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ARTICLE

Diet of endangered Steller sea lions (*Eumetopias jubatus*) in the Aleutian Islands: new insights from DNA detections and bioenergetic reconstructions

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Abstract: The endangered western stock of Steller sea lion (*Eumetopias jubatus* (Schreber, 1776)) still declines in the western Aleutian Islands and accurate diet information is vital to test leading hypotheses. We undertook the first bioenergetic diet reconstruction using both molecular and hard part prey identifications from >600 scats collected in March–April 2008 and 2012. Atka mackerel (*Pleurogrammus monopterygius* (Pallas, 1810)) remained a primary prey (17%–27% by energy), but large (mean 60 cm) Pacific cod (*Gadus macrocephalus* Tilesius, 1810) also emerged as important prey (20%–24%) in a more diverse diet than previously reported, with Cottidae and smooth lumpsucker (*Aptocyclus ventricosus* (Pallas, 1769)) also contributing ~10%. DNA detections highlighted a potentially important and previously underestimated prey, giant Pacific octopus (*Enteroctopus dofleini* (Wülker, 1910) (diet contribution 2%–15%, dependent on prey size assumptions). Although 504 unique DNA identifications resulted in significant increases for cephalopods, Pacific cod, and smooth lumpsucker, hard part alone species rankings were similar to composite ones and bioenergetic species rankings similar to occurrence-based ones. Retention or regurgitation of large cephalopod beaks, the removal of large cod heads, and skeletal fragility of lumpsuckers may explain these differences. DNA identifications provide valuable comparative and complementary prey occurrence data for pinnipeds, but composite diet estimates are optimal.

Key words: Steller sea lion, *Eumetopias jubatus*, diet, DNA, Aleutian Islands, scats.

Résumé : Le stock de l'Ouest d'otaries de Steller (*Eumetopias jubatus* (Schreber, 1776)), un stock en voie de disparition, est toujours en déclin dans la partie occidentale des îles Aléoutiennes, et de l'information exacte sur le régime alimentaire de ces animaux est d'importance vitale pour vérifier les hypothèses les plus populaires. Nous avons entrepris la première reconstitution bioénergétique du régime alimentaire en utilisant l'identification moléculaire et sur la base de parties dures de proies pour >600 échantillons d'excréments recueillis en mars et avril 2008 et 2012. Si le maquereau d'Atka (*Pleurogrammus monopterygius* (Pallas, 1810)) demeure la principale proie (17 % – 27 % en énergie), les grandes (en moyenne 60 cm) morues du Pacifique (*Gadus macrocephalus* Tilesius, 1810) ressortent aussi comme étant des proies importantes (20 % – 24 %) dans un régime alimentaire plus varié que ce qui avait été signalé auparavant, les cottidés et les poules de mer ventrues (*Aptocyclus ventricosus* (Pallas, 1769)) représentant également ~10 % de l'alimentation. La détection sur la base d'ADN fait également ressortir une proie potentiellement importante et jusqu'ici sous-estimée, la pieuvre géante du Pacifique (*Enteroctopus dofleini* (Wülker, 1910)) (contribution au régime alimentaire de 2 % – 15 %, selon les hypothèses sur la taille des proies). Si 504 identifications uniques d'ADN se traduisent par des augmentations significatives pour les céphalopodes, la morue de Pacifique et la poule de mer ventrue, les contributions d'espèces identifiées sur la seule base des parties dures sont semblables aux contributions composites et les contributions d'espèces établies à la lumière de données bioénergétiques sont semblables à celles établies sur la base de la présence. La rétention ou régurgitation de grands becs de céphalopodes, le retrait de grandes têtes de morues et la fragilité des squelettes de poules de mer ventrues pourraient expliquer ces différences. Si l'identification sur la base de l'ADN fournit des données comparatives et complémentaires utiles sur la présence de proies pour les pinnipèdes, les estimations composites du régime alimentaire sont toutefois optimales. [Traduit par la Rédaction]

Mots-clés : otarie de Steller, *Eumetopias jubatus*, régime alimentaire, ADN, îles Aléoutiennes, excréments.

