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REFERENCE AND BASELINE HEMATOCRIT MEASURES FOR THE THREATENED NEW ENGLAND COTTONTAIL (*SYLVILAGUS TRANSITIONALIS*) AND COMPARISON WITH SYMPATRIC EASTERN COTTONTAIL (*SYLVILAGUS FLORIDANUS*) RABBITS

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Abstract: From June 2014 to June 2015, capillary tube collections of blood were obtained concurrently with ear clips of trapped free-ranging, globally vulnerable New England cottontails (NEC; *Sylvilagus transitionalis*) and eastern cottontail rabbits (EC; *Sylvilagus floridanus*) in the Hudson Valley region of New York, United States. Species identification (NEC, EC) and sex (NEC) were determined genetically using a mitochondrial DNA assay and Y chromosome marker, respectively. Hematocrit values were obtained using a microhematocrit centrifuge. We provide the reference values 35.15–49.55 (2.5 and 97.5 percentiles) and 90% confidence intervals (CI) [lower: 33.00, 36.08; upper: 46.95, 51.00], for hematocrit of NEC. The mean hematocrit for NEC was 42.35% (SE = 0.58, n = 47) and a comparative contemporaneous mean in the same area for EC [39.96 (SE = 0.81, n = 26)], which was significantly different from NEC (P = 0.02). There was a significant sex difference for NEC [male: 43.99 (SE = 1.02, n = 28); female: 39.92 (SE = 0.78, n = 19), $P < 0.0001$], though not for EC.

Key words: Cottontail, hematocrit, New York, *Sylvilagus floridanus*, *Sylvilagus transitionalis*.

BRIEF COMMUNICATION

The New England cottontail (NEC; *Sylvilagus transitionalis*) is a shrubland obligate lagomorph.[11] Historically common throughout New England and eastern New York, the NEC has experienced drastic range contraction,[6] and is now found in five isolated populations at the edges of its historic range.[6] This decline prompted the United States Fish and Wildlife Service to list the NEC as a candidate species under the Endangered Species Act.

Loss of early successional forest is widely recognized as the driving factor in the decline of NEC.[2] However, the specific effects of different plant communities or dominance of invasive plant species on NEC survival have not been investigated. Additionally, little is known about the impacts of habitat structure and predator communities on fecundity, recruitment, and dispersal of NEC. Furthermore, competition and hybridization of NEC with nonnative eastern cottontails (EC; *Sylvilagus floridanus*) has been suggested as another contributing factor NEC decline.[12,15] The impacts of EC competition, either directly via resource competition for food or sheltering habitat, or through parasite-mediated competition, are thus far unknown. As part of a larger study in these landscapes, we seek to compare indices of health in the two species. A key measure of health in mammals is hematocrit, the measure of packed cell volume (PCV) in blood, which can be used to compare across sites and dates, or examine responses to potentially detrimental conditions or exposure. However, we found no reported baseline or reference values for hematocrit of NEC. We therefore report reference values of hematocrit for NEC, and compare these with measurements for EC in the same geographic area.

This study was conducted in the Hudson Valley of New York, United States, from June 2014 to June 2015. The site encompasses several public land areas, comprising native and nonnative vegetation associations, and which were previously assumed to be home to either one or both species. Rabbits were trapped using single door box traps baited with apple. Traps were placed under cover, near rabbit markings, or every 25 m in absence of markings. Traps were checked at dawn when maximum daytime temperatures were below 20°C, dawn and dusk daily at temperatures between 20 and 25°C, and closed midday when temperatures exceeded 25°C. Body condition was scored using methods adapted from Cardinali et

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al.,[4] by assessing loin and rump muscle, and bone protrusion on the hips and back, on a scale from 0 to 2; 0 is poor, 1 is normal, and 2 is very good. For each body area, scores were added, then collapsed to 3 categories: 1) very poor body condition (are or are nearly emaciated), 2) average body condition (healthy but no excess muscle or fat), and 3) excellent body condition.

