choana, and cloaca for pathogen surveillance.

Clinical pathology

White blood cell (WBC) counts, packed cell volume (PCV), total solids (TS), erythrocyte sedimentation rate (ESR), and fixed and stained blood smears were performed within 8 h of sample collection. We performed PCV and TS analysis using sodium heparinized hematocrit tubes (Jorgensen Laboratories, Inc., Loveland, Colorado, USA). Each hematocrit tube was centrifuged (2,910 × G for 5 min) and the PCV recorded. Total solids were determined with a hand-held refractometer (Amscope RHC-200ATC refractometer, National Industry Supply, Torrance, California, USA) using plasma from the hematocrit tubes, and ESR was performed using hematocrit tubes as described (Adamovicz et al. 2020). The WBC count was determined using avian leukopets (Vet Lab Supply, Palmetto Bay, Florida, USA) and Bright-line hemacytometers (Hausser Scientific, Horsham, Pennsylvania, USA) following the manufacturers' protocols. Fresh blood films were stained with a modified Wright's Giemsa stain, and 100 WBC differential counts were performed by a single observer (L.A.).

Plasma was obtained via centrifugation of LH blood samples at 4,180 × G for 10 min and frozen at −80 C for up to 4 mo before analysis. Plasma biochemistry profiles, including bile acids, calcium, phosphorous, uric acid, phosphorous, aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), and creatine kinase (CK), were performed using a commercial benchtop analyzer (AU680 Chemistry System, Beckman Coulter, Brea, California, USA) at the University of Illinois Veterinary Diagnostic Laboratory, Urbana, Illinois, US. Plasma protein electrophoresis was performed according to the procedure provided by the Helena SPIFE 3000 system with the use of Split Beta gels (Helena Laboratories, Inc., Beaumont, Texas, USA) at the University of Miami Miller Avian & Wildlife Laboratory, Miami, Florida, US. Protein fraction delineation was performed as previously described for Blanding's turtles (Andersson et al. 2021a).

Pathogen surveillance

We extracted DNA from oral and cloacal swabs using Qiagen DNA Blood mini kits (Qiagen, Valencia,

TABLE 1. Pathogens and copathogens tested in headstarted and wild Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA, and sources for quantitative PCR (qPCR) and PCR primers.

Pathogen	Source
FV3–ranavirus qPCR	Allender et al. (2013)
Ambystoma tigrinum virus–ranavirus qPCR	Picco et al. (2007)
Bohle iridovirus–ranavirus qPCR	Pallister et al. (2007)
Epizootic hematopoietic necrosis virus–ranavirus qPCR	Pallister et al. (2007)
Pan-ranavirus qPCR	Stillwell et al. (2018)
Mycoplasmopsis agassizii qPCR	Braun et al. (2014)
Mycoplasmopsis testudineum qPCR	Braun et al. (2014)
Box turtle *Mycoplasmopsis* sp. qPCR	In-house
Salmonella tymphimurium qPCR	Levin (2009)
Salmonella enteritidis qPCR	Malorny et al. (2007)
Intranuclear coccidia of Testudines qPCR	Alvarez et al. (2013)
Human-pathogenic *Leptospira* spp. qPCR	Smythe et al. (2002)
Coxiella *hurnetti* IS1111 qPCR	Klee et al. (2006a)
Coxiella *hurnetti* ICD qPCR	Klee et al. (2006b)
Testudinid herpesvirus 2 qPCR	Braun et al. (2014)
Emydid herpesvirus 1 qPCR	In house
Emydoidea blandingii herpesvirus 1 qPCR	Lindemann et al. (2018)
Emydomyces testavorans qPCR	Woodburn et al. (2019)
Consensus adenovirus	Wellehan et al. (2004)
Consensus *Mycoplasmopsis* sp.	Ossiboff et al. (2015)

California, USA), according to the manufacturer's protocol. The DNA concentration and purity was assessed using spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and DNA samples were stored at −20 C before PCR and quantitative PCR (qPCR) pathogen surveillance. Conventional PCR assays were run according to published protocols for adenoviruses and *Mycoplasmopsis* spp., inclusive of positive and negative controls (Wellehan et al. 2004; Ossiboff et al. 2015). Products were resolved on a 1% agarose gel. Samples producing bands of the appropriate size were treated with ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) and commercially sequenced in both directions. Quantitative PCR was performed using a Fluidigm platform to test for 16 pathogens using published or in-house TaqMan primer-probe assays (Table 1), as described previously (Archer et al. 2017). Following Fluidigm analysis, all positive samples were verified in a simplex reaction. Briefly, qPCR was performed using three technical replicates for each sample, negative control, and standard curve dilution (10^1–10^7 target copies) on a QuantStudio3 real-time thermocycler (Applied Biosystems, Carlsbad, California, USA) with associated software (QuantStudio Design and Analysis Software version 1.5.2, Applied Biosystems). Samples were considered positive if all three replicates had a lower cycle threshold (Ct) value than the lowest detected standard dilution.

Statistical analysis

Statistical testing was performed in R version 4.0.2 at an α value of 0.05 (R Core Team 2020). Data distributions were assessed for normality using histograms and Shapiro-Wilk tests, and transformation was performed to meet modeling assumptions, if necessary.

