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Effective Field Sampling of Rectoanal Mucosa–Associated Lymphoid Tissue for Antemortem Chronic Wasting Disease Testing in White-tailed Deer (*Odocoileus virginianus*)

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ABSTRACT: Chronic wasting disease (CWD) is a fatal prion disease of cervids that has spread across much of North America. Although gold standard CWD diagnostics involve postmortem testing of medial retropharyngeal lymph nodes or obex (brain stem), a key tissue sample for antemortem testing is rectoanal mucosa–associated lymphoid tissue (RAMALT). However, collection of an adequate sample (i.e., enough lymphoid follicles) may be affected by factors such as deer age, repeated sampling, skill of the sampler, and adverse conditions during collection. Here, we document the protocol used to train personnel for RAMALT collection in a large study of free-ranging white-tailed deer (*Odocoileus virginianus*) in Wisconsin, USA, and determine factors that contributed to the occurrence of inadequate RAMALT samples. Our training protocol included hands-on experience with postmortem tissues, as well as a mentored collection process in the field. Collection of RAMALT under field conditions was highly successful, with 763/806 (94.7%) samples deemed adequate for subsequent testing. Although inadequate samples were rare, they were more likely to occur with older deer and when samples were collected at dusk (i.e., limited ambient lighting). We conclude that RAMALT collection can be highly successful under adverse field conditions, including with technicians with limited prior veterinary experience, and we provide details of our training program to facilitate repeatability in other antemortem CWD testing efforts.

Key words: Biopsy, Cervidae, diagnostic testing, disease surveillance, field sampling, immunohistochemistry, *Odocoileus virginianus*, training.

Chronic wasting disease (CWD) is an infectious, fatal neurodegenerative disorder caused by a misfolded prion protein (PrP^{CWD}) affecting species within the Cervidae family. The disease was first observed in 1967 in captive mule deer (*Odocoileus hemionus*) in Colorado, USA, and continues to spread broadly over North America (Williams and Young 1980; Richards 2023). Because CWD threatens wildlife populations important for hunting, its economic impacts are considerable, and management of the disease is a priority (Bishop 2004; Chiavacci 2022).

An antemortem diagnostic test for CWD can offer direct insight into questions relating to wildlife health, population incidence, and disease transmission, and thereby inform disease management. Relative to postmortem testing, for which the gold standard is immunohistochemistry (IHC) testing of medial retropharyngeal lymph nodes or obex (brain stem), antemortem IHC analysis of peripheral lymphoid tissues such as tonsil and rectoanal mucosa–associated lymphoid tissue (RAMALT) has demonstrated variable sensitivity in cervids (68% sensitivity with RAMALT in white-tailed deer, *Odocoileus virginianus*; Thomsen et al. 2012; see also Wild et al. 2002; Monello et al. 2013; Haley and Richt 2017). Antemortem RAMALT biopsy is considered less invasive, requires fewer specialized tools—all of which can be disposable, reducing risk of iatrogenic transmission—and is easier to perform than tonsillar biopsy (Keane et al. 2008); RAMALT sampling has therefore become a prominent component of some research and herd management protocols. Because RAMALT biopsy is generally considered to be a surgical procedure (if a minor one), it necessitates effective training of sample collectors, particularly when those collectors have limited prior veterinary experience.

