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RESEARCH ARTICLE

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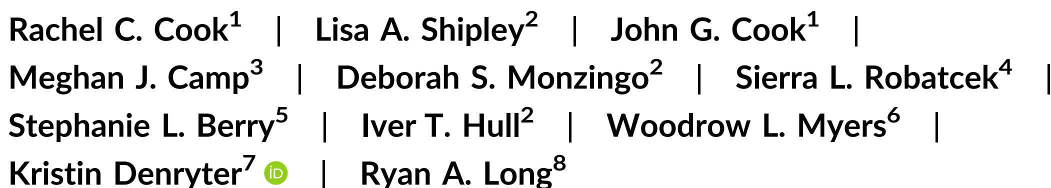
Sequential detergent fiber assay results used for nutritional ecology research: Evidence of bias since 2012



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Abstract

Forage quality surveys can provide a variety of insights into the nutritional well-being of herbivores. Even small differences in how much of the food eaten can be digested and used for life requisites (dry matter digestibility %; DMD) can affect performance of ruminants, and thus methods for determining forage digestibility must be accurate, repeatable, and robust among laboratories and over time. In 2013, we observed levels of DMD as determined from sequential detergent fiber assays with the filter bag method and the ANKOM fiber analyzer$^{200/200®}$ that were greater than expected given the plant species, season, and environmental setting. Using stock-piled and previously analyzed forage samples from Oregon, Washington, and Idaho, we evaluated whether fiber results had shifted since 2012, potential causes of the shifts, and whether results since 2012 aligned with data on ungulate performance. Beginning in 2012, our results indicated that sequential detergent fiber analysis began significantly overestimating DMD compared to the same samples analyzed using identical methodology prior to 2012. Magnitude of the difference increased as soluble fiber increased and overestimated DMD

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by up to 31 percentage points. We detected no changes in laboratory procedures (e.g., fiber bag design or origin of chemical solution) nor differences among laboratories using identical methodologies (e.g., Washington State University versus Dairy One) that might explain the shift, and thus were unable to pinpoint the cause. Results obtained prior to 2012 better explained ungulate performance (e.g., body fat, pregnancy, population trends) and thus we developed predictive equations ($0.59 \le r^2 \le 0.88$) to align post-2012 lab results with those obtained before 2012. Shifts in assay results and inconsistencies in laboratory methods have marked potential to affect inferences from studies of ruminant nutritional ecology. We provide recommendations for researchers using sequential detergent fiber analysis to measure DMD.

KEYWORDS
ANKOM Technology, dry matter digestibility, *in vitro* digestibility, nutritional ecology, ruminant nutrition, sequential fiber fractionation

Influences of nutrition on mammalian physiology are fundamental and cascading, with potentially important effects on reproduction, growth, and population dynamics (Crête and Huot 1993, Hjeljord and Histol 1999, Monteith et al. 2013, Hurley et al. 2014, Cook et al. 2018). Nutritional well-being of ruminants is generally a function of the concentration of useable energy and nutrients in forage and the amount of food consumed each day plus interactions between the 2 (Minson and Wilson 1994, Illius 1997, Owen-Smith 2002, Cook et al. 2016). Although forage quantity is most often associated with nutritional limitations in ruminants (i.e., through density-dependent pathways; Caughley 1970, 1976; Couturier et al. 1990; Crête and Huot 1993; Vucetich and Peterson 2004), numerous studies have shown that nutritional quality of forage can be inadequate to meet requirements of lactating females during the summer and autumn regardless of the quantity of food available (i.e., through density-independent pathways; Cook et al. 2016, 2018; Ulappa et al. 2020; Denryter et al. 2022).

Diets eaten by wild ruminants are lower in nutritional quality than those of carnivores because, in part, the plant cell wall contains relatively high amounts of structural carbohydrates (i.e., fiber) that requires extensive mastication and microbial fermentation to digest. Even then, only a portion of the energy and nutrients contained in the plant is available to the animal (i.e., is digestible). For example, digestibility ranged from 30 to 80% in ruminant diets during summer in the northwestern US (Cook 2002; Cook et al. 2014, 2016; Berry et al. 2019; Ulappa et al. 2020). Cell-wall fiber also increases rumination time and reduces passage rate of ingesta, thus decreasing the total amount of food ruminants can eat each day (Weston and Poppi 1987, Robbins 1993, Grey and Servello 1995, Hobbs 2003, Cook et al. 2004). Accordingly, low-quality (high fiber) forage, no matter the quantity, cannot substitute for high-quality forage (Cook et al. 2004, Hanley et al. 2012), and even small changes in digestibility of forages can have significant effects on the performance of ruminants (White 1983, Cook et al. 2004, Cebrian et al. 2008).

Dry matter digestibility (%; DMD) represents the portion of plant components (e.g., energy) that is available to ruminants and is influenced by (1) the amount and composition of cell wall, and to a lesser extent, (2) concentrations of condensed tannins that bind protein and create indigestible complexes (Robbins 1993). Three common methods for measuring DMD of forages are *in vivo* (i.e., in the animal), *in vitro* (i.e., in the lab), and sequential fiber analysis. Measuring DMD of forages directly via *in vivo* digestion trials (i.e., the difference between the amount of food

consumed by the animal and the amount it excretes in feces) is time consuming and logistically complex because it requires captive animals, separate trials for each food type examined, time for microbial and behavioral acclimation, and adequate supplies of each food type. *In vitro* methods that simulate the digestive process of ruminants using laboratory methods can be complicated and inconsistent (e.g., sources and quality of rumen fluid are often variable; Shipley et al. 2020). Sequential detergent fiber assays (Goering and Van Soest 1970) are an alternative method to estimate DMD that approximates *in vivo* digestibility of forages by using widely available chemical solutions and equipment for simulated digestion. Sequential detergent fiber assays produce estimates of digestibility of individual plant cell constituents (i.e., neutral detergent fiber [NDF], acid detergent fiber [ADF], acid detergent lignin [ADL], and acid insoluble ash [AIA]) that differ in digestibility (Appendix A). Values from individual constituents of sequential detergent fiber assays are related to *in vivo* digestibility through the summative equations of Robbins et al. (1987), which were developed and tested to predict DMD of forages for elk (*Cervus canadensis*) and deer (*Odocoileous* spp.; Hanley 1984, Hanley et al. 1992, Robbins 1993, Parker et al. 1999) and are based on the biological interactions between plant chemical composition and digestion in ruminants. For tannin-containing plants (primarily forbs and shrubs), the protein-binding capacity of tannins can reduce digestibility and is also included in the equation. The summative equations of digestibility have been used to study the nutritional ecology of a variety of ruminant species (Atkins et al. 2019, Berry et al. 2019, Sittler et al. 2019, Anderson 2020, Denryter et al. 2022).

Surveying forage quality or using captive animals to measure diet quality can provide insights into a variety of landscape management scenarios or can be used to evaluate success of habitat improvements (Wagoner et al. 2013, Cook et al. 2016, Hull et al. 2020, Ulappa et al. 2020). Particularly given that small differences in DMD can significantly affect performance of ruminants, deriving these management insights depends on comparing accurate estimates of DMD in available forage to levels required by the animal. As such, methods for determining forage digestibility need to be accurate, repeatable, and robust across space and time. Laboratory analysis, a key step in the process of evaluating forage quality, is broadly assumed to be accurate and consistent. This is especially true for sequential detergent fiber assays used to estimate DMD of forage because laboratories use reagents that are stringently manufactured. Since 1986, with their dietary fiber analyzer and filter bag technology, ANKOM Technology (www.ankom.com) has provided a state-of-the-art, mostly automated system for sequential fiber assays that is used in laboratories in ≥120 countries (Appendix A). The ANKOM system reduces labor and per sample costs, eliminates the use of vacuum flasks and crucibles, and uses reagents that are manufactured to exacting standards, all clear advantages over older, *in vitro* techniques (Vogel et al. 1999).

Collectively, starting in the late 1990s, we have conducted sequential detergent fiber analysis using the ANKOM dietary fiber analyzer and filter bag technology to estimate DMD of tens of thousands of samples using the Wildlife Habitat and Nutrition Laboratory at Washington State University (hereafter WSU) in Pullman, WA, USA. However, during 2013, we noticed patterns of DMD in several sets of samples that did not align with expectations for specific plant species and environmental and temporal settings. Thus, between 2013 and 2020, using multiple, large sets of stockpiled native vegetation samples that had already been analyzed using sequential detergent fiber assays (Table 1), we conducted the following steps: 1) reanalyzed a subset of stockpiled samples and directly compared results obtained before 2012 to results obtained after 2012; 2) evaluated samples analyzed before and after 2012 across various studies conducted in the northwestern U.S. to determine if patterns of DMD observed under objective one held for a larger, more diverse data set; 3) identified causes of differences in assay results; 4) analyzed a strategically chosen subset of samples to evaluate additional shifts in assay results since 2013 and possible effects of storage time; 5) evaluated which results (i.e., pre-2012 versus post-2012) were more biologically reasonable using *in vitro* DMD estimates and data on performance of wild ungulates from the same region; and 6) based on findings, reanalyzed a subset of stockpiled samples and developed equations to align post-2012 NDF, ADL, and AIA results to pre-2012 results (i.e., developed correction equations). Throughout our study process, we also learned that methodology for sequential fiber assays varied among laboratories. Therefore, we also investigated the degree to which each of the methodological inconsistencies could affect DMD. Herein, we discuss implications for the field of wildlife nutritional ecology and provide recommendations for wildlife professionals using sequential detergent fiber analysis.

TABLE 1 Sample type, years collected, sample size (*n*), location of samples collected, and methodology citation for plant samples analyzed from Idaho (ID), Oregon (OR), Washington (WA), USA, and British Columbia (BC), Canada. These samples were used to: 1) demonstrate a shift in sequential detergent fiber assay results since 2012; 2) test for what may have been causing these shifts; 3) compare pre- and post-2012 dry matter digestibility estimates with *in vitro* derived estimates; 4) develop correction equations to align post-2012 results with pre-2012 results; 5) validate the correction equations; and 6) evaluate the magnitude of effect on dry matter digestibility calculations of methodological inconsistencies found across laboratories.