Modeling was performed to identify differences in physical examination abnormalities, pathogens, and clinical pathology values based on rearing history. To facilitate this, a "turtle group" variable was created, and each assessment was classified as "pre-release headstart," "same-year post-release headstart," "1yr post-release headstart," "2 yr post-release headstart" (this group includes turtles sampled 2 yr or longer after release), or "wild-reared."

Continuous response variables (clinical pathology parameters, mass, BCI) were modeled using general linear mixed models (lme4 and lmerTest packages; Bates et al. 2015; Kuznetsova et al. 2017). Categorical response variables (presence or absence

TABLE 2. Population demographics, common physical examination abnormalities, and diagnostic testing for wild and headstarted Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA.

		Rearing group				
Parameter	Group	Pre-release headstart	Same-year post-release headstart	1 yr post-release headstart	2 yr post-release headstart	Wild juvenile
Age class	Juvenile	312	138	72	42	42
	Subadult	8	7	9	95	42
Sex	Male	133	59	46	86	20
	Female	129	55	34	51	45
	Unknown	58	31	1	0	19
Ectoparasites	Present	0	33	31	48	29
	Absent	320	112	50	89	55
Upper respiratory disease	Present	2	5	2	4	0
	Absent	318	140	79	133	84
Appendages	Normal	284	129	69	128	80
	Abnormal	36	16	11	19	4
Shell	Normal	69	22	36	25	15
	Abnormal	251	123	44	108	69
Integument	Normal	315	143	77	129	81
	Abnormal	5	2	4	8	3
Musculoskeletal	Normal	288	130	71	137	78
	Abnormal	32	15	10	0	6
Nares	Symmetrical	305	124	69	106	73
	Asymmetrical	15	21	12	31	11
No. hematologic assessments		176	129	53	78	50
No. erythrocyte sedimentation rate tests		302	141	75	133	78
No. plasma biochemistry panels		62	49	32	71	46
No. plasma protein electrophoresis panels		84	73	46	49	28
No. pathogen panels		148	95	45	41	28

of pathogens and physical exam abnormalities) were modeled using generalized linear mixed models (lme4; Bates et al. 2015). For all models, "turtle group" was the independent variable and turtle ID was the random effect. Overall predictor significance was assessed using *F*-tests (package lmerTest; Kuznetsova et al. 2017) or Wald tests (package car; Fox and Weisberg 2019). For statistically significant categorical predictors, post-hoc between-group differences were evaluated using the contrast function in the lsmeans package with a Tukey correction to control for multiple statistical comparisons (Lenth 2016).

RESULTS

We had data from 767 assessments from pre-release (n=320), same-year post-release (n=145), 1 yr post-release (n=81), and 2 yr post-release headstart groups (n=137); the 2 yr post-release group included turtles that were sampled 2 yr (n=27), 3 yr (n=14), 4 yr (n=29), 5 yr (n=29), 6 yr (n=14), 7 yr (n=11), 8 yr (n=6), 9 yr (n=4), 10 yr (n=2), and 13 yr (n=1) post-release. There were 84 wild turtle assessments included in this study. Survival data are presented elsewhere. All turtles received a complete physical examination, but other clinical pathology testing was frequently limited by the volume of blood that could safely be removed. Sample sizes for clinical pathology testing and pathogen surveillance are presented with demographic and physical examination findings in Table 2.

Body weight was significantly higher in 2 yr post-release and wild juveniles compared to all other groups (P<0.0001). The SCL increased significantly at each time point after release (P<0.0001), and there was no significant

TABLE 3. Physical examination parameters that differ significantly based on rearing history in juvenile Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA. Values presented are means±standard errors for each group, along with *P*-values for the overall significance of the rearing group variable. Significant between-group post-hoc comparisons are indicated by different superscripted letters within the same row.

		Rearing group					
Parameter	Unit	Pre-release headstart	Same-year post-release headstart	1 yr post-release headstart	2 yr post-release headstart	Wild juvenile	*P*-value
Ectoparasites	%	0.16 ± 0.2^{A}	22.9 ± 3.5^{B}	38.4 ± 5.4^{B}	35.1 ± 4.1^{B}	34.7 ± 5.2^{B}	<0.0001
Hemoparasites	%	0.28 ± 0.4^{A}	$0.39\pm0.5^{A,B}$	$2.7\pm2.2^{A,B}$	26.9 ± 5.0^{C}	$16.7\pm5.3^{B,C}$	<0.0001
Asymmetrical nares	%	4.8 ± 1.2^{A}	14.7 ± 2.9^{B}	15.2 ± 4.0^{B}	22.8 ± 4.0^{B}	13.5 ± 4.0^{B}	<0.0001
Appendage injuries	%	14.8 ± 2.0^{A}	$12.5\pm2.8^{A,B}$	$13.8\pm3.9^{A,B}$	$7.3\pm2.2^{B,C}$	$4.8\pm2.3^{B,C}$	0.03
Growth abnormalities	%	12.0 ± 1.8^{A}	19.5 ± 3.3^{B}	12.8 ± 3.7^{A}	1.8 ± 1.1^{C}	8.8 ± 3.0^{A}	<0.0001

difference in SCL ($P=0.9$) or mass ($P=0.2$) between 2 yr post-release headstarts and wild turtles. The BCI was significantly higher in 1 yr post-release, 2 yr post-release, and wild juveniles compared to pre-release and same-year post-release headstarts ($P<0.0001$). Ectoparasites, hemoparasites, and asymmetrical nares were significantly more common, while appendage injuries and growth abnormalities (e.g. shell conformation abnormalities, beak malocclusion) were less common in post-release turtles compared to pre-release turtles (Table 3).