Ears were clipped to provide tissue samples for genetic analyses and hematocrit. New gloves were worn for each rabbit, and a sterile razor blade and forceps were used to remove approximately 30 mg of ear tissue. Blood was immediately collected in 1–3 75-mm heparinized capillary tubes (100–200 μl), and sealed with Critoseal. No analgesia was used as the rabbits did not respond or show any sign of pain or distress during sampling. Rabbits were released after sampling. All work was approved by the SUNY-ESF IACUC (Protocol #120801). Within 8 hr of collection, tubes were centrifuged for 5 min on a LW Scientific LWS-M24 Microhematocrit Centrifuge machine at 12,000 rpm. Readings were taken visually and reported to the nearest percent. Several samples were lost to breakage or incomplete separation, leaving a total of 134 samples, representing 73 individuals (47 NEC, 26 EC).

For genetic analysis, <25 mg of ear tissue was digested overnight and DNA extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California 91355, USA). PCR was performed in 25 μL reaction volumes in AmpliTaq Gold® 360 Master Mix (Life Technologies, Grand Island, New York 14072, USA), with 0.25 μM of each primer and 3 uL of template DNA. Mitochondrial DNA was targeted for species identification with primers L15934 and H16498 of Litvaitis and Litvaitis.[13] The resulting product was restriction digested with Nla III (New England Biolabs, Ipswich, Massachusetts 01938, USA) as described by Kovach et al.,[9] and confirmed by restriction digest with Bfa I (New England Biolabs) as described by Litvaitis and Litvaitis.[14] For genetic confirmation of sex, we used the SRY marker primers for INRACCDDV0326 from Chantry-Darmon et al.[5] with a HEX labeled forward primer and same PCR conditions as above. The resulting product was analyzed on the 3730xl 96-Capillary Genetic Analyzer (Applied Biosystems, San Francisco, California 94080, USA).

Reference values for NEC hematocrit were established by calculating the central 95% interval (2.5–97.5 percentiles), according to the International Federation of Clinical Chemistry (IFCC) protocol;[18] with $n > 40$ samples, this was acceptable. A 90% confidence interval (CI) was calculated for each reference percentile, by bootstrapping on 1,000 data replicates, as the sample size was still small relative to the necessary population of samples to establish the CIs. We report Efron's nonparametric bias-corrected and accelerated (BC_a) bootstrap method CIs (R package "boot"), consistent with the IFCC protocol requirement of nonparametric estimates. This yielded reference values 35.15–49.55 (2.5 and 97.5 percentiles) and 90% confidence intervals (CI) [lower: 33.00, 36.08; upper: 46.95, 51.00], for hematocrit of NEC.

Shapiro-Wilk tests for normality of PCV measurements by species failed to reject normality assumptions (NEC: $W = 0.9837$, $P = 0.7486$; EC: $W = 0.9753$, $P = 0.9753$). We collapsed individual sample measures to means, as all blood tubes were collected simultaneously. We examined differences in means between species and by sex, within species, controlling for body condition, using a two-way analysis of variance (ANOVA), and post-hoc Tukey Honestly Significant Differences (HSD) tests in R (ver 3.1.3). We established baseline mean hematocrit for NEC [42.35% (SE = 0.58, $n = 47$)], and for EC of 39.96 (SE = 0.81, $n = 26$), which were significantly different ($F = 7.353$, $P = 0.009$). Although PCV differed significantly by sex with species pooled ($F = 18.947$, $P < 0.0001$), within species this only held for NEC [male 43.99 (SE = 1.02, $n = 28$); female 39.92 (SE = 0.78, $n = 19$), $P < 0.0001$; two-way ANOVA Tukey HSD], and not EC [male 41.63 (SE = 1.53, $n = 12$); female (38.54, SE = 1.04, $n = 14$), $P = 0.55$; two-way ANOVA Tukey HSD].