Sample type	Year collected	*n*	Location	Source	Use in manuscript[a]
Elk diet composites	2006	30	eastern OR	Cook et al. unpubl. data	Pre-2012 vs Post-2012
Elk diet composites	2005–2012	719	eastern OR	Cook et al. unpubl. data	Pre- and Post-2012
Deer diet composites	2009	144	eastern WA	Wagoner et al. 2013	Pre-2012 distribution
Elk diet composites	2005–2007	258	eastern OR	Cook et al. unpubl. data	Pre-2012 distribution
Elk diet composites	2004–2006	1,484	eastern OR	Cook et al. 2014	Pre-2012 distribution
Elk diet composites	2000–2002	1,364	western OR/WA	Cook et al. 2016, 2018	Pre-2012 distribution
Elk diet composites	1998	132	eastern OR	Cook unpubl. data	Pre-2012 distribution
Individual plants species	2003–2004	210	western WA	Lopez Perez 2006	Pre-2012 distribution
Individual plants species	2009	178	eastern WA	Wagoner et al. 2013	Pre-2012 distribution
Individual plants species	2004–2005	478	eastern WA	Myers et al. unpubl. data	Pre-2012 distribution
Individual plants species	2000–2002	1,070	western OR/WA	Cook et al. 2016, 2018	Pre-2012 distribution
Individual plants species	2004–2006	1,174	eastern OR	Cook et al. 2014	Pre-2012 distribution
Deer diet composites	2014–2019	374	eastern WA	Berry et al. 2019, Hull et al. 2020	Post-2012 distribution
Elk diet composites	2006–2012	461	eastern OR	Cook et al. unpubl. data	Post-2012 distribution
Deer diet composites	2012–2013	459	western WA	Ulappa et al. 2020	Post-2012 distribution
Individual plants species	2014–2019	564	eastern WA	Berry et al. 2019, Hull et al. 2020	Post-2012 distribution
Individual plants species	2012–2013	264	western WA	Ulappa et al. 2020	Post-2012 distribution
Plant species/plant groups	2016–2017	3,058	north-central ID	Monzingo 2020	Post-2012 distribution
Elk diet composites	2006	30	eastern OR	Cook et al. unpubl. data	Fiber bags, laboratory
Elk diet composites	2000–2007	36	OR and WA	Cook et al. 2014, 2016, unpubl. data	NDF, ADF solution
Elk diet composites	2006	30	eastern OR	Cook et al. unpubl. data	Sodium sulfite
Plant species/plant groups	2016–2017	2,003	southern ID	Robatcek 2019	AIA
Individual plants species	2015	30	northeastern BC	Cook et al. unpubl. data	1.0 g vs 0.5 g
Elk diet composites	2006	30	eastern OR	Cook et al. unpubl. data	Dairy One *in vitro*
Elk diet composites	2000–2007	50	OR and WA	Cook et al. 2014, 2016, unpubl. data	CSU[b] *in vitro*
Elk diet composites	2006	11	eastern OR	Cook et al. unpubl. data	*In vitro* comparison
Elk diet composites	2000–2006	100	OR and WA	Cook et al. 2014, 2016, unpubl. data	Correction equations

TABLE 1 (Continued)

Sample type	Year collected	*n*	Location	Source	Use in manuscript[a]
Elk diet composites	2011	47	Western OR	Cook et al. unpubl. data	Correction validation
Plant groups	2004–2005	24	Eastern WA	Myers et al. unpubl. data	Correction validation
Elk diet composites	2001–2013	17	OR	Cook et al. 2014, 2016	Standards
Caribou diet composites	2013	7	northeastern BC	Denryter et al. 2020, 2022	Standards

[a]ADF, acid detergent fiber; AIA, acid insoluble ash; NDF, neutral detergent fiber.

[b]CSU, Colorado State University.

METHODS

To determine if sequential detergent fiber assay results had shifted since 2012, whether these differences in fiber results were consistent across a suite of research projects, and what may have caused these differences, we acquired a variety of data from samples that were originally collected and analyzed from 1998 to 2019 (Table 1). Researchers in all studies handled and analyzed all forage samples identically and all samples were analyzed by WSU except where noted. All were samples of native vegetation and were either composite diet samples collected during captive ruminant foraging studies (i.e., a mix of plant species representing diets consumed in one sample), a single plant species per sample (whole or separated by part such as leaf and stem), or a mix of plant species by life form group (e.g., deciduous shrubs, perennial forbs). When samples were originally collected, researchers placed clipped forage in plastic freezer bags and immediately buried bags under ice for ≤4 days before being transferred to a freezer. Frozen forage samples were freeze-dried, ground in a Wiley mill to pass through a 1-mm screen and stored in plastic or paper envelopes.

The standard sequential fiber methodology used for all samples, except where noted, was conducted as follows. Using ~0.5 g (usually between 0.495–0.505 g) of each sample per replicate, WSU calculated percent NDF, ADF, ADL, and AIA using sequential fiber fractionation with filter bags, alpha amylase, and the ANKOM fiber analyzer$^{200/200®}$ (ANKOM Technology, Fairport, NY, USA; Goering and Van Soest 1970). Washington State University used NDF and ADF powdered concentrate purchased from ANKOM and added enough distilled water (and triethylene glycol for NDF and 1 N sulfuric acid for ADF) to make 20 L of each respective solution (ANKOM Technology; Goering and Van Soest 1970). Full batches of solution were made at one time and a stir bar was used to remix the solution and prevent settling. Washington State University added 20 g sodium sulfite to the ANKOM fiber analyzer at the NDF step for all composite diet samples and for plant samples that likely contained tannins. Grasses, lichens, and plants in the *Asteraceae* family lack tannins and thus were assayed without sodium sulfite (Mould and Robbins 1981, Hanley et al. 1992; Appendix A).

For all samples analyzed for the objectives herein, WSU assayed each sample in duplicate. We averaged each fiber component (i.e., NDF, ADF, ADL, and AIA) across duplicates and used the averages to predict DMD as per the summative equation of Robbins et al. (1987). If we detected differences in predicted DMD between duplicates >2.5 percentage points, WSU analyzed a triplicate of the sample and we averaged each fiber component across triplicates before predicting DMD. For the purposes of this paper, we did not include the effect of tannins in our calculations (i.e., tannin level was set to 0) unless noted otherwise. All results are reported on a dry-matter basis.

Detecting a shift in assay results

We evaluated whether fiber assays began estimating different levels of NDF, ADF, ADL and AIA since 2012 in 2 steps. First, we chose 30 composite diet samples previously stockpiled and originally analyzed prior to 2012 (Pre-2012 vs Post-2012 row in Table 1) that had at least 5 g of sample remaining and that represented a wide range

of variation in NDF, ADF, ADL, AIA, and DMD. The 30 samples ranged from 30.6–65.5% NDF ($\bar{x}$ = 49.3), 20.1–41.6% ADF ($\bar{x}$ = 32.5), 3.7–13.2% ADL ($\bar{x}$ = 7.1), 0.5–6.9% AIA ($\bar{x}$ = 2.8), and 42.9–67.1% DMD ($\bar{x}$ = 55.4) (based on original, pre-2012 results). We reanalyzed these samples in 2014 and directly compared pre-2012 results to post-2012 results.

Second, to determine if differences in sequential fiber assay results evident in step one held for larger, more diverse data sets, we compared distributions of fiber assay results using 2 data sets from previous studies analyzed either before or after 2012 (Table 1). First, we evaluated a data set from the Starkey Experimental Forest in northeastern OR (Pre- and Post-2012 row in Table 1) where captive female elk were transported to the same permanent enclosures at the same time of year (early August) from 2005 to 2012. Composite diet samples from the captive elk trials collected from 2005 to 2007 were analyzed by WSU during 2008 (1–3 years after collection) whereas samples collected from 2008 to 2012 were analyzed during 2013 (1–5 years after collection). The second set of data were less coupled in space and time but were collected in forest and rangeland communities only in Oregon, Washington, and northern Idaho, only during the growing season (April–October), and only included current annual growth to reduce effects of environmental variation on results (Pre-2012 distribution rows in Table 1 versus Post-2012 distribution rows in Table 1). For both data sets in step 2, we present 99% confidence intervals (CIs) to evaluate differences in mean DMD pre- versus post-2012. If differences between pre- and post- 2012 assay results identified in step one were consistent using the broader data sets in step 2, the combined results would confirm that differences in assay results were pervasive and consistent across a wide variety of environmental settings.

Potential causes of shift in assay results

If a shift in NDF, ADF, ADL, AIA and DMD values was detected, we attempted to determine the cause using the same 30 composite diet samples described in step one above that had been previously stockpiled and analyzed. We first considered the most obvious sources of error: a change in protocol or methodology by a specific laboratory technician or a miscalculation in the assay calculation spreadsheet. We manually checked the calculation spreadsheet and found no errors. To test for laboratory-specific issues, we submitted these 30 composite diet samples to Dairy One (Ithaca, NY, USA) working to ensure that their methodology matched exactly that used by WSU (fiber bags, laboratory row in Table 1). In addition, we requested the specific mass measurements from each fiber step to manually calculate NDF, ADF, ADL, and AIA ourselves. We chose Dairy One because it is one of the commercial labs that ANKOM rigorously monitors for quality control (B. Layton, ANKOM Technology, personal communication).

Second, differences in laboratory equipment or reagents could cause a shift in assay results. Since 2012, ANKOM changed the design of their fiber bags and digestion trays (B. Layton, personal communication). Thus, using portions of the same 30 composite samples analyzed by WSU and Dairy One, WSU also estimated NDF, ADF, ADL, and AIA using fiber bags originally purchased in 2005 and again using fiber bags originally purchased in 2012 (fiber bags, laboratory row in Table 1). Because WSU was not using the new tray design, we assumed we had accounted for any effect of this equipment change with samples submitted to Dairy One.