All clinical pathology parameters differed based on rearing history except PCV and uric acid; several of these differences persisted into the 2 yr post-release group. Trends in the data are presented in Tables 4–6 and Figures 1–3.

All pathogen testing was negative except for adenoviruses, *Mycoplasmopsis* sp., and *Emydomyces testavorans*. Adenoviruses were detected in nine samples including same-year post-release headstarts (2/95, 2.1%), 1 yr post-release headstarts (5/45, 11.1%), and 2 yr post-release headstarts 2/41, 4.9%). Three of the adenovirus positive individuals were tested more than once; one turtle was negative at the pre-release assessment but positive at the same-year and 1 yr post-release exams, another was negative at both pre-release and same-year post-release evaluations but positive at its 1 yr post-release exam, and a third was negative at pre-release, then positive at its same-year post-release evaluation. Of the nine adenovirus positive turtles, two had asymmetrical nares, two were missing digits, and five had shell abnormalities (flaking, erosions, focal discoloration). It is unknown if these abnormalities are related or merely concurrent with positive status. All adenovirus DNA polymerase gene sequences (275 bp) were identical to each other and were 92% homologous to *Terrapene adenovirus 1* sequences in GenBank. This Blanding's turtle adenovirus, tentatively named *Emydoidea* adenovirus 1, was deposited in GenBank (accession number MQ561636).

Mycoplasmopsis sp. was detected in a single 1 yr post-release subadult turtle that was also positive for adenovirus; no signs of upper respiratory disease were noted. The sequence from the 478 bp *16S-23S* ribosomal RNA intergenic spacer was >99% identical to multiple *Mycoplasmopsis* sp. from emydid turtles. *Emydomyces testavorans* was detected in two pre-release and one same-season post-release headstart. Both positive pre-release turtles had erosions on the carapace and plastron, and neither was found again following release. Erosions were also noted on the plastron of the post-release individual; however, these had not been present at its pre-release examination.

DISCUSSION

The goals of this study were to characterize how the health of headstarted Blanding's turtles changed following release and to compare the overall health of headstarted turtles to their

TABLE 4. Hematology parameters based on rearing history in juvenile Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA. Values presented are means±standard errors for each group, along with *P*-values for the overall significance of the rearing group variable. Significant between-group post-hoc comparisons are indicated by different superscripted letters within the same row.

Parameter	Units	Rearing group					*P*-value
		Pre-release headstart	Same-year post-release headstart	1 yr post-release headstart	2 yr post-release headstart	Wild juvenile	
Erythrocyte sedimentation rate	mm	7.73 ± 0.268^{A}	$7.77\pm0.392^{A,C}$	$6.91\pm0.478^{A,C}$	$6.45\pm0.343^{B,C}$	6.14 ± 0.422^{B}	0.003
Packed cell volume	%	17 ± 0.41	15.7 ± 0.59	15.6 ± 0.8	16.3 ± 0.63	17.1 ± 0.8	0.2
Total solids	g/L	$38.5^{A}\pm0.60^{A}$	$26.9\pm0.9^{B,C}$	25.0 ± 1.2^{B}	30.2 ± 1.0^{C}	36.2 ± 1.2^{A}	<0.0001
White blood cell count	Cells×10^9/L(cells/mL)	11.26 ± 0.56^{A} ($11,256^{A}\pm560$)	10.06 ± 0.59^{A} (10,059±585)	13.74 ± 1.25^{A} (13,739±1,251)	23.21 ± 1.73^{B} (23,210±1,734)	21.97 ± 2.05^{B} (21,973±2,049)	<0.0001
Heterophils	Cells×10^9/L (cells/mL)	1.23 ± 0.13^{A} (1,230±134)	$0.85\pm0.11^{A,B}$ (853±108)	1.50 ± 0.3^{A} (1,501±298)	$0.52\pm0.09^{B,C}$ (521±86)	$1.18\pm0.24^{A,B}$ (1,184±243)	<0.0001
Lymphocytes	Cells×10^9/L (cells/mL)	6.45 ± 0.37^{A} (6,454±368)	6.10 ± 0.41^{A} (6,100±407)	7.75 ± 0.81^{A} (7,748±808)	17.11 ± 1.48^{B} (17,114±1,481)	15.15 ± 1.63^{B} (15,147±1,626)	<0.0001
Monocytes	Cells×10^9/L (cells/mL)	0.24 ± 0.04^{A} (242±35)	0.09 ± 0.02^{B} (92±16)	0.47 ± 0.12^{A} (465±124)	0.39 ± 0.09^{A} (393±87)	0.41 ± 0.11^{A} (408±112)	<0.0001
Eosinophils	Cells×10^9/L (cells/mL)	0.87 ± 0.07^{A} (871±66)	$0.76\pm0.07^{A,B}$ (765±68)	$0.52\pm0.07^{B,C}$ (519±72)	0.86 ± 0.1^{A} (861±99)	0.95 ± 0.14^{A} (974±139)	0.009
Basophils	Cells×10^9/L (cells/mL)	1.20 ± 0.12^{A} (1,199±115)	1.07 ± 0.12^{A} (1,06±120)	$1.55\pm0.27^{A,C}$ (1,546±272)	$2.25\pm0.33^{B,C}$ (2,253±326)	$1.48\pm0.27^{A,C}$ (1,479±267)	0.0008
Heterophil: lymphocyte	(Ratio)	$0.623\pm0.081^{A,C}$	$0.439\pm0.066^{A,B}$	1.521 ± 0.366^{C}	0.181 ± 0.037^{D}	$0.268\pm0.066^{B,D}$	<0.0001