Additionally, a key uncertainty with RAMALT sampling is the impact of various animal and sampler characteristics on the subsequent diagnostic suitability of the collected sample; namely, that the biopsy must contain adequate lymphoid follicles for reliable CWD testing (typically six follicles for IHC; Keane et al. 2009; Thomsen et al. 2012). Rates of sufficient follicle collection seem to be variable: Thomsen et al. (2012) reported collection of adequate RAMALT samples in 56–91% of sampled white-tailed deer across different herds. Even when collecting RAMALT samples from deceased free-ranging white-tailed deer, Keane et al. (2009) reported that samples from 21% of individuals were inadequate. Cervid age and repeated sampling can affect the likelihood of collecting an adequate RAMALT sample, as lymphoid follicle counts or numbers affected can be reduced in older individuals or those subject to repeated biopsies (elk, *Cervus elaphus canadensis*: Spraker et al. 2009; mule deer: Geremia et al. 2015). Collecting an adequate RAMALT sample may also become more difficult in field conditions, where lighting or visibility may be poor, weather conditions may be challenging, and prior sampling experience may be limited. Sources of variation in successful RAMALT sampling rates in white-tailed deer have not been described, and the effects of different samplers or environmental conditions on RAMALT collection are unknown. These knowledge gaps leave uncertainty in how best to implement RAMALT sampling in order to maximize collection success, especially in high-volume efforts where many inexperienced samplers may be employed.

As part of ongoing CWD research in southwestern Wisconsin, the Wisconsin Department of Natural Resources (WDNR) trapped 773 unique white-tailed deer (hereafter, deer), from which they collected 806 RAMALT biopsies (25 deer were trapped and sampled two times, and four were sampled three times; resampling occurred in different years). Captures occurred from January to March in 2017–2020. Field conditions were often cold, with limited ambient light, representative of conditions common to cervid captures in much of temperate North America. Our objective in this communication is to describe our training process and success rate for collecting adequate RAMALT biopsies when following previously described procedures (Wolfe et al. 2007), and to determine what sampling and environmental factors contributed to the probability of collecting an inadequate sample in the field.

Before the onset of deer trapping, all sample collectors underwent RAMALT tissue collection training with the WDNR wildlife veterinarian (see Supplementary Material). This 4-h training was based on experience provided by Colorado Parks and Wildlife staff to the WDNR wildlife veterinarian and a WDNR project fieldwork coordinator. Sample collectors were field technicians with backgrounds typically in wildlife biology. Training consisted of a presentation with visuals, handouts that outlined anatomic features relevant for sampling, hands-on practice with tissues from dead deer, and in-field observations and guidance. A minimum of two anuses with rectums were provided to trainees from hunter-harvested deer (as described by Keane et al. 2009); one specimen could then be longitudinally incised and opened for full visualization of the mucocutaneous junction and other sampling landmarks, and the other could be processed as would occur antemortem (as described by Wolfe et al. 2007). Deer carcasses with known cause of mortality (e.g., vehicle collision) were also collected for sampling training to provide more similar conditions to antemortem testing. Before their first RAMALT sampling, trainees observed more-experienced samplers conducting the procedure on live animals in the field. Additionally, more-experienced samplers observed and guided less-experienced samplers during their first field collections.

During field seasons, deer were trapped in Iowa, Grant, and Dane counties, Wisconsin, USA, using either clover traps or drop nets. Deer were then chemically immobilized using intramuscular injections of butorphanol 27.3 mg/mL, azaperone 9.1 mg/mL, medetomidine 10.9 mg/mL (BAM, ZooPharm, Laramie, Wyoming, USA) at 1–2 mL per deer depending on age and estimated body weight; see Gilbertson et al. 2022 for additional capture information. For individuals >20 mo old, an incisiform tooth was collected during processing for aging by cementum annuli (Storm et al. 2014). RAMALT samples were typically collected toward the end of processing following the protocol given by Wolfe et al. (2007), but using Moredun RecSpec RAMALT speculums (Veterinary Instrumentation, Sheffield, UK). With an assistant retracting the deer's tail and holding the speculum in place, initial RAMALT samples were taken from the "six o'clock" position; resampling biopsies were always taken from a different position (three, nine, or 12 o'clock). In all cases, an approximately 2-cm^2 tissue sample (as in Thomsen et al. 2012) was taken about 1–2 cm cranial to the anorectal junction. All biopsies were collected using single-use equipment to prevent transmission between individuals. After sampling, atipamezole was administered for partial reversal (25 mg/mL, with 25 mg atipamezole administered per 10 mg of medetomidine; ZooPharm) and deer were released once able to stand and walk normally. Deer capture and handling protocols were approved under WDNR's Animal Care and Use Committee (Protocol: 16-Storm-01).