Third, the solution used for NDF and ADF can be purchased in 3 forms: pre-mixed liquid solution from ANKOM to use as-is, pre-mixed powdered concentrate from ANKOM that is mixed with distilled water at the laboratory before use (the preferred protocol of WSU), or individual components that are mixed at the lab as per Goering and Van Soest (1970). Ideally, we would have used this same set of 30 samples to compare NDF, ADF, ADL, and AIA using the pre-mixed commercial solutions versus those when the solutions were mixed from individual chemicals. However, because sample material was continually depleted for each test (~0.5 g of sample was used for every test; 1.0 g in duplicate) we replaced several of the original 30 samples with new composite diet samples previously stockpiled from a different region in Oregon or Washington that had been originally analyzed prior to 2012. We also added 6 additional composite diet samples that had originally been analyzed prior to 2012 to expand the range of variation for all fiber steps and DMD (NDF, ADF solution row in Table 1). These 36 samples ranged from

18.4–74.1% NDF ($\bar{x}$ = 48.3), 9.4–61.2% ADF ($\bar{x}$ = 32.1), 1.0–34.7% ADL ($\bar{x}$ = 8.5), 0.02–11.4% AIA ($\bar{x}$ = 2.7), and 17.5–78.8% DMD ($\bar{x}$ = 54.5; based on original, pre-2012 results). When assaying the samples using solution mixed from individual chemicals, WSU did not add decalin (decahydronapthalene; an anti-foaming agent used by Goering and Van Soest [1970]), as per instructions from ANKOM Technology (B. Layton, personal communication) because foaming is not a problem with the pressurized instrument.

Finally, we chose 24 composite diet samples stockpiled and analyzed previously as standards to track any additional shifts in sequential detergent fiber assays that may have occurred from 2016 to 2020 (Standard rows in Table 1). We chose samples that had an excess of ground plant material (usually >10 g), were collected across a wide range of years (2000–2013) in case length of time samples were stored was a confounding issue, and that spanned a wide range of DMD (21.4–76.9%). We analyzed these samples specifically for their use as standards first in 2016 and used those results of DMD as a baseline for subsequent comparison to track additional changes across time. We then analyzed these same samples in May 2018, May 2019, February 2020, and September 2020, calculated DMD and graphed each of these results against the 2016 baseline data. If no additional shifts in NDF, ADF, ADL, and AIA (and thus, DMD) occurred over that time, we expected all lines would overlap with a slope of 1.0 and an intercept of 0. In addition, grouped by length of storage time, we graphed differences from the 2016 baseline DMD (baseline DMD minus each successive DMD estimate in percentage points) for each sample. If storage time of samples was a factor, we expected differences to be consistently >0 and to change as storage time increased.

Additional inconsistent methodological considerations

When determining what factors may have caused the shift in sequential fiber assay results since 2012, we discovered several methodological inconsistencies that could potentially affect results across laboratories. Using results and methodology from WSU as a basis for comparison, we evaluated the degree to which these methodological inconsistencies could potentially impact DMD estimates.

Estimating AIA

When requesting sequential fiber analysis from Dairy One, the NDF, ADF, and ADL calculations were comparable to WSU calculations. We determined, however, that AIA calculations differed between the 2 laboratories (M. Reuter, Dairy One, personal communication). The AIA component used in the summative equations of Robbins et al. (1987) is estimated from an aliquot of forage sample that has been through the NDF, ADF, and ADL stages (i.e., the fourth step of the sequential fiber assay; Appendix A). Because of different objectives, many laboratories that analyze agricultural samples, including Dairy One, calculate AIA on a separate aliquot of sample that has not been through the previous stages of the sequential fiber assay, which can influence the DMD results. We used the data set that led us to discover this methodological difference to assess the effect this calculation had on both AIA and DMD (n = 2,003; AIA row in Table 1).

Sodium sulfite

Washington State University Laboratory uses sodium sulfite only for composite diets and plants that are known to contain tannins (e.g., shrubs, conifers, ferns, and some forbs; Mould and Robbins 1981, Hanley et al. 1992). However, debate exists in the literature regarding whether sodium sulfite should be used in sequential analysis (Krueger et al. 1999), which has led to inconsistencies in methodologies among labs. To determine the potential effect of not adding sodium sulfite to the fiber analyzer at the NDF stage for tannin-containing plants, we analyzed the same 30 composite

diet samples used above for testing differences due to specific laboratories or equipment (Sodium sulfite row in Table 1) with and without the addition of 20 g of sodium sulfite and reported the difference in estimated DMD.

Sample mass

The amount of sample placed in the fiber bag, particularly for native vegetation, has the potential to influence the degree to which solutions can penetrate the ground plant matter, i.e., if the fiber bag is packed full of sample, the various solutions will not penetrate all sample matter equally (B. Layton, personal communication). ANKOM recommends 0.5 g of sample per replicate, but their methodology does not specify the consequences of using more or less sample. Both WSU and Dairy One use ~0.5 g of sample per replicate, but a commercial lab in Alaska used 1.0 g of sample (K. Sittler, Wildlife Infometrics, personal communication). Using 30 stockpiled samples collected during 2015 near Fort St. John, British Columbia (1.0 g versus 0.5 g row in Table 1), we analyzed each sample using ~1.0 g of sample per replicate and again using ~0.5 g of sample per replicate and reported the difference in estimated DMD. We expected plant volume would affect results, particularly given the volume to weight ratio of native vegetation as compared to grain and cured hay (i.e., lower-density plant samples require a significantly greater volume to reach 0.5 g than higher-density plant samples). For this reason, we specifically chose plant species from a variety of plant groups that would provide a range of DMD and a broad range of volume to mass ratios: arboreal lichens, terrestrial lichens, evergreens including conifers, grasses, ferns, perennial forbs, deciduous shrubs, a rock-borne foliose lichen, mushrooms, and berries. As per general protocol (Mould and Robbins 1981, Hanley et al. 2012), we added 20 g sodium sulfite to the fiber analyzer at the NDF step only for tannin-containing plants (berries, evergreens, ferns, perennial forbs, and deciduous shrubs) when running both 0.5 g and 1.0 g of sample.

Statistical analysis

Detecting a shift in assay results and potential causes

We evaluated 4 questions with our main analysis: 1) Were post-2012 lab results significantly different than pre-2012 lab results?; 2) If so, what aspect of the relationship was different: were differences consistent (slope = 0), or were they related to the pre-2012 lab result value for NDF, ADF, AD:, AIA, or DMD (i.e., slope for trend line ≠ 0), and were differences associated with the other test factors such as different bags, labs, or chemical solutions?; 3) Given the sequential nature of the assays (Appendix A), did the relative magnitude of any difference increase from NDF to ADF to ADL to AIA?; and 4) Was there evidence that any factor we tested (e.g., different bags, labs, chemical solutions) produced results that differed from the current (post-2012) WSU results? To answer these questions simultaneously, we used linear regression (R version 4.4; R Core Team 2021) with the difference from pre-2012 NDF, ADF, ADL, AIA, and DMD values (pre-2012 minus post-2012) as our response variable. We included pre-2012 values of NDF, ADF, ADL, AIA, and DMD as predictor variables in the corresponding models. We also included test type (i.e., 2005 fiber bag, 2012 fiber bag, different laboratory, chemical solution) as a second, categorical predictor, along with the interaction between the 2 variables (pre-2012 value × test type). Because we used a different set of samples to compare NDF and ADF solutions that we developed from individual chemicals versus those that were pre-mixed by the manufacturer, we ran 2 separate analyses to test for these effects. To evaluate a potential laboratory effect (i.e., Dairy One results) and the different fiber bags collectively, we fit 5 models (one each for NDF, ADF, ADL, AIA, DMD; hereafter referred to collectively as fiber steps) to data from 120 samples. To evaluate if fiber results differed depending on the origin of the chemical solution (i.e., pre-mixed versus not), we fit 5 models (again, one for each of the fiber steps) to data from 72 samples. In all models we used the post-2012 WSU assay results as the basis for comparison for each test type. We tested for evidence of a relationship between the pre-2012 fiber step values (predictor) and the difference between the pre-2012 and post-2012 values (response) by calculating

90% CIs (equivalent to alpha = 0.1) for the coefficients of each fiber step. If the 90% CIs for the slope included 0, we concluded that there was no evidence of a difference between pre-2012 and post-2012 fiber values. Similarly, we calculated 90% CIs for coefficients for test type (varying intercepts), and the interaction between test type and each fiber step (varying slopes). If 90% CIs for coefficients for predictor variables (slopes, or in the case of categorical variables, intercepts) or their interactions included 0, we concluded that there was not a significant effect. We were more concerned about the risk of not detecting relationships in differences between pre- and post-2012 laboratory values and test types when a relationship existed (Type II error) than with incorrectly identifying at least one statistically significant relationship (Type I error), because even small differences in nutrition can have biologically meaningful effects on ruminant performance. We evaluated adherence to assumptions of the linear models using residual plots and QQ plots prior to calculating CIs.

Development of correction equations

To develop equations to correct for differences in sequential detergent fiber results obtained after 2012, we used 100 composite elk diets collected during 2000–2002 in western Washington and Oregon ($n = 45$) and during 2004–2006 in eastern Oregon ($n = 55$) to build separate correction equations for NDF, ADL, and AIA (Correction equations row in Table 1). Original assays for the stockpiled samples were conducted between 2003–2007 at WSU. Current assays were conducted during 2016 at WSU.

We used residual plots and QQ plots to evaluate adherence to model assumptions (e.g., normality and homoscedasticity of errors). If model assumptions were violated, we converted the percentages to proportions and fit a beta regression model (Ferrari and Cribari-Neto 2004). The beta distribution is flexible enough to accommodate highly variable proportion data in the interval (0,1), especially when the variance increases with the mean (Ferrari and Cribari-Neto 2004) but does not guarantee better predictions. We fit beta regression models using the betareg package in the R statistical environment (Cribari-Neto and Zeileis 2010, R Core Team 2021), and we evaluated the model fit using raw, Pearson, and deviance residuals (McCulloch et al. 2008). We also compared predictions from the beta regression models to those from the standard linear regression models.

We secured 2 independent sets of forage samples previously stockpiled that had been analyzed before 2012 (Correction validation rows in Table 1) to help validate our correction equations. Both sets of samples had been originally analyzed by WSU either in 2006 or early in 2012 with the same methodology as described above. We chose a subset of those samples to represent a wide range in variation in DMD ($n = 47$ composite diet samples from southwestern Oregon with pre-2012 DMD ranging from 35.8–69.5% and $n = 24$ samples mixed by plant group from northeastern Washington with pre-2012 DMD ranging from 29.0–65.8%) and reanalyzed them during 2016 or 2018 to estimate DMD. We graphed predicted DMD (as estimated by correcting NDF, ADL, and AIA separately) and current observed DMD (2016 or 2018) against pre-2012 observed DMD. If our predictive models were robust, we expected a slope close to 1.0 and an intercept close to 0.0 in the regression of predicted versus pre-2012 DMD.