Introduction

The western Steller sea lion (*Eumetopias jubatus* (Schreber, 1776)) has an extensive breeding range across most of the North Pacific Ocean (Figs. 1*a*, 1*b*). This distinct population segment (DPS) was listed as "endangered" in 1997 under the U.S. *Endangered Species Act* due to a persistent decline in overall abundance since at least the mid-1970s (NMFS 2008). Beginning in the early 2000s, the overall abundance of the western DPS in Alaska has increased at 2.2%/year,

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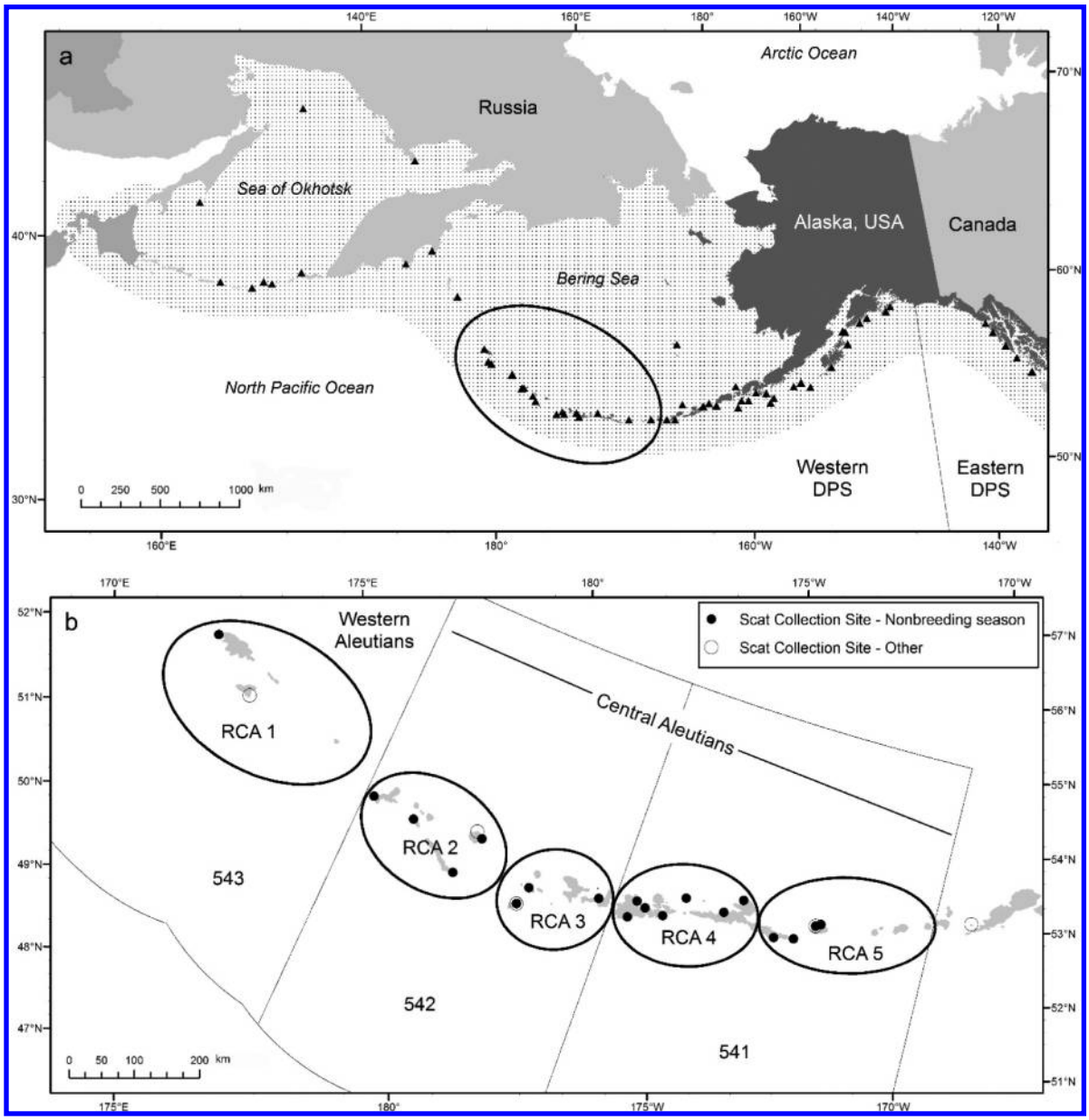
Fig. 1. (*a*) Distribution of terrestrial breeding sites (rookeries) and general at-sea range (stippled area) of the western distinct population segment (DPS) of Steller sea lion (*Eumetopias jubatus*) in Alaska, USA, and Russia. The 144°W line separates the western and eastern DPS and the study area in the Aleutian Islands (Alaska, USA) is circled. (*b*) Location of Steller sea lion terrestrial sites in the Aleutian Island study area where scats were collected during the nonbreeding season, as well as other collection sites (throughout the year) used for comparison of hard part and DNA prey identifications. Other areas in the Aleutian Islands shown include western Steller sea lion recovery areas (western and central Aleutians; NMFS 2008), rookery cluster areas (RCAs) 1–5 used for population trend analysis and diet assessment, and NMFS fishery management areas (541–543).