wild-reared counterparts. We found that pre-release headstarted turtles had multiple differences in abnormalities detected on physical examination, clinical pathology parameters, and the presence of parasites and pathogens compared to wild-reared turtles. Pre-release headstarts had more appendage injuries than wild-reared counterparts, which is consistent with reports in densely housed headstarted green turtles and desert tortoises (*Gopherus agassizii*) and is attributed to conspecific aggression (Kanghae et al. 2016; Mack et al. 2018). Higher rates of musculoskeletal abnormalities in headstarts, including more beak malocclusions and shell conformational abnormalities compared to wild-reared turtles, might be attributable to dietary imbalances in calcium, phosphorous, or protein or inadequate access to ultraviolet light for vitamin D synthesis with subsequent development of nutritional secondary hyperparathyroidism (Meyer and Selleri 2019). It may also be due to the much faster growth rates of juvenile Blanding's turtles from this headstarting facility, which routinely reach the size of a 2-yr-old wild-reared juvenile by 1 yr of age and continue to grow faster than their wild counterparts for up to 6 yr post-release (Golba et al. 2022). Rapid growth coupled with favorable environmental conditions such as elevated overnight temperatures has been associated with the development of musculoskeletal abnormalities including pyramiding and might be playing a role in the abnormalities identified in the present study as well (Heinrich and Heinrich 2016). Limited exposure to infectious diseases may explain the reduced occurrence of asymmetrical nares in pre-release headstarts, as this is frequently a sign of chronic upper respiratory disease due to *Mycoplasmoidales* spp. and other pathogens (Rodriguez et al. 2018). Lack of exposure also explains the higher prevalence of ectoparasites and hemoparasites in post-release headstarts compared to pre-release headstarts.

The increased bile acids, total protein, prealbumin, albumin, and albumin:globulin (A:G) ratio of pre-release headstarts may be due to a combination of more frequent feedings, higher nutritional plane, and reduced ecto- and

TABLE 5. Plasma biochemistry parameters based on rearing history in juvenile Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA. Values presented are means±standard errors for each group, along with *P*-values for the overall significance of the rearing group variable. Significant between-group post-hoc comparisons are indicated by different superscripted letters within the same row.

		Rearing group					
Parameter	Units	Pre-release headstart	Same-year post-release headstart	1 yr post-release headstart	2 yr post-release headstart	Wild juvenile	*P*-value
Calcium	mmol/L	1.68 ± 0.08^{A}	1.89 ± 0.09^{A}	1.20 ± 0.12^{B}	1.84 ± 0.08^{A}	1.75 ± 0.11^{A}	<0.0001
Phosphorous	mmol/L	1.1 ± 0.07^{A}	0.61 ± 0.04^{B}	0.48 ± 0.04^{B}	0.85 ± 0.05^{C}	$1.02\pm0.07^{A,C}$	<0.0001
Calcium:phosphorous	(Ratio)	1.82 ± 0.14^{A}	3.99 ± 0.16^{B}	3.82 ± 0.2^{B}	2.7 ± 0.13^{C}	$2.18\pm0.16^{A,C}$	<0.0001
Uric acid	mmol/L	71.38±2.97	71.38±2.97	82.68±4.16	75.54±2.97	75.54±3.57	0.2
Bile acids	mmol/L	5.55 ± 0.44^{A}	3.02 ± 0.27^{B}	2.7 ± 0.3^{B}	2.68 ± 0.2^{B}	3.29 ± 0.32^{B}	<0.0001
Glutamate dehydrogenase	U/L	4.63 ± 0.46^{A}	2.83 ± 0.31^{B}	2.29 ± 0.32^{B}	4.38 ± 0.4^{A}	4.79 ± 0.55^{A}	<0.0001
Creatine kinase	U/L	421 ± 40^{A}	381 ± 41^{A}	360 ± 48^{A}	739 ± 67^{B}	763 ± 86^{B}	<0.0001
Aspartate aminotransferase	U/L	$58.2\pm3.8^{A,B}$	54.4 ± 4.0^{B}	51.9 ± 4.9^{B}	73.4 ± 3.9^{C}	$71.9\pm4.9^{A,C}$	0.003

endoparasite burden (Anderson et al. 2011; Rosser 2022). The hematologic differences of lower WBC count and higher heterophil:lymphocyte ratio driven by decreased lymphocyte counts, and higher ESR and alpha globulins in pre-release headstarts compared to wild-reared turtles may indicate increased physiologic stress (due to normal age-related changes or husbandry) or slightly increased levels of acute inflammation in headstarted turtles compared to wild individuals, potentially attributable to high stocking density, resource competition, and conspecific aggression as reported in other species of headstarted chelonia (Redrobe and MacDonald 1999; Kanghae et al. 2016; Mack et al. 2018; Mumm et al. 2019; Adamovicz et al. 2020; Andersson et al. 2021a; Cray 2021).