RESULTS

Detecting a shift in assay results

All analyses indicated that post-2012 assays resulted in markedly lower estimates of NDF, ADF, ADL and AIA, and thus markedly higher estimates of DMD than pre-2012 assays. First, using identical samples analyzed pre-2012 and again post-2012, we found a strong linear relationship between pre-2012 values calculated in each step of the

sequential detergent fiber assay (NDF, ADF, ADL, AIA) and the difference between those values and post-2012 values; a similar relationship was also evident for DMD (Table 2, Figure 1). In addition, the slope of the relationship increased across the sequential steps (from NDF to ADF to ADL to AIA) indicating that all lab assays conducted after 2012 produced significantly different fiber values than assays conducted before 2012, and that as fiber content increased, so too did this difference.

Second, 99% CIs of mean DMD from 461 composite diet samples analyzed before 2012 (DMD ranged from 42.9–67.1%; $\bar{x}$ = 56.4%; 99% CIs = 55.6–57.1%) did not overlap 99% CIs from samples collected from the same location in the same month but in different years and analyzed after 2012 (DMD ranged from 53.7–77.2%; $\bar{x}$ = 67.8%; 99% CIs = 67.4–68.3%; Figure 2A). Similarly, 99% CIs of mean DMD from 6,492 samples analyzed before 2012 did not overlap 99% CIs of 5,180 samples analyzed after 2012 (Figure 2B,C). Mean DMD of composite diet samples analyzed before 2012 was 58.5% (99% CIs = 58.2–58.8%) versus 66.2% (99% CIs = 65.7–66.8%) for those analyzed after 2012; mean DMD of forage samples analyzed before 2012 was 56.4% (99% CIs = 55.9–56.9%) versus 67.7% (99% CIs = 67.4–68.1%) for samples analyzed after 2012.

We found no evidence of additional shifts in fiber assay results from the WSU laboratory from 2016 to 2020 (Figure 3A), nor any evidence that as storage time increased, the difference in DMD from 2016 baseline values averaged >0 or consistently changed over time (Figure 3B). Mean change in DMD from baseline (2016 values) across 4 years was 0.44 percentage points for samples stored one year, −0.53 percentage points for samples stored for 3 years, −0.43 percentage points for samples stored for 10 years, and 0.99 percentage points for samples stored for 12–15 years.

TABLE 2 Slope coefficients (β), standard errors (SE), degrees of freedom (df), and 90% confidence intervals (CI) for the main effects of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), acid insoluble ash (AIA) and dry matter digestibility (DMD) when evaluating the difference between pre-2012 results and post-2012 results. The same forage samples were analyzed pre- and post- 2012 and we evaluated the difference in the results as a function of pre-2012 NDF, ADF, ADL, AIA, DMD, the test for potential factors (e.g., fiber bags, different lab, solutions), and the interaction. We used linear regression and set the post-2012 WSU assay results as our comparison level. If the 90% confidence intervals (CI) for the slope included 0, we concluded that there was no evidence of a difference between pre-2012 and post-2012 fiber values. Starred β values indicate when an interaction between pre-2012 results and test type was significant (i.e., slope of one of the tests for potential factors was significantly different than post-2012 WSU results).

Main effect (pre-2012)	β	*SE*	df	CI
NDF[a]	0.239	0.049	112	0.158–0.320
ADF[a]	0.389*	0.046	112	0.313–0.465
ADL[a]	0.393	0.074	112	0.270–0.516
AIA[a]	0.745*	0.042	112	0.675–0.815
DMD[a]	0.498	0.068	112	0.385–0.611
NDF[b]	0.245	0.051	68	0.194–0.330
ADF[b]	0.297	0.062	68	0.235–0.400
ADL[b]	0.218	0.048	68	0.170–0.298
AIA[b]	0.798	0.025	68	0.773–0.840
DMD[b]	0.696	0.078	68	0.618–0.826

[a]Tests for 2005 fiber bag, 2012 fiber bag and different laboratory (i.e., Dairy One).

[b]Tests for pre-mixed NDF and ADF solutions versus mixed from individual chemicals.

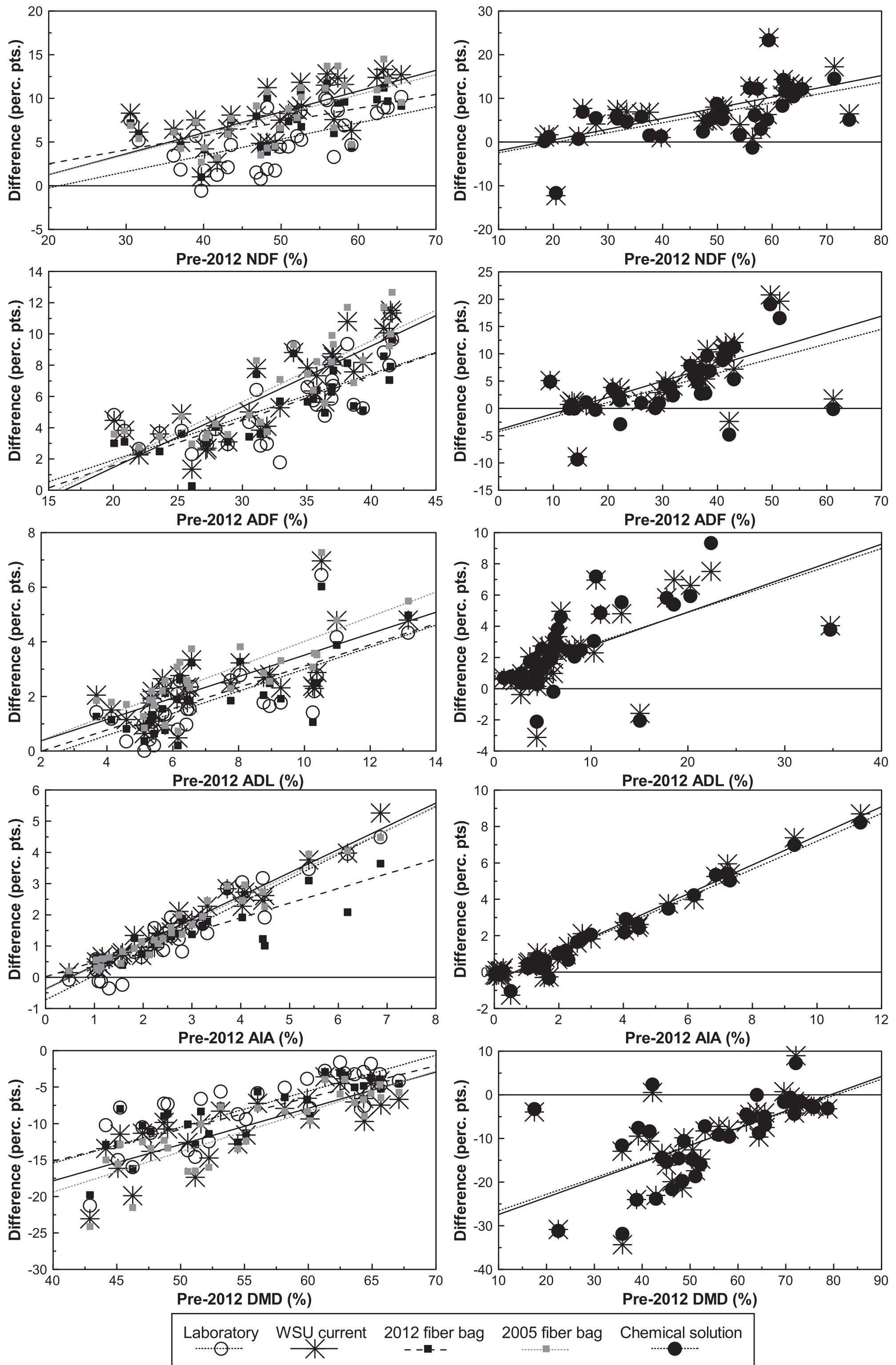

FIGURE 1 (See caption on next page)

Potential causes of shift in assay results

Tests for effects of 2005 fiber bag, 2012 fiber bag, and laboratory

Using 30 composite diet samples, we found no evidence of different intercepts or slopes across the individual tests (e.g., 2005 bag, 2012 bag, and use of a different laboratory) from assays conducted at WSU (all 90% CIs included 0) for NDF, ADL, or overall DMD. For ADF, the slope of the regression line was flatter for assays conducted at Dairy One versus those conducted at WSU (0.276 versus 0.389; SE = 0.064, df = 112; CI = 0.170–0.382) but we found no evidence of different intercepts or slopes when using fiber bags purchased in 2005 or 2012. For AIA, the intercept of the regression line for assays conducted at Dairy One and for assays conducted using a fiber bag purchased in 2012 differed from WSU results (Dairy One = −0.720; 2012 bag = 0.021; WSU = −0.374). In addition, the slope of the regression line was flatter for assays using a fiber bag purchased in 2012 than for post-2012 WSU results (0.476 versus 0.745; SE = 0.059, df = 112; CI = 0.377–0.575) (Table 2, Figure 1).

Tests for chemical NDF and ADF solution

Using 36 composite diet samples, we found no evidence of different intercepts or slopes between pre-mixed NDF and ADF concentrate solutions versus creating the solution from individual chemicals (all 90% CIs included 0) for NDF, ADF, ADL, AIA, or overall DMD (Table 2, Figure 1).

Development of correction equation

Given that we identified differences from the same samples between results obtained before 2012 versus after 2012, we developed predictive equations to bring laboratory results obtained since 2012 into alignment with those obtained before 2012. We chose to correct post-2012 to pre-2012 values because post-2012 levels of DMD violated well-established thresholds of requirements relative to ruminant performance (National Research Council 1984, 1985, 2007; Cook et al. 2004). We found evidence of non-normality and heteroscedasticity in the residuals only in the AIA model. The residual plots from beta regression did not show evidence of lack of fit but the beta regression model over-predicted pre-2012 AIA values in a crucial portion of its range (0–1.0%) compared to standard linear regression. Thus, because we were more concerned

FIGURE 1 Relationships between sequential detergent fiber assay results conducted before 2012 and the difference between pre- and post-2012 results (conducted on the same sample) as a function of 5 potential factors that could have caused a shift in laboratory results since 2012. Results are shown for each fiber step: percent neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), acid insoluble ash composition (AIA), and overall percent dry matter digestibility (%) as calculated using Robbins et al. (1987) but setting tannins to 0. Using 30 composite diet samples collected from elk in eastern Oregon during 2006 and analyzed pre- and post-2012, the left column of graphs shows the difference from Washington State University (WSU) Wildlife Habitat and Nutrition Laboratory's current results for Dairy One's results, WSU using a fiber bag purchased in 2012, and WSU using a fiber bag purchased in 2005. Using 36 composite diets samples collected from elk in Oregon and Washington during 2000–2007 and analyzed pre- and post-2012, the right column of graphs shows the difference from WSU current results using a pre-mixed powdered concentrate NDF and ADF solution from WSU results but mixing NDF and ADF solution from individual chemicals.