but there has been considerable regional variability in population trends: abundance has increased at ~4%/year in the Gulf of Alaska and eastern Bering Sea (east of 170°W) but decreased at ~2%/year in the Aleutian Islands (west of 170°W; Fritz et al. 2016). If Aleutian abundance trends are investigated at a finer spatial scale (at the level of rookery cluster areas (RCAs)), further longitudinal variation is revealed, with the steepest declines (–8%/year) occurring in the western Aleutian Islands (RCA 1), and generally improving trends to the east (–4%/year and –3%/year in RCAs 2 and 3, respectively, and stable in RCAs 4 and 5; Fritz et al. 2016).

Accurate dietary information is a vital component of population monitoring and population recovery mitigation, as diet and diet diversity are central to leading hypotheses regarding impact of fisheries, nutritional stress, and lack of recovery in this portion of the range (see Trites et al. 2007; NMFS 2008; Rosen 2009; Fritz et al. 2013; Sinclair et al. 2013). Atka mackerel (*Pleurogrammus monopterygius* (Pallas, 1810)) is the most abundant resident fish (Lowe et al. 2013) and is also the species most frequently consumed by Steller sea lions in the Aleutian Islands (Sinclair and Zeppelin 2002; Sinclair et al. 2013). Atka mackerel dominates the Steller sea lion breeding season diet, with >90% frequency of occurrence in fecal (scat) samples (May–August; Sinclair and Zeppelin 2002; Sinclair et al. 2013). In the nonbreeding season for Steller sea lions (September through April), Atka mackerel remains the dominant prey species (frequency of occurrence >60%), but the diet is supplemented by winter-spawning gadids (walleye pollock, *Theragra*

chalcogramma (Pallas, 1814); Pacific cod, *Gadus macrocephalus* Tilesius, 1810) and other species (e.g., rockfish genus *Sebastes* Cuvier, 1829; Irish lord sculpin genus *Hemilepidotus* Cuvier, 1829; smooth lumpsucker, *Aptocyclus ventricosus* (Pallas, 1769)), resulting in a more diverse diet than during the breeding season (as is typical throughout its range; Sinclair and Zeppelin 2002; McKenzie and Wynne 2008; Sinclair et al. 2013).

The majority of dietary information for Steller sea lions has been based on a conventional, but potentially biased, method — morphological identification of diagnostic prey skeletal remains and other hard parts (hence termed "hard part identification") recovered from stomachs or scat samples. The key concern with the sole use of morphological hard part identification is not detecting (or severely underestimating) important prey contributions. This may occur if soft-bodied prey are not represented by hard parts (e.g., Elasmobranchii; see Olesiuk et al. 1990), if only the fleshy parts of large or spiny prey are consumed, if the heads of large prey are discarded (e.g., large gadids or salmon), or if a prey's hard parts are preferentially retained or regurgitated (e.g., cephalopod beaks; see Bigg and Fawcett 1985; Gudmundson et al. 2006). Furthermore, prey with robust skeletal elements (e.g., walleye pollock) may be over-represented compared with prey with fragile skeletons (e.g., salmon, myctophids, and lumpsuckers), due to differential rates of digestion (e.g., Jobling and Breiby 1986). In addition, a number of commercially and trophically important prey taxa (notably salmon, rockfish, Elasmobranchii, Cephalopoda (cephalopod), and crustaceans) can typically only be identified using hard parts to the family or genus level, rather than the species level. In contrast, DNA has been shown to improve species-level resolution for these taxa (Tollit et al. 2009), as well improved species detection (King et al. 2008; Tollit et al. 2009). Therefore, the first hypothesis this study tests is whether diet determined using traditional techniques (prey hard part identification) is the same as that determined using DNA prey detection methods alone or determined using a composite approach of both methods.