We could not find previous reports of hematocrit measures in NEC. However previous reports of hematocrit for EC, while scarce, contain a range of values far exceeding those we found for either species, at both higher and lower percentiles (see Table 1, Eastern cottontails). Expanding our comparison to other rabbit species (Table 1), we similarly see that our range for NEC and EC are consistent with other rabbits. We found that NEC had a higher mean hematocrit than EC in our study site. Because we did not have literature comparisons for NEC, we can only infer that if the two species are similar in their physiology, the NEC may be performing better on their native landscape. Most rabbits of both species in traps had ectoparasites (ticks, fleas), which we have collected opportunistically, but not analyzed in detail. We saw no apparent difference in health status between the two species evaluated. It may

Table 1. Measures of hematocrit found in a selection of previous studies of lagomorphs.

Study	Species	Packed cell volume
This study	New England cottontail (*Sylvilagus transitionalis*) Eastern cottontail (*Sylvilagus floridianus*)	Mean: 42.35 (SE = 0.58) reference range: 35.15-49.55 (2.5 and 97.5 percentiles) [90% CI: l: 33.00, 36.08; u: 46.95, 51.00] mean: 39.96 (SE = 0.81)
Lepitski and Woolf, 1991	Eastern cottontail (*Sylvilagus floridianus*)	Mean: 37.2, range 18–49
Jacobson et al., 1978	Eastern cottontail (*Sylvilagus floridianus*)	Range: 29.2–40.2 (Fort Pickett, Virginia, United States), range: 37.2–45.6 (Radford, Virginia, United States)
Black et al., 2009	Riparian brush rabbits (*Sylvilagus bachmani riparius*)	Reference range: 29.7–45.8
Calvete et al., 2005	European rabbits (*Oryctolagus cuniculus*)	Mean: 38.213 (SD = 0.564)
Moore et al., 2015[a]	New Zealand white rabbits (*Oryctolagus cuniculus*)	Adult male range: 33–50, adult female range: 31.0–48.6; age 1–3 mo range: 38.1–44.1
Moore et al., 2015	Dutch belted rabbits (*Lepus europaeus*)	Range: 34.8–48.9
Moore et al., 2015	Jackrabbit (*Lepus californicus*)	Range: 42–53

[a] Data represent summaries of previous studies, collapsed into single ranges (from: Moore DM, Zimmerman K, Smith SA. Hematological assessment in pet rabbits: blood sample collection and blood cell identification. Hematology. 2015;18(1)9–19)

instead be that NEC naturally have higher PCV, or there is a species-level difference in the reaction to stress, and splenic contraction is greater in NEC. We found that male rabbits of both species had higher mean hematocrit than females, although this was only significant in NEC. In previous work, Jacobson[8] found that EC males had higher hematocrit than females, as did Lepitzki and Woolf,[10] although not significantly. In a study[1] of brush rabbits (*Sylvilagus bachmani riparius*), males also had higher hematocrits. Our samples were taken across all seasons, and without finer resolution, it is hard to tease apart the contributions to the variation we saw. ECs were found to have higher PCV in winter in two previous studies.[8,10]

Hematocrit is an important physiological measurement of condition and health, providing an indicator for stressful or suboptimal conditions, nutritional status, and immunological response. In rabbits, PCV levels in quarantined European rabbits (*Oryctolagus cuniculus*) were negatively correlated with mortality risk, allowing for recommendations on management in quarantine conditions,[3] and a diet study in juvenile ECs demonstrated a direct relationship between manipulated crude dietary protein levels (CP) and PCV, reflected strongly in weight gain or loss.[17] The impact of stress due to capture method (trapping or shooting) on PCV was also examined for ECs;[7] trapped rabbits had higher PCV, attributed to splenic contraction—a stress response—than the shot rabbits. As ours is a trapping study, we suggest that our PCVs may be elevated above baseline. Rabbits were in traps less than 24 hr when daily maximum temperature was less than 20°C, and less than 12 hr when maximum daily temperature exceeded 20°C; however, as cottontails were infrequently trapped between sunrise and sunset, rabbits were rarely in traps longer than 12 hr.

In order to provide a means to measure the health of EC and NEC, we need a useful and comparable index to condition. As pointed out by Black et al.,[1] establishing reference values for threatened or endangered species is often complicated by a lack of access to sufficient samples under appropriate field conditions. We hope that this study will not only provide us a reference and baseline for this particular population in New York, but can serve as a reference for other work on other populations of this vulnerable species.

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