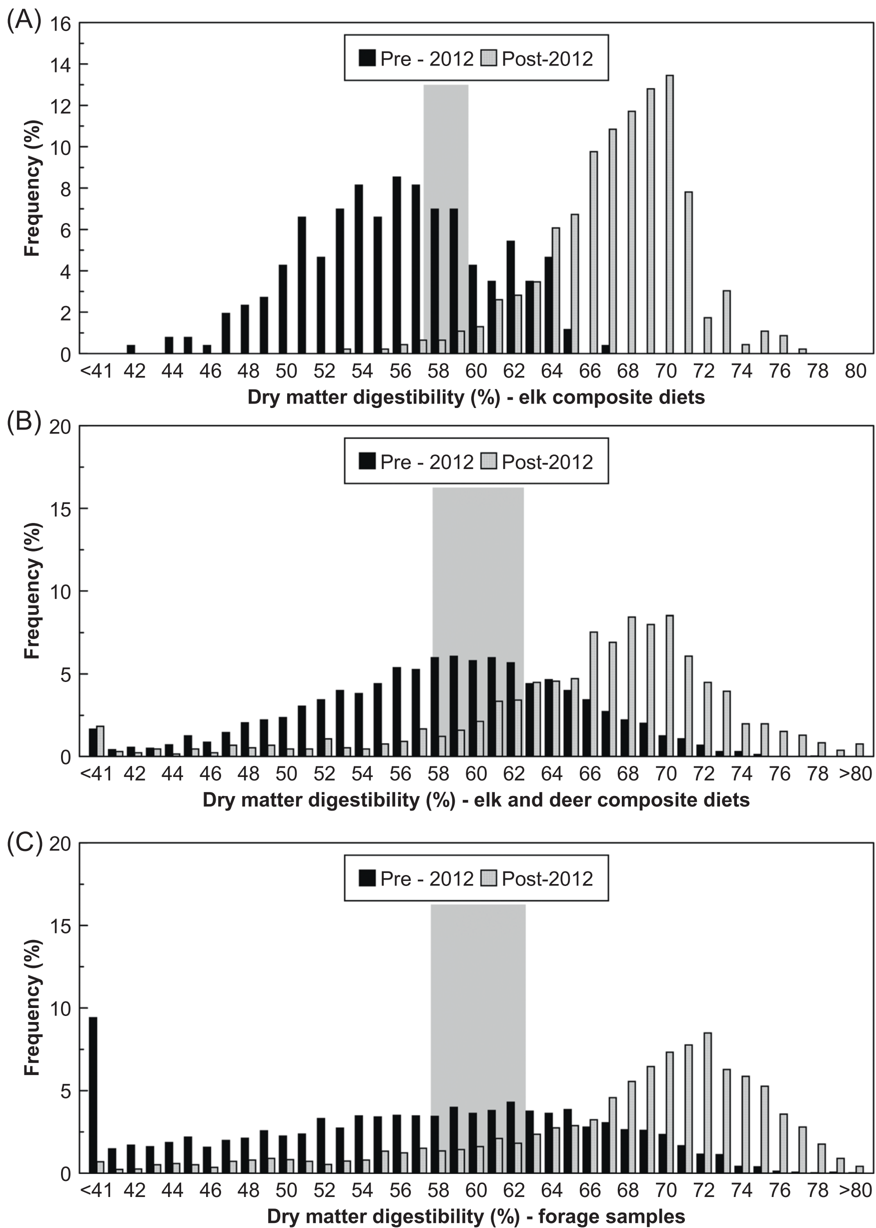

FIGURE 2 Comparison of the distribution of forage or composite diet samples collected during the growing season (April–October) and analyzed for dry matter digestibility (%; DMD) either before 2012 or after 2012. A) Composite diet samples collected from elk during August at Starkey Experimental Forest at permanent enclosures each year from 2005 through 2012. Samples collected during 2005–2007 were analyzed by Washington State University (WSU) Wildlife Habitat and Nutrition Laboratory in 2008 whereas samples collected during 2008–2012 were analyzed in 2013 by the same lab. B) Elk and deer composite diet samples. C) Forage samples collected from a variety of studies across Oregon, Washington, and Idaho, USA, during 1998–2019. All methodologies for sample collection and storage were identical and all samples were analyzed identically by WSU. The gray bar represents the approximate DMD requirement for lactating deer and elk during summer.

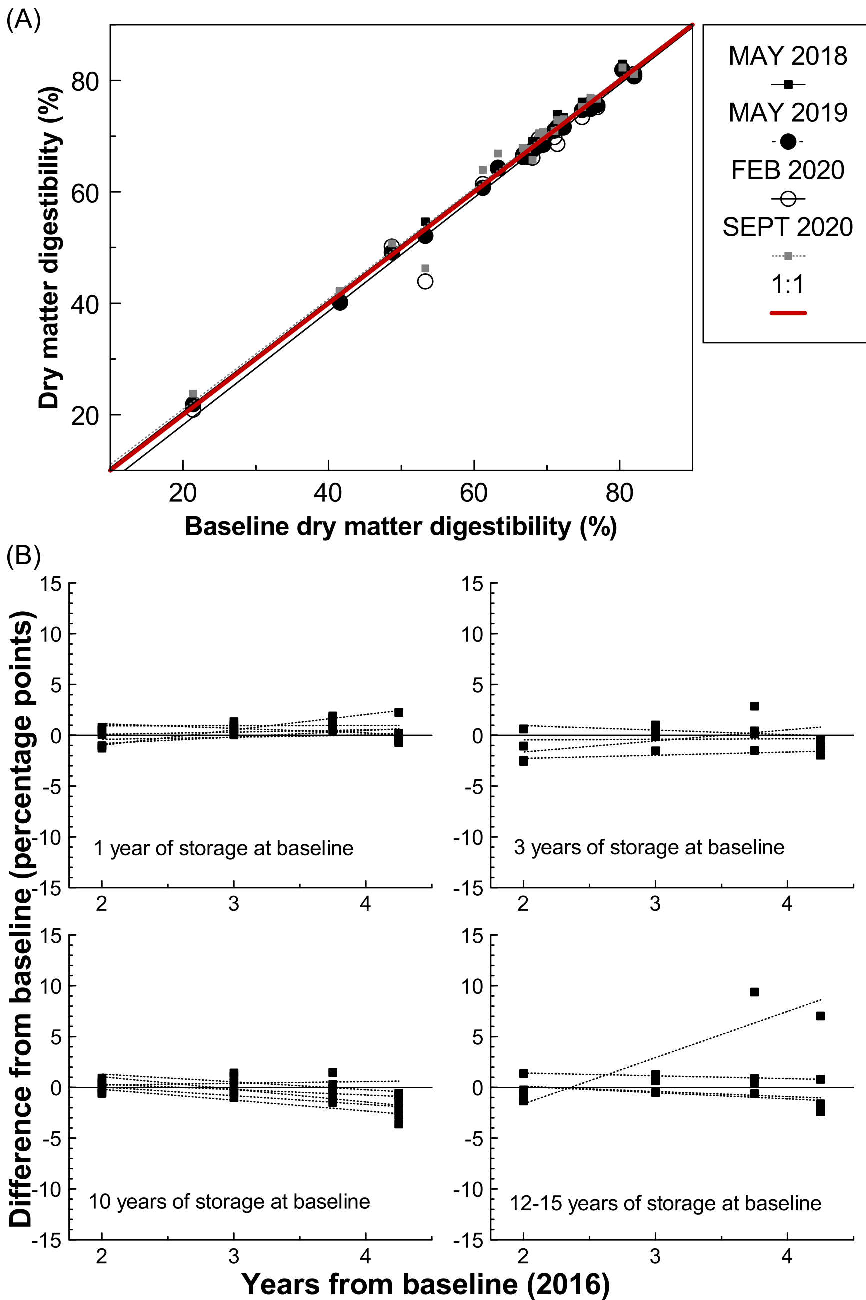

FIGURE 3 A) Relationship between dry matter digestibility (%; DMD) for 24 composite diet samples collected from elk in Oregon during 2001–2013 and caribou in British Columbia during 2013 and analyzed during 2016 (baseline) versus each successive time the same samples were analyzed across a 4-year period. If no additional changes in sequential fiber results were detected, we expected baseline values from 2016 and each successive run to have a 1:1 relationship (red line). B) Grouped by length of storage time, difference from baseline DMD (baseline DMD minus each successive DMD in percentage points) for each successive time the same samples were analyzed across a 4-year period. If storage time of samples was a factor, we expected differences to be consistently >0 and differences to get larger each year the samples were stored.