Furthermore, Steller sea lion diet has been largely described using occurrence methods (Sinclair and Zeppelin 2002; McKenzie and Wynne 2008; Sinclair et al. 2013), a technique considered most useful for geographic and temporal comparisons. Estimating diet, however, is a quantitative endeavor that is best achieved using a biomass or ideally a bioenergetic approach (Hyslop 1980; Laake et al. 2002). Captive feeding studies (Tollit et al. 2007; Phillips and Harvey 2009) and computer simulations (Joy et al. 2006) have shown that traditional occurrence indices can perform poorly compared with biomass reconstruction methods if certain techniques at reducing known limitations are employed. These techniques include using multiple diagnostic prey structures rather than just fish otoliths and cephalopod beaks (Olesiuk et al. 1990; Cottrell and Trites 2002), the application of numerical correction factors (NCF) to account for interspecific differences in the proportion of prey remains surviving digestion (Tollit et al. 2003, 2007, 2015; Grellier and Hammond 2006; Phillips and Harvey 2009), and the application of digestion correction factors (DCF) to account for size reduction of hard remains due to acidic erosion (e.g., Tollit et al. 1997, 2004, 2015; Phillips and Harvey 2009). Independent scientific reviews of Steller sea lion diet studies in Alaskan waters have recommended increased utilization of biomass reconstruction techniques (Bowen et al. 2001; NPFMC 2001). Therefore, the second hypothesis this study tests is whether diet estimated using frequency of occurrence methods is the same as that determined using biomass reconstruction techniques.

This study formed part of a larger NOAA-led multidecadal project investigating the relationship between Steller sea lion abundance, diet, diet diversity, and prey availability across the Aleutian Islands (Fritz et al. 2016). In summary, this study firstly compared and combined traditional prey hard part identifications and molecular prey DNA identifications (molecular methodology outlined in Tollit et al. 2009) to describe the diet of Steller sea lions in the western and central Aleutian Islands using scats collected in the nonbreeding season of 2008 and 2012 (hypothesis one). A combination of methods is now widely thought to be a best practice to not only characterize the diet, diet diversity, and fisheries interactions, but also to understand the scale of key biases (e.g., missing or under-represented prey taxa) when using conventional hard part methods (e.g., Casper et al. 2007*a*, 2007*b*; Bowen and Iverson 2013; Thomas et al. 2014). Secondly, this study integrated DNA and hard part prey identifications with prey counts, size estimates, DCFs, and prey energetic density data to better describe the composite diet of Steller sea lions in terms of bioenergetic contribution. This was then used to provide a direct comparison with previous frequency of occurrence dietary information (hypothesis two). This study represents the first bioenergetic dietary reconstruction for Steller sea lions in a crucial part of their range — the Aleutian Islands (Figs. 1*a*, 1*b*).

Materials and methods

Scat collection and prey hard part identification analysis

Steller sea lion scats were collected at 19 sites in the Aleutian Islands in March–April 2008 and 2012. Scat collections were made at haul-outs within five RCAs, which are pre-defined areas used to assess population trends (Fritz et al. 2016; Figs. 1*a*, 1*b*). In 2008, 297 scat samples were collected at 10 sites in the eastern RCAs 4 and 5 in April, whereas in 2012, 309 samples were collected at 13 sites in RCAs 1–5 in March. Additional samples were collected at a single site east of 170°W in March 2012 and throughout the Aleutian Islands in summer 2012 (n = 93), resulting in a total of 699 scats available for a comparative analysis of prey hard part and DNA prey identification. Subsamples of scat soft part matrix for DNA analysis were collected by gently pressing homogenized scat slurry through individual 0.5 mm plastic mesh using a disposable spatula and 2–3 mL of matrix material scraped from the underside (i.e., no hard parts were collected) and placed in ~15 mL of 95% nondenaturing ethanol (2012 samples) or simply stored frozen (2008 samples) prior to analysis by Fisheries and Oceans Canada's Molecular Genetics Laboratory at the Pacific Biological Station, Nanaimo, British Columbia.