Following collection, RAMALT samples were stored between foam pads in individual histology cassettes in formalin and submitted to the Wisconsin Veterinary Diagnostic Laboratory, Madison, Wisconsin, USA, for CWD testing via IHC. Test results were either "positive," "not detected," or, if a sample lacked adequate lymphoid follicles (i.e., fewer than six follicles), "insufficient follicles" (ISF, or "insufficient sample"). If a sample was initially deemed ISF, it was often resectioned and retested; for the purposes of this study, a sample was deemed insufficient or inadequate if it was classified as ISF even after retesting.

To identify predictors of the occurrence of insufficient RAMALT samples (ISF), we fitted logistic regressions in R v4.2.0 (R Core Team 2018). Models were hypothesis driven (Table 1), and we used degrees of freedom spending to avoid overfitting (Giudice et al. 2012). Predictor variables included deer age, sex, sampler identity, the sampler's RAMALT collecting experience, the sampler's number of days of sampling experience, ambient light at the time of reversal injection (a proxy for the ambient lighting conditions at the time of RAMALT sampling), the number of RAMALT collections in a capture event (a proxy for the number of deer being processed at one time), the number of capture events in one day, and the minimum daily temperature (mean across Iowa County weather stations; National Oceanic and Atmospheric Administration n.d.). Adult deer of uncertain age (n=36) were conservatively estimated to be 2 yr old at initial capture. Ambient lighting was based on sun phases from the *suncalc* package in R (Thieurmel and Elmarhraoui, 2022), and was specific to capture coordinate locations, date, and daylight saving time changes. Model selection was by Akaike information criterion (Akaike 1974). The occurrence of inadequate samples was rare, which can bias coefficient estimates in logistic regressions (King and Zeng 2001); we therefore followed King and Zeng's (2001) approach for correcting coefficients and standard errors for rare-event bias.

A total of 32 unique samplers were trained for and performed RAMALT biopsy in the field, with samplers performing a mean of 25.12 biopsies (range, 2–93). Of the 806 RAMALT samples collected, 725 (90.0%) were deemed sufficient on initial CWD testing; another 38 samples (4.7%) were deemed adequate upon resectioning of tissue samples and retesting. Thus, a total of 763 or 94.7% of samples were ultimately adequate for CWD testing. Of the 33 RAMALT samples collected from previously sampled individuals, only 1 was classed as ISF (3.0%). The rarity of inadequate samples from previously sampled individuals precluded robust inference, but implied that resampling did not have a substantial impact on collecting inadequate samples. We did not observe any morbidity or mortality as a result of RAMALT collection (Gilbertson et al. 2022).

Our top model for the occurrence of insufficient RAMALT samples included deer age, sampler RAMALT collecting experience (not statistically significant), and ambient lighting at sample collection (Fig. 1). This model found an increased probability of an insufficient sample with increasing deer age and when the sample was collected at dusk (Table 2). To better understand the relationship between ambient light and adequate RAMALT samples, we fit an additional ad hoc model with deer age and the combined light conditions at induction and reversal (four categories with lighting at induction listed first, followed by lighting at reversal): day-day, day-dusk, dusk-dusk, and dusk-night. We excluded night-night as this

TABLE 1. Hypotheses for predictors of insufficient rectoanal mucosa–associated lymphoid tissue (RAMALT) samples from chemically immobilized free-ranging white-tailed deer (*Odocoileus virginianus*).