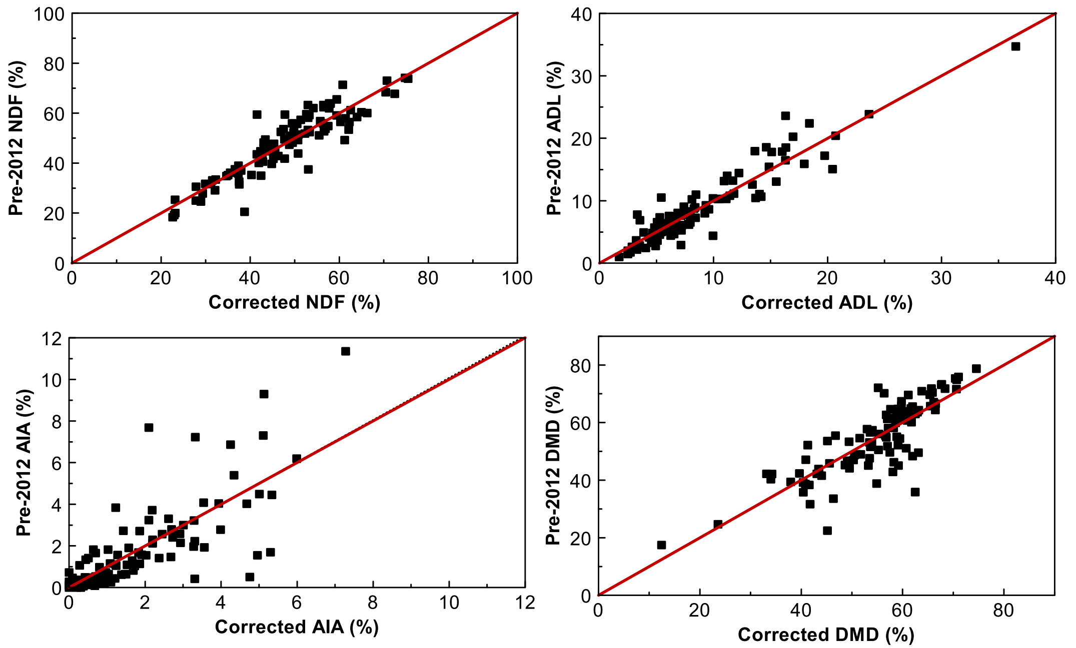

FIGURE 4 The relationship between corrected (i.e., predicted) and pre-2012 (i.e., observed) percent neutral detergent fiber (NDF), acid detergent lignin (ADL), acid insoluble ash composition (AIA), and overall dry matter digestibility (DMD). One hundred composite diet samples from elk collected during 2000–2006 in Oregon and Washington, USA, were used to develop correction equations. The red line indicated a 1:1 relationship.

with providing a robust predictive equation to adjust post-2012 fiber values (i.e., current) to pre-2012 values, we used the linear regression model to build correction equations for NDF (Equation 1), ADL (Equation 2), and AIA (Equation 3; Figure 4). We did not derive a correction equation for ADF because that value is not used in the summative equations of Robbins et al. (1987).

$$\text{CorrectedNDF} = 1.03334 \times (\text{current NDF}) + 4.84719;\ F_{(1,99)} = 498.59,\ P < 0.0001, r^2 = 0.83; S_{y\cdot x} = 5.3 \quad (1)$$

$$\text{CorrectedADL} = 1.14617 \times (\text{current ADL}) + 1.34973;\ F_{(1,99)} = 709.21,\ P < 0.0001, r^2 = 0.88; S_{y\cdot x} = 2.05 \quad (2)$$

$$\text{CorrectedAIA} = 2.8689 \times (\text{current AIA}) - 0.35145;\ F_{(1,99)} = 140.66,\ P < 0.0001, r^2 = 0.59; S_{y\cdot x} = 1.38 \quad (3)$$

Both sets of independent samples used to evaluate the correction equations indicated a strong correlation between predicted DMD estimates (corrected as per Equations 1 through 3) and estimates obtained before 2012 ($r^2 = 0.90$–0.93), slope estimates close to 1.0 (1.02 and 0.98), and intercept estimates close to 0.0 (−1.56 and 0.36; Figure 5). In addition, when 4,806 samples analyzed after 2012 (Figure 1B,C) were corrected (using Equations 1 through 3), average DMD for diets declined from 66.1% to 59.4% (versus 58.5% before 2012) and average DMD for forage samples declined from 67.7% to 61.3% (versus 56.4% before 2012; Figure 6).

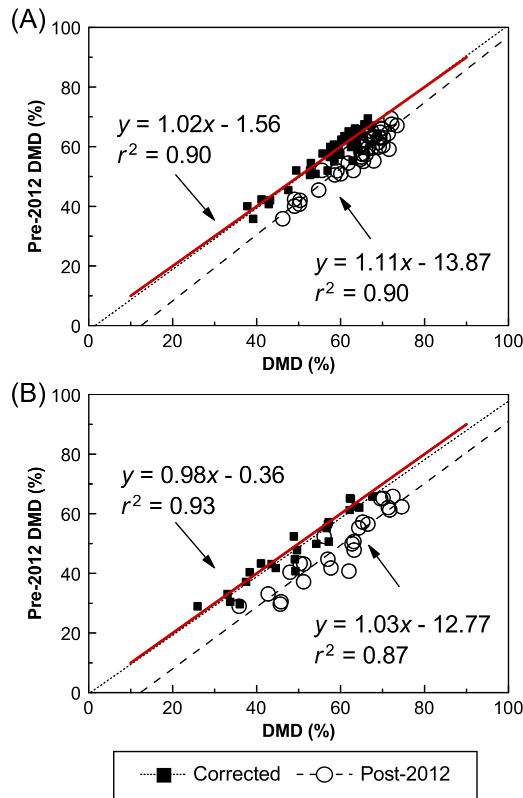

FIGURE 5 Relationships between corrected (i.e., predicted) and pre-2012 (i.e., observed) percent dry matter digestibility (%; DMD) versus the relationship between post-2012 and pre-2012 (i.e., observed) DMD for 2 independent data sets: composite diets collected from elk in southwest Oregon, USA, during 2011 (A) and forage samples collected by plant life group in northeastern Washington, USA, during 2004–2005 (B). We calculated corrected DMD by correcting percent neutral detergent fiber (NDF), acid detergent lignin (ADL), and acid insoluble ash composition (AIA) using Equations 1–3 (this study) and then using equations from Robbins et al. (1987) to predict DMD. The red line indicated a 1:1 relationship.

Additional inconsistent methodological considerations

We corrected NDF, ADL, and AIA using Equations 1, 2, and 3 before estimating DMD (Robbins et al. 1987). Using these corrected values, we evaluated the potential effect of each inconsistent methodology (i.e., separate aliquots for AIA, exclusion of sodium sulfite, use of 1.0 g instead of 0.5 g).

Estimating AIA

We found that using a separate aliquot of forage sample for AIA rather than one that had been through the previous stages of the sequential analysis (Appendix A) influenced DMD calculations. The absolute mean difference in corrected AIA from Dairy One as compared to WSU calculations on the same samples was 2.3 percentage points but ranged from −2.7 to 42.1 percentage points resulting in a mean underestimation of DMD of 4.5 (min-max = −72.7–4.5 percentage points; Figure 7A). We supply instructions on calculating AIA correctly for use in the

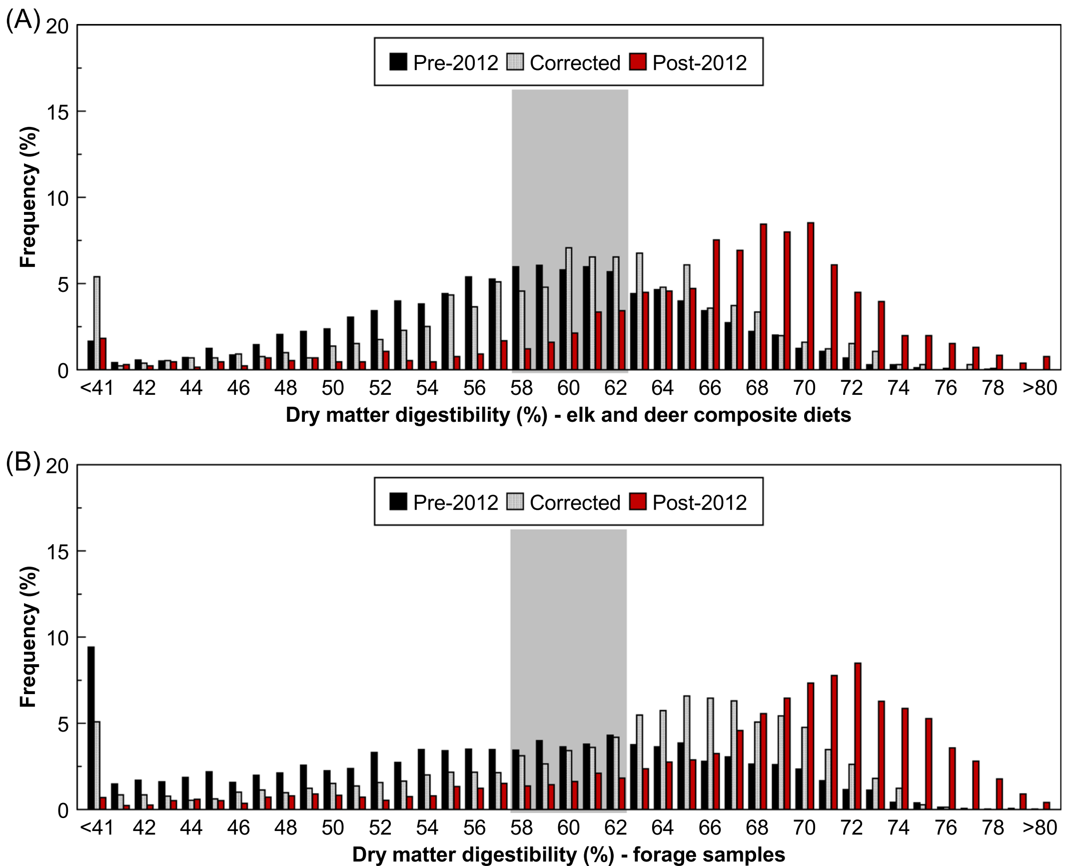

FIGURE 6 Comparison of the distribution of dry matter digestibility (%; DMD) for samples analyzed pre-2012 versus post-2012 versus post-2012 but with DMD estimates corrected (Corrected). Samples included elk and deer composite diets collected in Oregon and Washington, USA, during 1998–2019 (A), or forage samples collected during the growing season (April–October; 2000–2019) in Idaho, Oregon, and Washington (B). We calculated corrected DMD by correcting percent neutral detergent fiber (NDF), acid detergent lignin (ADL), and acid insoluble ash composition (AIA) using Equations 1–3 (this study) and then using equations from Robbins et al. (1987) to predict DMD. All methodology for sample collection and storage were identical and all samples were analyzed by Washington State University Wildlife Habitat and Nutrition Laboratory with identical methodology. The gray bar represents the approximate DMD requirement for lactating deer and elk during summer.

equations of Robbins et al. (1987) when submitting samples to Dairy One (and potentially additional agriculture-based laboratories; Method S1, available in Supporting Information).