The changes noted in biochemical parameters (significantly lower bile acids, GLDH activity, phosphorous, total protein, and all electrophoresis fractions, as well as an increase in the calcium: phosphorous ratio) within 1 mo of release

TABLE 6. Plasma protein electrophoresis parameters based on rearing history in juvenile Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA. Values presented are means±standard errors for each group, along with *P*-values for the overall significance of the rearing group variable. Significant between-group post-hoc comparisons are indicated by different superscripted letters within the same row.

		Rearing group					
Parameter	Units	Pre-release	Same-year post-release headstart	1 yr post-release headstart	2 yr post-release headstart	Wild juvenile	*P*-value
Total protein	g/L	45.4 ± 1.1^{A}	$31.3\pm1.2^{B,C}$	28.4 ± 1.5^{B}	$30.7\pm1.5^{B,C}$	37.0 ± 2.0^{C}	<0.0001
Prealbumin	g/L	0.7 ± 0.08^{A}	$0.5\pm0.06^{B,C}$	$0.6\pm0.09^{A,C}$	$0.4\pm0.06^{B,C}$	$0.4\pm0.09^{A,C}$	0.003
Albumin	g/L	12.7 ± 0.3^{A}	$7.5\pm0.3^{B,C}$	6.3 ± 0.4^{B}	6.8 ± 0.4^{B}	8.8 ± 0.5^{C}	<0.0001
Alpha 1 globulins	g/L	9.2 ± 0.3^{A}	7.4 ± 0.3^{B}	6.3 ± 0.4^{B}	2.6 ± 0.4^{C}	4.0 ± 0.5^{C}	<0.0001
Alpha 2 globulins	g/L	9.1 ± 0.3^{A}	6.5 ± 0.3^{B}	5.3 ± 0.4^{B}	5.6 ± 0.4^{B}	6.6 ± 0.5^{B}	<0.0001
Beta globulins	g/L	8.5 ± 0.2^{A}	5.5 ± 0.3^{B}	5.7 ± 0.3^{B}	8.2 ± 0.3^{A}	9.1 ± 0.4^{A}	<0.0001
Gamma globulins	g/L	4.8 ± 0.2^{A}	3.6 ± 0.2^{B}	$4.1\pm0.2^{A,B}$	7.0 ± 0.3^{C}	7.7 ± 0.3^{C}	<0.0001
Albumin:globulin	(Ratio)	0.439 ± 0.007^{A}	0.363 ± 0.008^{B}	0.32 ± 0.009^{C}	0.312 ± 0.009^{C}	$0.348\pm0.012^{B,C}$	<0.0001