Predictor	Description	Hypothesis or rationale	Mean (range)[a]
Deer age	Continuous integer age in years	Hypothesize that older individuals have fewer lymphoid follicles; expect higher rates of insufficient RAMALT samples for older individuals	1.4 (<1–16)[b]
Deer sex	2 categories: female and male	Determine if rates of insufficient RAMALT samples vary by sex	Female: n=490 Male: n=313
Sampler identity	Identity of the person who collected a given RAMALT sample	Hypothesize that some samplers may be more or less skilled than others; expect one or more samplers to have higher rates of insufficient samples than others	32 unique samplers
Sampler's RAMALT sampling experience	nth RAMALT sample collected by a sampler (e.g., first RAMALT collected = 1)	Hypothesize that experience improves RAMALT sample collection; expect reduced rates of insufficient samples with increasing experience	20.4 (1–93)
Sampler's days of experience	nth day of RAMALT sample collection by a sampler (e.g., first day of RAMALT collection = 1)	Hypothesize that experience improves RAMALT sample collection; expect reduced rates of insufficient samples with increasing experience	13.9 (1–62)
Ambient light at time of reversal injection	A proxy for the ambient lighting at the time of RAMALT collection	Hypothesize that ambient lighting affects technique during sample collection; expect increased rates of insufficient samples when collections occur in night/dark conditions	Day: n=329 Dusk: n=367 Night: n=107
Number of RAMALT collections in capture event	A proxy for the number of deer being processed at one time	Hypothesize that sampler stress in managing multiple immobilized deer at once affects RAMALT collection; expect increased rates of insufficient samples with increased deer being processed	1.5 (1–5)
Capture events in a given day	Representation of the effort of capture crews in a given day	Hypothesize that long or exhausting days with many capture events affect sample collection technique; expect increased rates of insufficient samples on days with more capture events	2.7 (1–14)
Minimum daily temperature	Mean across Iowa county weather stations	Hypothesize that cold temperatures affect dexterity; expect increased rates of insufficient samples with lower temperatures	−10.4 C (−28.4–4.5)

[a] Means, with ranges in parentheses, are provided for continuous variables. For categorical variables, we provide sample sizes per stratum (sex and ambient light at time of reversal) or the number of unique strata (sampler identity).
[b] Adults of uncertain age (n=36) were conservatively estimated to be 2 yr old; as such, the actual mean age is probably higher than reported here.