Sodium sulfite

As expected, given sodium sulfite solubilizes small amounts of lignin at the NDF stage (Mould and Robbins 1981, Hanley et al. 1992), neglecting to add sodium sulfite resulted in an average increase of ADL by 12% (min-max = −1.0–31%; *n* = 30 composite diet samples). The increase in ADL resulted in an average underestimation of DMD of 6.5 percentage points (min-max = 2.4–13.5 percentage points). In addition, the effect of sodium sulfite increased as fiber content of the sample increased (Figure 7B).

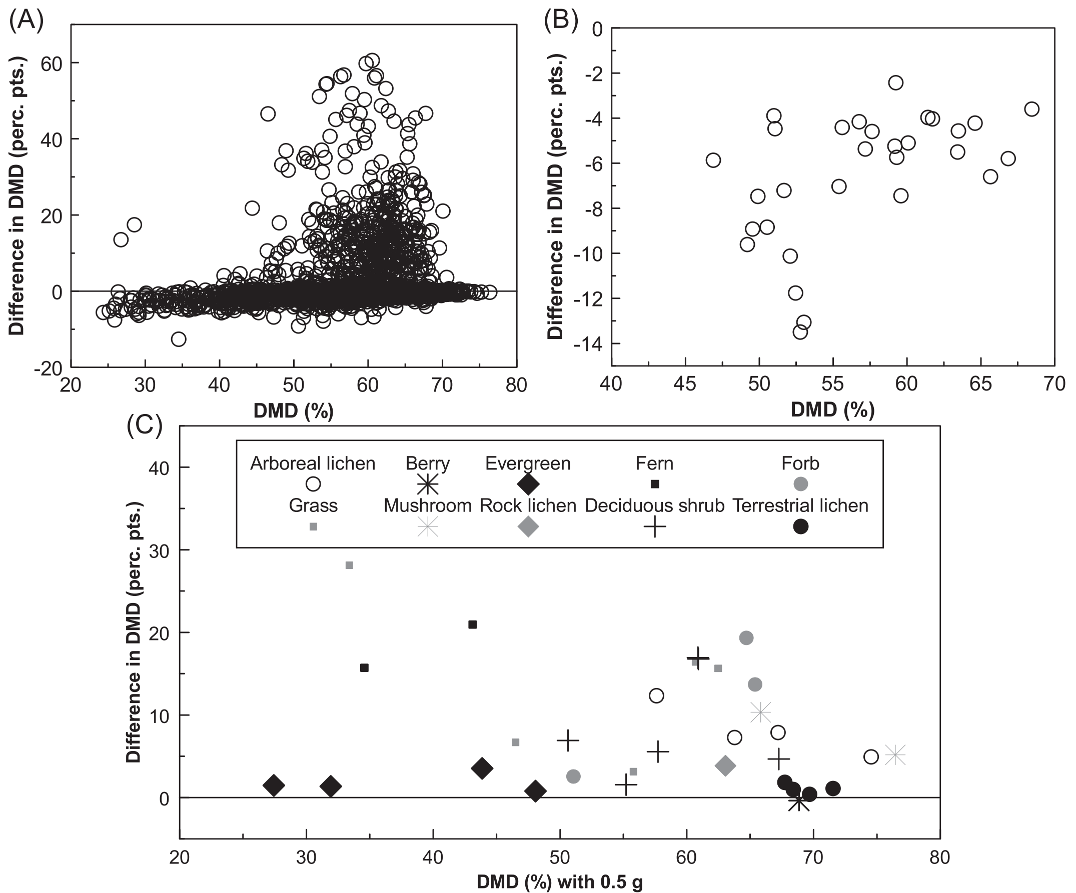

FIGURE 7 Differences in dry matter digestibility (percentage points; DMD) for 3 methodological inconsistencies found during the course of our study (i.e., difference = results from Washington State University [WSU] Wildlife Habitat and Nutrition Laboratory minus results using a different methodology): A) the difference in DMD with percent acid insoluble ash composition (AIA) calculated as per WSU versus AIA calculated as per Dairy One (i.e., using a new aliquot of sample for calculating AIA) using forage samples collected in Idaho, USA, during 2016–2017; B) the difference in DMD in 30 composite diet samples collected from elk in Oregon, USA, during 2006 when sodium sulfite was added to the fiber analyzer at the neutral detergent fiber step (NDF) as per WSU methodology versus not adding sodium sulfite; and C) the difference in DMD when ~0.5 g of sample was used as per ANKOM Technology's methodology (and WSU's) versus when ~1.0 g of sample was used from a variety of plant species collected in British Columbia, Canada, during 2015 that varied in volume to mass ratios. We corrected percent neutral detergent fiber (NDF), acid detergent lignin (ADL), and acid insoluble ash composition (AIA) using Equations 1–3 (this study), and then used equations from Robbins et al. (1987) to predict DMD for all the data presented in these graphs.

Sample mass

For every sample except one (berries), using 1.0 g ($\bar{x}$ = 1.005 g; min-max = 0.9354–1.0308 g) instead of the standard 0.5 g ($\bar{x}$ = 0.5055 g; min-max = 0.4910–0.5402 g) of plant sample underestimated DMD by 0.8–28.1 percentage points (Figure 7C). On average, underestimation of DMD was greatest for ferns (n = 2; $\bar{x}$ = 18.3 percentage points), perennial forbs (n = 3; $\bar{x}$ = 11.9 percentage points), and grasses (n = 4; $\bar{x}$ = 14.0 percentage points); was moderate for deciduous shrubs (n = 6; $\bar{x}$ = 8.7 percentage points), arboreal lichens (n = 4; $\bar{x}$ = 6.7 percentage points), and

mushrooms ($n = 2$; $\bar{x} = 7.8$ percentage points); and was lowest for the rock-borne foliose lichen ($n = 1$; 3.9 percentage points), terrestrial lichens ($n = 4$; $\bar{x} = 1.1$ percentage points), berries ($n = 1$; −0.4 percentage points) and evergreens ($n = 4$; $\bar{x} = 1.8$ percentage points).

DISCUSSION

Our analysis confirmed that circa 2012, sequential detergent fiber assays with the filter bag method and the ANKOM fiber analyzer$^{200/200®}$ began significantly underestimating NDF, ADF, ADL, and AIA, and consequently overestimating DMD compared to samples analyzed with the same methodology prior to 2012, regardless of laboratory. The magnitude of the difference increased with each step of the sequential analysis and as soluble fiber increased in the sample, resulting in overestimation of DMD by up to 30.9 percentage points for the samples we analyzed. The bias we identified has marked potential to affect inferences regarding adequacy of nutritional resources in studies of ruminant nutritional ecology. We found no evidence of additional shifts through 2020 for assay results generated by WSU and thus were able to develop predictive equations to align post-2012 lab results with those obtained before 2012. Nonetheless, at least 3 important questions arise from our results: 1) What caused the shift in fiber results beginning 2012 and can these be corrected in the laboratory?; 2) Which results are right and why does this matter?; and 3) Where does that leave us now?

In evaluating which factors may have caused the shift in fiber results beginning 2012 (e.g., laboratory, chemical solution, fiber bag design), we found slight differences between results from WSU and Dairy One and when using a bag purchased in 2012 versus bags purchased in 2005 or 2015, but the magnitude of these differences was minor and differences were inconsistent across fiber steps. In our correspondence with ANKOM Technology, 2 additional concerns surfaced that we were unable to directly evaluate (B. Layton, personal communication). First, improper use of NDF and ADF solutions can cause erratic results. For example, if laboratories use concentrate solutions to make their own working solutions and make batches smaller than the recommended 20 L volume, the solution could contain inaccurate ratios of constituent chemicals. In addition, if labs purchase solution rather than the concentrate, the chemicals can precipitate from the solution during transportation in cold temperatures. Neither of these potential problems were relevant to our analyses. Washington State University only buys concentrated solution, makes the 20 L volume, and stirs the solution before use. In addition, we found that making the NDF and ADF solution from individual chemicals rather than purchasing the pre-mixed product had no influence on assay results.

Second, length of time samples were stored or the storage method itself could have caused a shift in fiber results over time. Although we cannot rule out this factor completely, our combined results suggest that time of sample storage was not a factor. Throughout testing, we used samples that had been stored in sealed plastic bags or paper envelopes indoors in temperature-controlled environments, and we found no evidence that any of the vegetation samples we used had degraded (i.e., no moisture, mold, or fungal growth). Data from the Starkey Experimental Forest also refuted the hypothesis that sample storage time caused the shift in fiber results given the similar range of time that elapsed between sample collection and analysis for samples analyzed before versus after 2012. In addition, we chose a set of standards that represented a wide range of time from collection to analysis to track any further changes at the WSU lab. With these samples, we again found no indication that sample degradation explained the shift in fiber results. Thus, despite 4 years of conducting assays to investigate several laboratory-based causes of shifts in assay results, we were unable to satisfactorily explain why these shifts occurred.

Researchers who have estimated DMD using ANKOM's dietary fiber analyzer and filter bag technology since 2012 face a challenging dilemma regarding which set of results (pre- or post-2012) are biologically most relevant to ruminant nutrition. To evaluate this issue, we first compared results of *in vitro* digestibility assays versus pre-2012 and post-2012 ANKOM results (Analysis S1, available in Supporting Information). Overall, *in vitro* DMD estimates were poorly correlated to DMD calculated from sequential fiber analysis, on average were between pre-2012 and post-2012 results (although

were slightly more similar to pre-2012 results) and were inconsistent across the 2 different labs assaying the same samples. Thus, our *in vitro* comparisons provided little guidance regarding which fiber results were most reliable and reinforced the problems with using *in vitro* methodology (i.e., sources and quality of rumen fluid are often variable, and the methodology does not replicate effect of tannins on digestion; Shipley et al. 2020).