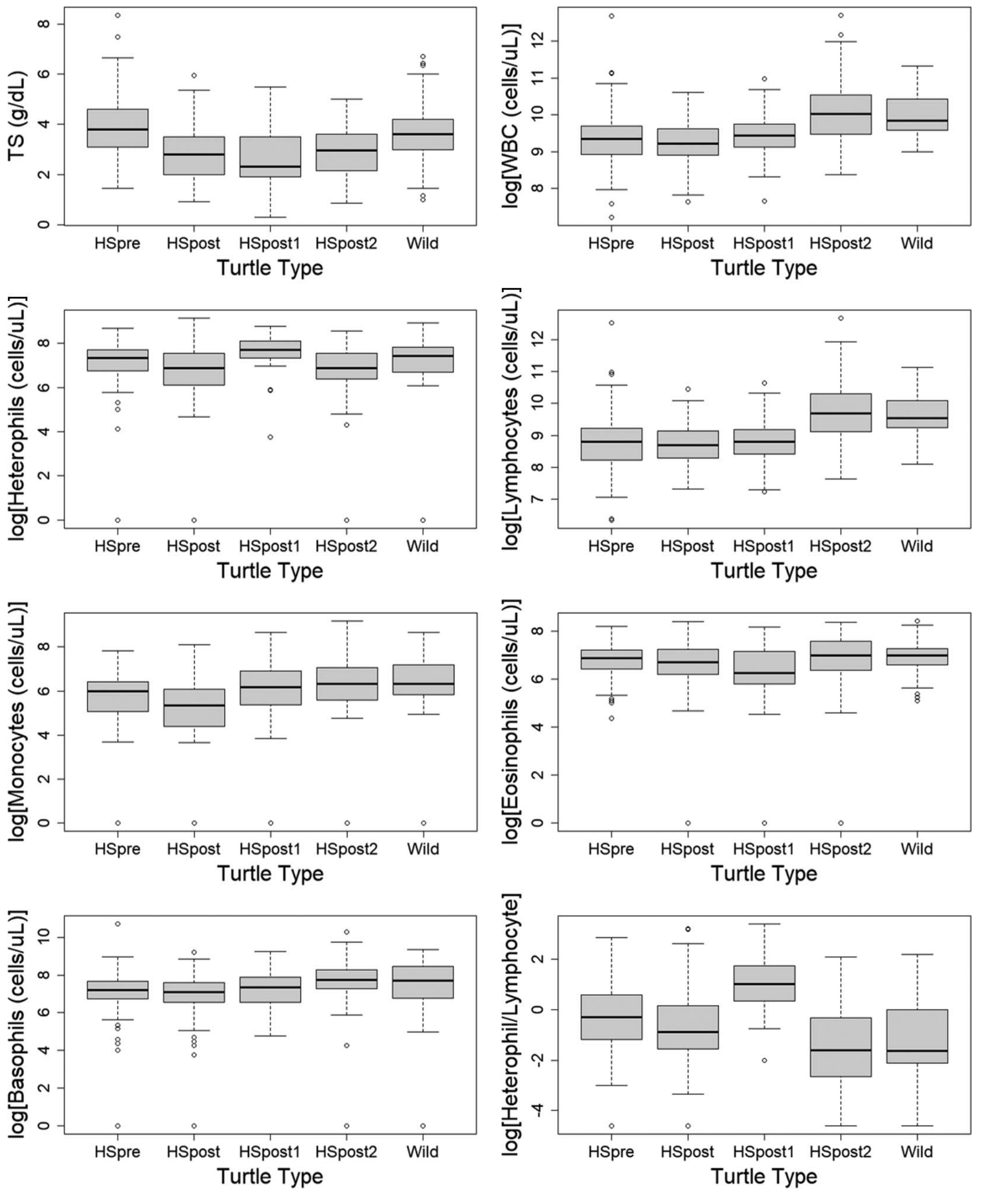

FIGURE 1. Hematologic parameters which significantly differ based on rearing history in headstarted and wild-reared Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA. TS = Total solids, HSpre = pre-release headstarts, HSpost = same-year post-release headstarts, HSpost1 = 1 yr post-release headstarts, HSpost2 = 2 yr post-release headstarts.

probably reflect sudden shifts in diet, environment, and exposure to pathogens and parasites (Heatley and Russell 2019; Cann 2021). Several of these changes persisted for up to 2 yr following release, indicating that rearing history can have lasting impacts on turtle health. This is supported by a smaller study in Blanding's turtles that identified shifts in sodium, chloride,

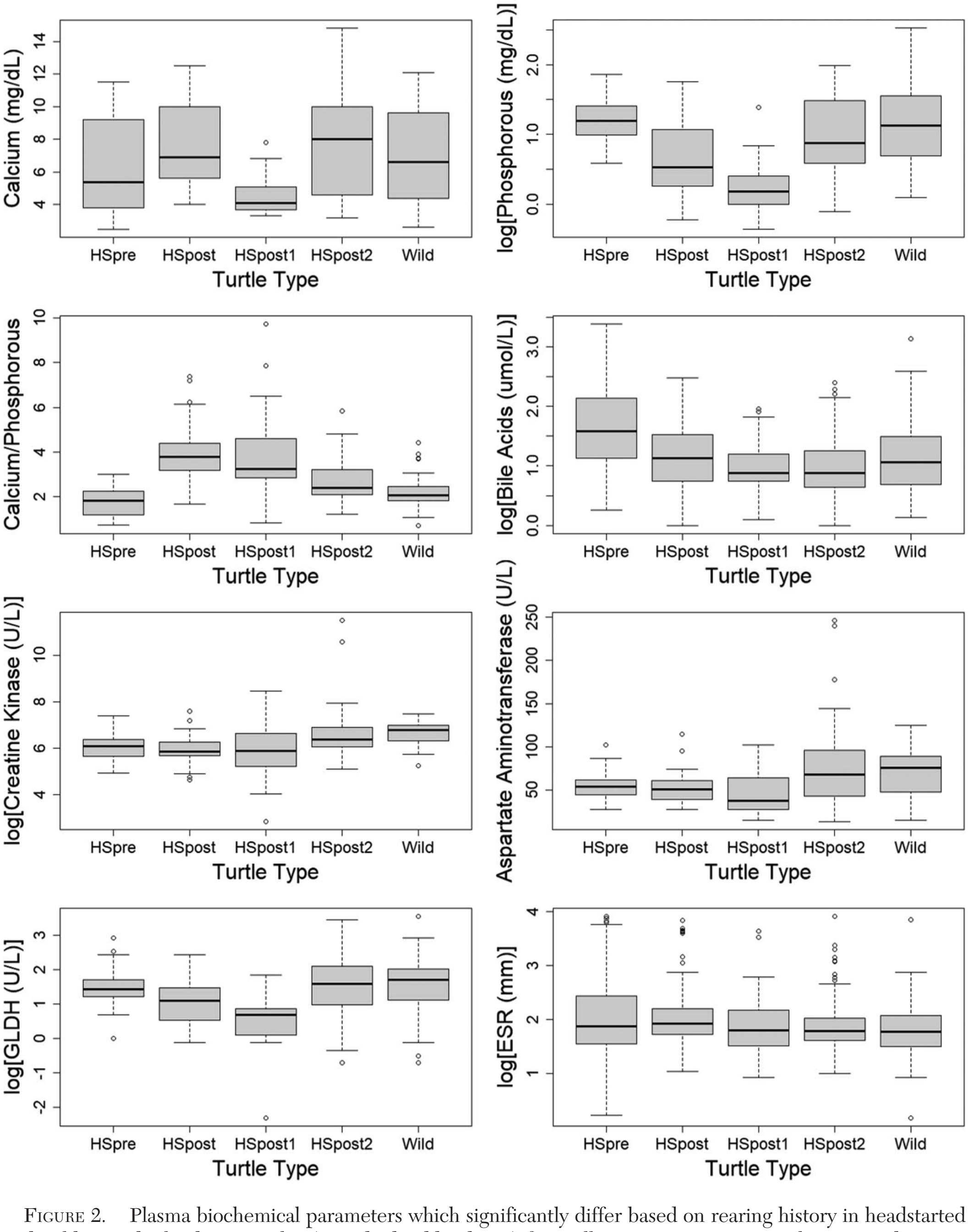

FIGURE 2. Plasma biochemical parameters which significantly differ based on rearing history in headstarted and wild-reared Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA. ESR = erythrocyte sedimentation rate, HSpre = pre-release headstarts, HSpost = same-year post-release headstarts, HSpost1 = 1 yr post-release headstarts, HSpost2 = 2 yr post-release headstarts.