All methods for sample processing and prey identification were identical to those described by Sinclair and Zeppelin (2002). Individual scats were machine-washed (Orr et al. 2003) or rinsed through nested sieves and all retained hard parts were identified to the lowest possible taxonomic group based on diagnostic morphological criteria developed by Pacific IDentifications Inc. (scats collected in 2008) or by National Marine Mammal Laboratory (NMML) scientists (scats collected in 2012), both using comprehensive comparative reference skeletons (e.g., see Olesiuk et al. 1990). Scat samples with no prey remains or only the remains of polychaetes or crustaceans were excluded from further dietary analysis.

Molecular techniques methodology

The PCR–DGGE molecular technique used has been proven to be able to identify a wide range of potential prey species (i.e., fish, cephalopods, and crustaceans) in various aged scats collected from wild sea lion populations (Tollit et al. 2009). The DNA from scats is expected to be somewhat degraded (particularly from the more aged samples) and contain a range of concentrations derived from prey and host sources. Therefore, PCR primers must amplify small DNA fragments (approximately 200–300 bp) in nested (two internal secondary primers) or seminested (one internal secondary primer) primer sequences for two rounds of amplification to obtain enough specific product for visualization. Tollit et al. (2009) used the well-characterized 3′ end of the mitochondrial 16S gene to allow species identification confirmation through sequencing and submission to the National Centre for Biotechnology Information (NCBI, Bethesda, Maryland, USA) Basic Local Alignment

Search Tool (BLAST). DGGE is a sequence dependent electrophoretic technique separating amplification products based on their melting behavior as they denature.

A mean of 240 mg (100–500 mg range) was subsampled from each soft scat matrix and extracted using a new high throughput approach. The preliminary steps were performed in a single format with modifications to the protocol used in Deagle et al. (2005), while the filtration and wash steps were performed using the 96-well DNeasy 96 blood and tissue kit protocol (Qiagen, Germantown, Maryland, USA). All centrifugation steps were carried out at room temperature. ASL Buffer (1.6 mL) was added to each scat sample, vortexed thoroughly, and centrifuged at 20000*g* for 2 min. Supernatant (1.4 mL) plus an Inhibitex tablet was vortexed for 1 min, incubated at room temperature for 1 min, then centrifuged at 20000*g* for 6 min, transferred to a new tube immediately, and centrifuged again at 20000*g* for 4 min. Supernatant from this step was then transferred to another new tube containing 25 μL of proteinase K. Buffer AL (600 μL) was added to the sample, vortexed for 15 s, and incubated at 70 °C for 10 min. Prior to loading the lysate onto the DNeasy 96 plate, 100% EtOH (600 μL) was added to each sample and this mixture was transferred to a 96-well microtube collection tray. The final DNA was eluted in 100 μL of AE Buffer.

Prey standard tissue samples from 75 potential sea lion prey species, including fish (68), cephalopods (3), and crustaceans (4), were used in Tollit et al. (2009) to validate PCR primers, develop optimal species resolution conditions, and provide prey standards for prey identification. Prey standards were expanded for this study to optimize identification. Ten additional region-specific fish prey tissues samples (n = 7 species) were supplied by the University of Washington, Seattle, Washington, USA, and 11 additional cephalopod prey tissue samples (n = 11 species) were supplied by NMML, Alaska Fisheries Science Center (AFSC), Seattle, Washington, USA (Supplementary Table S1).[1] All tissue samples were extracted using the DNeasy blood and tissue kit.