A long history of research has established relationships between forage quality and ruminant performance (National Research Council 1984, 1985, 2007; Cook et al. 2004). Controlled feeding studies on captive wild and domesticated ruminants indicate that DMD requirements range from 58 to 62% for lactating elk and deer during summer (e.g., Cook 2002, Cook et al. 2004, National Research Council 2007, Hanley et al. 2012). These studies show that forage DMD levels that fall just 3–4 percentage points below requirements have substantial, significant effects on ruminant performance (e.g., reduced pregnancy rates, juvenile growth rates, and body fat levels in autumn and early winter; Cook et al. 1998, 2001, 2004). Using these guidelines, a series of studies over the last 2 decades in the northwestern U.S. clearly indicate that poor to marginal nutritional resources (relative to the requirements of cervids) dominate summer ranges. In Oregon and Washington, lactating elk and moose (*Alces alces*) had autumn body fat indicative of moderate (<12% body fat; Cook et al. 2004) to severe (<6% body fat; Cook et al. 2004) nutritional limitations (Cook et al. 2013, 2021; Johnson et al. 2019). Many of these populations displayed depressed pregnancy rates (some even below 50%), delayed conception, and at least 3 showed evidence of adult and juvenile starvation mortality (Stussy 1993, Cook et al. 2013, Johnson et al. 2019). Of 9 elk populations in western Washington and western Oregon, Cook et al. (2018) reported that the extent of depressed pregnancy rates and autumn body fat levels was significantly related to the levels of digestible energy of forage on each of their ranges. In elk populations, Horne et al. (2019) provided evidence of summer nutritional limitations on juvenile survival in Idaho and Proffitt et al. (2016) reported effects of inadequate summer nutrition on populations in western Montana. In mule deer populations, Hurley et al. (2014) found strong correlations between remotely-sensed surrogates of summer and autumn nutrition and recruitment from juvenile to adult age classes in Idaho, and Merems et al. (2020) reported early winter body fat levels below the threshold for maintaining positive population growth in eastern Oregon.

Given depressed ungulate performance in the region, we would expect DMD levels of forage on summer ranges in the northwestern U.S. to routinely average near or below nutritional requirements, particularly after mid-summer. Studies using lab assay results conducted before 2012 have documented this to be the case; DMD levels in surveys of forage quality across cervid ranges or in cervid diets typically average at or below basic nutritional requirements of lactating females during summer and autumn (Cook et al. 2014, 2016, 2018). A variety of older studies also documented DMD levels that typically failed to satisfy nutritional requirements in the region, particularly during late summer (Schommer 1978, Leslie et al. 1984, Merrill et al. 1995, Alldredge et al. 2002, Cook 2002).

In stark contrast, forage and dietary quality data from Oregon, Washington, and Idaho analyzed after 2012 indicated that most of the available forage in these areas met or, in most cases, greatly exceeded nutritional requirements for lactating deer and elk during summer, an inference that is markedly inconsistent with cervid performance data in the region. In addition, data collected from repeated sampling across multiple years from enclosures at the Starkey Experimental Forest provided striking evidence of a shift in the range of DMD values with pre-2012 values being consistent with ecological conditions and elk performance and post-2012 values being inconsistent with those conditions. For diet samples collected during 2005–2007 and analyzed prior to 2012, elk were able to achieve diets that met nutritional requirements (≥58% DMD) in only 36% of the samples and no samples had DMD ≥70%. These results matched our expectations because samples were collected in August from dry forest communities in eastern Oregon that are prone to drought after early summer (Franklin and Dyrness 1988) and because elk exhibited behavior indicative of nutritional stress and rapidly declined in body condition (R. Cook, National Council for Air and Stream Improvement, unpublished data). Despite similar patterns in body condition and behavior of elk, fiber assay results for diet samples collected during 2008–2012 but analyzed after 2012, indicated that female elk were able to achieve diets that met nutritional requirements in 98% of the samples (DMD ≥58%) with 30% of the samples having DMD ≥70%.

Our results indicated clear shifts in assay results after 2012 that resulted in higher DMD estimates that are inconsistent with well-established thresholds of requirements relative to ruminant performance. Thus, we urge all researchers using sequential detergent fiber analysis and summative equations (e.g., Robbins et al. 1987, Hanley et al. 2012) to strongly consider our corrections for any forage samples analyzed from 2013 to present time (2021) and potentially beyond. We chose composite diets with a mix of plant species when developing these corrections based on the assumption that the equations would be more robust across types of vegetation than would corrections based on samples of single species or life form groups; our validation analysis suggests we achieved this goal. We recognize that using correction equations introduces additional variation into the DMD estimates, and thus some may argue that evaluating trends in the data would suffice as a basis for biological inference particularly if simply comparing across treatments, years, or study areas. However, our data indicate both absolute values and trends would be significantly affected because the magnitude of bias increases as soluble fiber increases. When post-2012 NDF, ADF, and AIA estimates were corrected, average diet quality was more consistent with measured levels of ruminant performance in the region. Although average DMD for individual forage samples analyzed after 2012 was reduced to a more biologically reasonable level when the correction was applied, DMD of post-2012 estimates still tended to exceed those from pre-2012. However, sampling strategy differed in the data sets before and after 2012. Before 2012, researchers collected all plant species including those that deer and elk avoided, and stems and leaves were often combined. After 2012, sampling emphasized plants that deer and elk readily consumed, with an effort to separate samples into stems and leaves (high and low-quality portions; e.g., Monzingo 2020). Thus, we would expect slightly higher post-2012 DMD values on average due to sampling strategy independent of the shift in fiber assay results from pre-2012 to post-2012 documented herein.

Our results also indicate a need for developing and analyzing a set of lab standards that represent the expected range of variation in native vegetation. ANKOM employs a quality control program for their reagents and equipment and thus many commercial and private labs do not routinely run standards for sequential detergent fiber analysis (WSU included). However, ANKOM uses only 6 standards that are exclusively feedstuffs (i.e., not native vegetation) and generally only tests at the NDF stage (B. Layton, personal communication). For native vegetation and sequential fiber analysis, our results suggest this baseline set of samples may be inadequate. Not only did we find a greater deviation in samples analyzed after 2012 from those analyzed before 2012 as samples increased in soluble fiber, but the magnitude of the lab assay effect increased for each fiber step with the least relative difference at the NDF step and the greatest at the AIA step (i.e., NDF < ADF < ADL < AIA). Using a sample subset for quality control or for justifying a change in methodology or equipment requires careful consideration: the samples should accurately reflect not only the variation in vegetation quality, but also the physical characteristics (e.g., varying mass-specific volume, fiber content) of the vegetation itself. Thus, we also recommend that all laboratories, commercial or private, collect ample dry matter from at least 25 plant samples expected to range in DMD from very low (i.e., stems, dead grass, etc.) to very high (i.e., new vegetation regrowth, berries, flowers, leaves etc.) to serve as a standard set for all future fiber analysis to make certain no additional shifts are occurring. This strategy would be most effective if a pool of standards were created for common use, thereby providing a means to evaluate differences among laboratories. We recognize that this is a daunting task and are unsure what organization would be most appropriate for undertaking this effort. Nevertheless, such an effort would add credibility and reduce uncertainty regarding potential bias and inaccurate results among and within laboratories over time. For those researchers using WSU, we can confirm that no additional shifts have been detected since 2013. We also report studies that have either applied our correction to data sets analyzed after 2012 or that were analyzed before 2012 but published after 2012 (Table S1, available in Supporting Information).

We urge researchers to be routinely skeptical of laboratory results. Certainly, depending on the number of reagents or steps involved and how automated the process is, the risk of error or the risk of using different methods that affect results can be significant (Shipley et al. 2020). Researchers can guard against this in 2 ways. First, gain a full understanding of the laboratory technique (e.g., fiber assays vary widely depending on the application) and the ways in which error can be introduced for each assay before submitting samples. Second and most importantly, researchers should explore laboratory

results in detail to judge whether data are biologically reasonable given the plant (group) and plant part, the time of year, the location, and the nutritional requirements of herbivores. We avoid detailed guidelines herein, but we highlight a few trends that would be expected in temperate forests. In dry ecoregions or plant communities, downward trends in DMD as the growing season progresses are expected with grasses and annual forbs declining earlier and more sharply than perennial forbs or deciduous shrubs (e.g., Cook et al. 2014, Monzingo et al. in press). During the growing season, leaves are expected to have significantly higher DMD than stems for shrubs, grasses, and large forbs (Ulappa 2015, Monzingo 2020). Only plants containing minerals like silica (e.g., grasses) should have AIA values significantly >0; contaminants like dirt, road dust, or sand can artificially inflate AIA estimates. We recommend skepticism of samples with DMD substantially greater than ungulate requirements except early in the growing season, but note that in autumn and even in winter, some plants that resume growth with autumn rains may have DMD substantially higher than that of dormant plant material (Cook et al. 2014). Field notes that provide a record of phenology of these early greening plants may help identify this pattern.

Finally, individual labs may employ different methodologies than recommended by ANKOM that may affect results (e.g., when 1.0 g rather than the recommended 0.5 g of sample is used; how sodium sulfite is used). Our results suggested authors need to provide greater detail regarding the methods used for lab assays than has been customary in the past. We recommend that when reporting methods of analyzing fiber content of forages, authors include additional details on the amount of sample used, the number of replicates analyzed (e.g., single, duplicates, triplicates), what lab analyzed the samples and in what year, what type of NDF and ADF solution was used, and which samples had sodium sulfite added to the NDF step.

With increasing evidence that nutritional deficiencies, including those in summer, have important limiting effects on ungulate populations and that seemingly small differences in forage quality, particularly DMD, has disproportionately large effects on ruminant performance (White 1983, Cook et al. 2004, Cebrian et al. 2008), biologists and managers increasingly require reliable and accurate estimates of forage quality on large ungulate ranges. Forage quality surveys can provide insights for wildlife and land managers about whether landscape management that features improvement of forage resources should be considered. Further, estimates are also needed to evaluate success of habitat improvements conducted to improve DMD and other metrics of forage quality. Yet these insights depend on comparing estimates of DMD (and estimates derived from DMD, such as digestible energy) to levels of DMD required by the animal. Consistency and accuracy are vitally important for reliable assessment of forage resources and bottom-up limitations, for identifying differences across space and time and, more generally, for advancing the field of nutritional ecology.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

No ethical information provided.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website. We provide: 1) methodology and results comparing pre–2012 and post-2012 sequential fiber assay results to *in vitro* digestion trial results; 2) methods to calculate acid insoluble ash (AIA, %) for use in summative equations from Robbins et al. (1987) if analyzing plant samples at Dairy One (Ithaca, NY) or potentially any other laboratory that primarily runs agriculture-based samples; and 3) a list of manuscripts published after 2012 but forage samples were analyzed prior to 2012 and thus no correction was required, or forage samples were analyzed after 2012 but were corrected to align to results from before 2012.

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APPENDIX A: STEPS FOR SEQUENTIAL DETERGENT FIBER ANALYSIS WITH THE FILTER BAG METHOD AND THE ANKOM FIBER ANALYZER$^{200/200®}$.