calcium, blood urea nitrogen, and osmolality in headstarted turtles following release, with differences in sodium, chloride, and plasma osmolality persisting over time when compared to a cohort of headstarts that had been released the previous year (Cann et al. 2021). Most clinical pathology values for 2 yr post-release and wild-reared juvenile turtles were similar

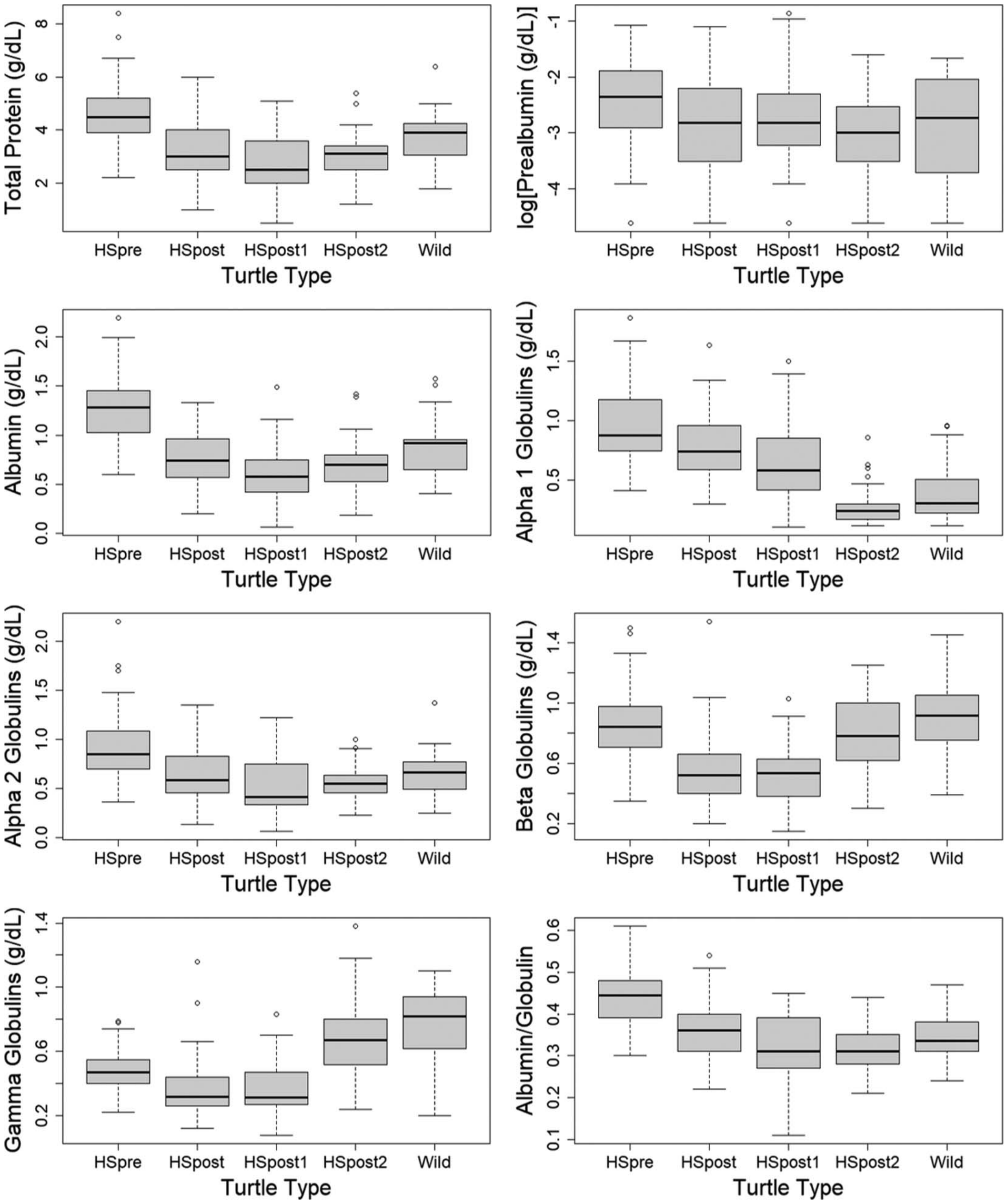

FIGURE 3. Plasma protein electrophoresis parameters which significantly differ based on rearing history in headstarted and wild-reared Blanding's turtles (*Emydoidea blandingii*) from Illinois, USA. HSpre = pre-release headstarts, HSpost = same-year post-release headstarts, HSpost1 = 2 yr post-release headstarts, HSpost2 = 2 yr post-release headstarts.

to previously published values for wild juveniles, indicating that headstart health status eventually comes to mirror the health of wild-reared juveniles (Mumm 2019; Andersson et al. 2021a).