For fish prey identification, the extracted soft scats were first amplified with a general PCR primer pair (16Sf1 and 16SallR), which amplifies both fish and cephalopods (for primer sequences and PCR conditions see Tollit et al. 2009). Seminested PCRs were subsequently performed using 2 μL of the primary PCR reaction as template, with forward primers fluorescently labeled with 6-FAM (Operon Biotechnologies, Inc., Huntsville, Alabama, USA) for visualization of products (Tollit et al. 2009). The PCRs were then resolved via electrophoresis on two DGGE gels, one at 56 °C and the other at 60 °C (for gel and running conditions see Tollit et al. 2009). Running two variations in temperature aided in the resolution of prey using DGGE techniques. Over both temperatures, the migration banding patterns of prey items per scat were compared with the migration of prey standards run in nine lanes of the gels. Scat bands matching a prey standard under both running conditions were tentatively identified as matches (and a subsample subsequently confirmed with sequencing). Bands that did not match prey standards at one or both conditions were labeled "unknowns" and their relative migratory positions to the standards were noted. Unknowns were classified based on known prey standard profiles at both temperatures. Representatives of each unknown (up to two from each original DGGE gel) were re-run on new DGGE gels alongside all others in the same grouping and were surrounded by standards and other known prey items that had similar migration profiles. After all of the unknowns were re-run and re-classified, the new identification was extrapolated to any other unknowns that had the same migration profile on the two original DGGE gels.

To confirm DGGE prey assignments and identify bands that did not migrate with standards (i.e., the unknowns), representative bands were excised from the gels, purified with Exo_SAP (Affymetrix, Ohio, USA), and sequenced. To accomplish this, each scat sample was re-amplified with the appropriate primer sets and re-run on DGGE leaving a lane between samples. The excised gel slices were added to 50 μL of sterile dH_2O, exposed to freeze thawing, and 2 μL of each was used as template in a subsequent seminested PCR (for conditions see Tollit et al. 2009). To confirm band identity, each was re-run next to their corresponding scat sample as a control.

For cephalopod prey identification, two seminested PCRs were performed on all samples using 2 μL of the primary PCR reaction as template, with forward primers (16ScephF or 16ScephFb) fluorescently labeled with NED (Applied Biosystems, Foster City, California, USA) for visualization of products (for primers and PCR conditions see Tollit et al. 2009). These were electrophoresed on 2.0% agarose gels with 1× TAE buffer for 110 V and 33 min. In this primary identification step, samples were scored and deemed positive based on fluorescent label incorporation at appropriate sizes (223 bp for giant Pacific octopus (*Enteroctopus dofleini* (Wülker, 1910)) and 189 bp for other species).

In a secondary confirmatory analysis, cephalopod positive samples from both primer sets were pooled into groups. Some positive cephalopod samples were either not visualized or not well separated on the DGGE gels based on the previous conditions (Tollit et al. 2009). Therefore, new DGGE conditions were determined using amplifications of the pooled samples on a 15%–90% perpendicular denaturing gradient gel (7.5% acrylamide) at 60 V for 15 h at 56 °C. Various products were visualized and three ranges were identified to resolve these (15%–30%, 30%–45%, and 45%–60%). Both of the cephalopod primer sets were re-run under these three DGGE conditions and all unique bands were excised and sequenced. New cephalopod standards were developed based on the migration profiles of the sequenced pooled cephalopod samples, as well as the provided prey standards. The individual positive samples (pooled where both PCRs were positive) were subsequently resolved on 15%–30% DGGE at 60 V for 15 h at 58 °C and classified based on their migration profile. In cases where samples were not visualized under the 15%–30% DGGE conditions but were deemed positive based on agarose gels, a second set of DGGE conditions was used. Those not visualized on DGGE were excluded from diet estimates.

Identification of salmon (Salmonidae) and rockfish (Scorpaenidae) species was achieved by amplifying all samples with nested Salmonidae (MHC class II B2) and Scorpaenidae (cytochrome *b*) specific primers (see Tollit et al. 2009). These were electrophoresed on 2.0% agarose gels and 1× TAE buffer for 110 V and 33 min. Samples were deemed positive based on fluorescent label incorporation and appropriate size. These were subsequently resolved on DGGE and classified based on their migration profile. All unique bands were sequenced.