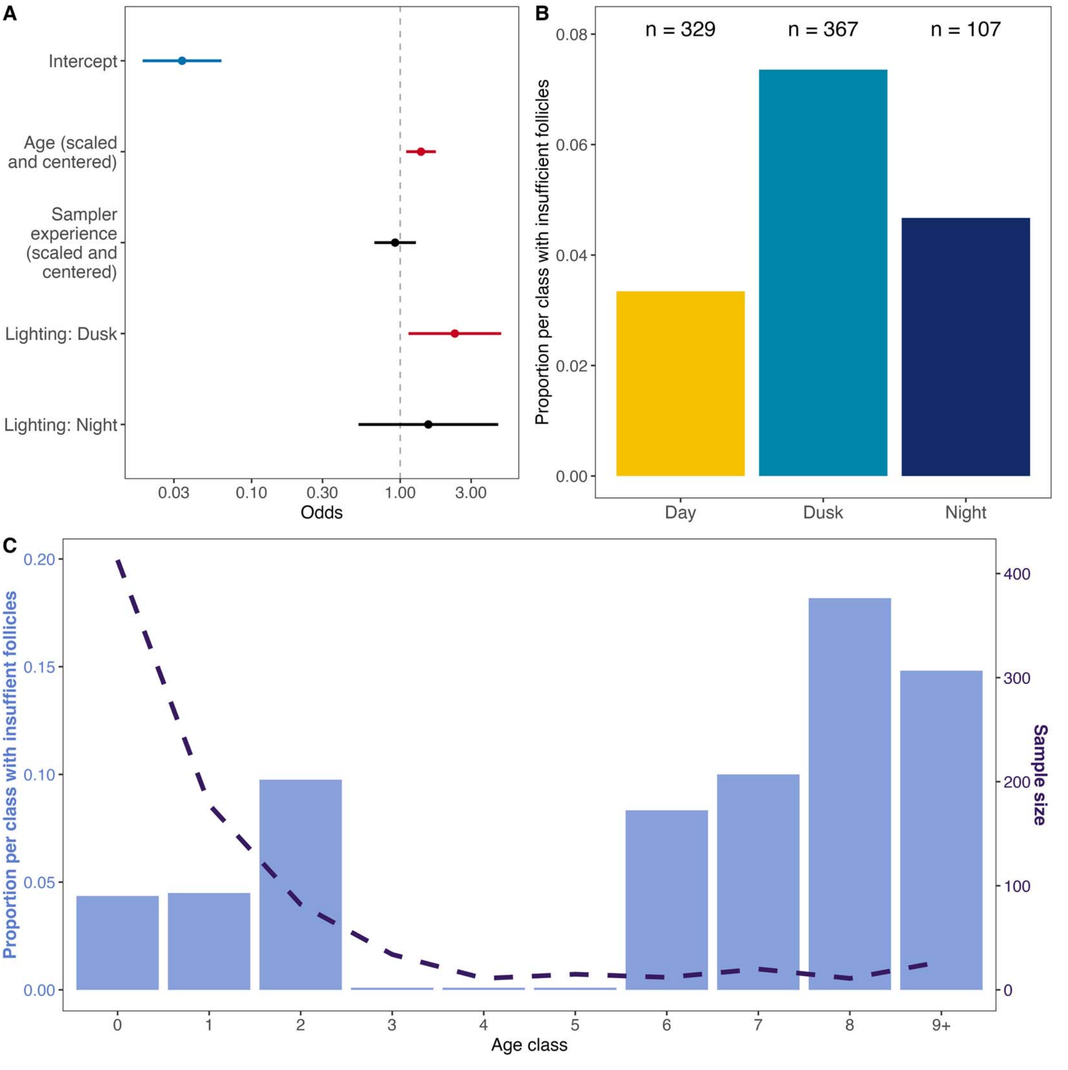

FIGURE 1. Occurrence of insufficient rectoanal mucosa–associated lymphoid tissue (RAMALT) samples from white-tailed deer (*Odocoileus virginianus*) under field conditions in Wisconsin, USA. Panel (A) gives results from a logistic regression for occurrence of insufficient RAMALT samples, with predictors on the y axis and odds estimates (points) and 95% confidence intervals (horizontal lines) corrected for rare event bias. Confidence intervals for significant predictors do not include 1 (vertical dashed line) and are colored by relationship direction (blue=negative, red=positive). Panels (B) and (C) show the proportion of insufficient RAMALT samples per ambient light class and age class, respectively. Sample sizes are given above each bar in (B). In (C), sample size per age class is given by the dashed purple line and right-hand y axis. In addition, no samples were insufficient for the 3–5 yr age classes, but bars are set to 0.001 for visibility. Note that adults of uncertain age were conservatively estimated to be 2 yr old, which may increase apparent rates of insufficient samples for this age class in (C), but makes the observed age effect in (A) conservative.

class included only 14 samples, none of which were inadequate, so we lacked heterogeneity in outcomes to make robust inference. This model found that samples collected at captures initiating in daylight and ending at dusk had the highest probability of insufficient samples (Table 2). We did not detect an effect of any of our other predictors on the occurrence of insufficient RAMALT samples.

Our results indicate that RAMALT samples suitable for antemortem CWD testing can be collected with a high degree of success, even by

TABLE 2. Results of logistic regressions for the occurrence of insufficient rectoanal mucosa–associated lymphoid tissue (RAMALT) samples from chemically immobilized free-ranging white-tailed deer (*Odocoileus virginianus*). Predictors that were considered statistically significant are shown in bold. All estimates and confidence intervals were corrected for rare-event bias. For the ad hoc model, ambient light predictors reflected lighting conditions at induction (listed first) and reversal (listed second); day-day was the reference category and night-night was excluded because of small sample sizes. In the top model, "day" was the reference ambient light category; Adults of uncertain age were conservatively estimated to be 2 yr old, making the age effect estimate in both models conservative.