Our study indicates a significant and prolonged shift in health parameters following release. While it is tempting to posit that post-release headstart health improves over time, it is also possible that only the strongest animals survive and that this is why an improvement in clinical pathology parameters toward recognized species reference intervals over time is observed, rather than an improvement in individual health. This could explain the nadir in health status for 1 yr post-release turtles, characterized by low total solids, total protein, albumin, beta globulins, calcium, phosphorous, and glutamate dehydrogenase, and increased heterophil:lymphocyte ratio and calcium:phosphorous ratio compared to other groups, followed by an apparent rebound by 2 yr post-release. Individual health would need to be evaluated long term to ultimately determine the relationship between these parameters and survival, which was outside the scope of this study.

Mycoplasmopsis sp. and *Emydoidea* adenovirus 1, detected in post-release headstarts and wild-reared juveniles, have both previously been detected in adult Blanding's turtles from Illinois (Winter et al. 2020). Emydid *Mycoplasmoidales* have been suggested as host-adapted organisms of little clinical consequence without an underlying comorbidity (Sandmeier et al. 2009; Jacobson et al. 2014; Ossiboff 2015). Emydid adenoviruses appear to be endemic in multiple wild chelonian populations and are probably host-adapted, with clinical disease occurring only in young or immunocompromised animals or secondary to environmental stressors, coinfection with other infectious agents, or infection of aberrant hosts (Franzen-Klein et al. 2020). The pathogen-positive juveniles in the present study did not present with active clinical signs of illness such as discharge or swelling, potentially indicating that *Mycoplasmopsis* sp. and *Emydoidea* adenovirus 1 have limited impacts in otherwise healthy Blanding's turtles. Nevertheless, adenovirus prevalence was highest in 1 yr post-release headstarts, corresponding to the time period with the poorest overall health, meriting additional research into the epidemiology of this pathogen. Additionally, the detection of *Emydoidea* adenovirus 1 in two 1 mo post-release headstarts demonstrates how quickly these pathogens can be acquired in the wild, or possibly how quickly previously-undetected pathogens might recrudesce and be shed following release.

The keratinophilic onygenalean fungus *Emydomyces testavorans*, detected in two pre-release and one post-release headstart in 2021, is associated with skin and shell lesions in freshwater turtles, including pitted depressions, discoloration and flaking of scutes, thickening and exudation along scute margins, traumatic perforations, and epithelial inclusion cysts (Woodburn et al. 2019). While the biology and epidemiology of *E. testavorans* are currently uncharacterized (Haman et al. 2019), shell disease associated with this pathogen can cause significant morbidity and has tremendously impacted conservation initiatives for the western pond turtle in Washington state (Barten 2006; Rodriguez et al. 2018; Woodburn et al. 2019). Detection of *E. testavorans* within the Illinois Blanding's turtle headstarting program caused an immediate cessation of releases; details of outbreak management will be described in subsequent publications. This underscores the value of infectious disease surveillance within headstarting programs and supports its continued use in this and other chelonian conservation initiatives (Dodd and Seigel 1991; Jacobson 1993; Smith 2015).

Some changes to headstarting protocols might further improve the health of pre- and post-release turtles. Examining husbandry conditions (stocking density), including the nutritional composition of the diet (calcium, vitamin D), access and proximity to UV light (supplementary UV lighting and soft release outdoors), and environmental temperature range, may be beneficial to reduce the incidence of musculoskeletal abnormalities and improve the dynamic changes in bloodwork observed in this study. Adjustments to rearing time may also affect headstart survival. There have been opposing studies about whether larger juveniles have higher survivability: some show that body size is positively associated with survival (Daly et al. 2018; Tetzlaff 2019), whereas others have demonstrated body size does not affect survivorship (Carstairs et al. 2019). Although size was not considered a driver of health changes in this study, surviving turtles with normal bloodwork showed growth over time.

Headstarting has shown success in supplementing populations in the Galapagos tortoise (*Chelonoidis niger*; Gibbs et al. 2014; Aguilera et al. 2015), Duvaucel's geckos (*Hoplodactylus duvaucelii*; Bell and Herbert 2017), western pond turtles (Vander Haegan et al. 2009), Mona Island iguanas (*Cyclura stejnegeri*; Pérez-Buitrago et al. 2008), and Chiricahua leopard frogs (*Rana chiricahuensis*; Sprankle 2008). In Illinois, Blanding's turtle headstarting programs have successfully and positively shifted population demographic structure and bolstered population sizes (Thompson et al. 2020). Headstarted Blanding's turtles have been shown to successfully reproduce and have similar movement ecology, growth, and survival compared to wild-reared juveniles (Starking-Symanski et al. 2018; Carstairs et al. 2019; Thompson et al. 2020; Golba et al. 2022). Our findings indicate that pre-release headstart health appears adequate, but there is a significant post-release decrease in plane of health that may persist for at least 2 yr. Adjustments to current headstarting protocols may support the health of turtles both pre- and post-release, while continued infectious disease surveillance will allow for rapid identification of and response to the introduction of novel pathogens. Pairing health data with survival data may allow for identification of health parameters that predict survival in pre- and post-release Blanding's turtles, generating a survival index to further benefit species conservation as in other species (Li et al. 2015). A multipronged approach to conservation that includes considerations for health and disease will further support Blanding's turtle populations in Illinois and other states, and our data should aid in current and future Blanding's turtle conservation efforts.

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