Model	n[a]	Predictor	Exp(est)[b]	95% CI[c]
Top model	802	Intercept	0.03	(0.02–0.06)
		Age (scaled and centered)	**1.38**	**(1.10–1.74)**
		Sampler experience[d] (scaled and centered)	0.92	(0.67–1.27)
		Light: dusk	**2.33**	**(1.14–4.78)**
		Light: night	1.55	(0.52–4.56)
Ad hoc model	788	Intercept	0.03	(0.02–0.06)
		Age (scaled and centered)	**1.36**	**(1.08–1.70)**
		Light: day-dusk	**2.79**	**(1.20–6.48)**
		Light: dusk-dusk	2.09	(0.94–4.63)
		Light: dusk-night	1.80	(0.61–5.30)

[a] n=the sample size for the given model.
[b] Exp(est)=exponentiated coefficient estimate for each predictor.
[c] 95% CI=exponentiated 95% confidence interval.
[d] Sampler experience=the nth number of RAMALT collections completed by the sampler, to that particular sample collection date.

samplers with limited veterinary experience. In the interest of animal health, we did not compare our hands-on training program with one based on only lecture or video instruction. Nevertheless, clinical skills training in human and veterinary medicine highlights elements of our approach that aligned with literature-supported practices. For example, training with models (as in the harvested rectums used here) can be effective for training clinical skills, even when fidelity to the real tissue or material is low (Malone 2019). In addition, incorporating "near peers" as instructors (e.g., in-field mentoring performed by our experienced samplers) can facilitate effective training for both the new learner and the peer instructor (Malone 2019). This may help explain why we found no effect of sampler identity or experience: Novice samplers were guided through the process, ensuring that they were correctly following protocol. Based on clinical skills training research, future RAMALT biopsy training efforts could incorporate more video material in prelaboratory instruction to improve material retention (Langebæk et al. 2016; Malone 2019). In addition, if cadavers or harvested rectums are not available, other "lower-fidelity" models (i.e., less realistic) would probably still be useful for training technicians (Aulmann et al. 2015; Caston et al. 2016; Malone 2019).

Inadequate RAMALT samples were rare in our study, with our success rate (94.7%) higher than those reported elsewhere (56–91%; Wolfe et al. 2007; Keane et al. 2009; Thomsen et al. 2012). This does not appear to be explained by any differences in our biopsy protocol, as we closely followed the methods described in Wolfe et al. (2007) and Thomsen et al. (2012). When our samples were inadequate, they appeared to be associated with older deer and the ambient light conditions during collection. The observed age effect confirms findings in other studies (Spraker et al. 2009; Geremia et al. 2015). Because deer of uncertain age were conservatively estimated to be 2 yr old, the strength of the age effect may be even greater than we observed in our models. The effect of ambient light conditions on successful sample collection may represent underutilization of supplementary lighting (e.g., headlamps). This is especially likely given that the highest odds for collecting an inadequate sample occurred when captures were initiated in daylight and ended at dusk. In these cases, the capture team may have had adequate lighting for all other processing activities and therefore underestimated the lighting limitations for RAMALT sampling. To mitigate the effects of ambient lighting on RAMALT biopsy collection, project leaders could ensure that samplers always wear headlamps during RAMALT biopsies, regardless of the perceived lighting conditions.

Our study demonstrated that, with appropriate training, RAMALT biopsy can be a practical sample type for high-volume antemortem CWD testing using a large team of technicians with limited veterinary background, even in challenging conditions with low environmental temperatures and multiple deer being processed simultaneously, although supplementary lighting is advisable to promote adequate sample collection. This should help research, management, and production agencies to implement antemortem CWD testing protocols, even when faced with challenging field conditions.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/JWD-D-24-00020.

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