All samples were also amplified with nested crustacean (16S) specific primers (see Tollit et al. 2009) and electrophoresed on 2.0% agarose gels and 1× TAE buffer for 110 V and 33 min. Samples were deemed positive based on fluorescent label (Rox) (Applied Biosystems) and appropriate size. All sequencing reactions were performed using Big Dye Terminator version 3.1 Cycle sequencing kits (Applied Biosystems) and resolved on the ABI 3730 XL capillary sequencer. Nucleotide sequences were edited and assembled with Sequencher version 5.1 (Gene Codes Corp, Ann Arbor, Michigan, USA) and the sequences of the prey standards, as well as isolated bands of the soft scats (fish, cephalopods, and unknowns), were submitted to the NCBI BLAST and compared for identity

[1]Supplementary tables are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjz-2016-0253.

confirmation. High scoring matches (98% or greater) were considered species identities.

Data analysis of prey occurrences using hard part and soft part DNA identifications

Diet composition (fish and cephalopods; henceforth termed "prey") using morphological hard part identification and prey DNA identified from scat soft part matrix were compared using occurrence (presence–absence) measures. Analysis of prey occurrence data was performed using custom-written R code (R Core Team 2016) to determine diet composition estimated using percent split-sample frequency of occurrence (% SSFO; Olesiuk et al. 1990). In simple terms, this method weights prey species depending on how many prey species were present in the same scat (i.e., assumes all prey were consumed in equal volumes within each scat). The % SSFO dietary index has been frequently used to calculate diet diversity indices to compare with population trends (Merrick et al. 1997; Trites et al. 2007). To allow comparison with previous NMML diet assessments, we calculated prey counts, percent frequency of occurrence, and percent modified frequency of occurrence (Bigg and Perez 1985), whereby percent frequency of occurrence values are down-weighted so that, summed across all prey types, they total 100%. Percent SSFO gives relatively lower weighting to those species found in a mixed meal compared with percent modified frequency of occurrence and higher weighting for species found singly within a scat. Prey species were grouped and then collapsed into 20 primary prey "types" to allow ease of comparison when species-specific information was not consistently available across both techniques or for very uncommon species occurrences (prey type groupings were selected based on >1% frequency of occurrence over all 1990–2000 diets; for full species listing see Fritz et al. 2016). Species types typically included appropriate family groupings (e.g., Cottidae, Liparidae, Salmonidae), subfamily groupings (Pleuronectidae (flatfish spp.)), and ecological or residual groups of species. Sandfish spp., snailfish spp., skate spp., and myctophid spp. were subsequently collapsed into an "other key fish" grouping for ease of presentation. Additional species-specific resolution information based on individual DNA identifications was provided for seven prey types typically difficult to identify using prey hard part identification, such as salmon and rockfish.

All Steller sea lion scats collected in March–April 2008 and 2012 were used to describe the nonbreeding season diet composition based on (*i*) prey hard parts alone, (*ii*) on prey DNA alone, and (*iii*) a combined dual or "composite" method, noting that not all scats with hard part remains were subsampled for DNA or had prey DNA successfully extracted. All scats with positive DNA prey presence and (or) prey hard parts were included in estimating the overall composite diet. Statistical comparison for each primary prey type was made separately using contingency tests on count data (Wright 2010) and the overall % SSFO diet estimates compared using a Spearman's rank-order correlation test on the 10 most prevalent prey types. Dietary data were partitioned by collection year (2008 and 2012), by three RCA groupings (1–3, 4, and 5), and by scat collection site. Dietary diversity indices (DDI) were determined using techniques similar to those documented by Merrick et al. (1997) and Trites et al. (2007), noting % SSFO was weighted evenly across sites. A Shannon's diversity index (*H*) was also calculated (Sinclair and Zeppelin 2002).

To equitably compare the two different identification techniques, we also compared prey occurrences on an individual "matched" scat by scat basis to determine how often species occurrences matched and to what extent the inclusion of prey DNA data increased species richness in a scat (i.e., additional prey species incidences for which hard part identification had found no evidence) and also what species DNA was not identified that had been identified using hard parts. This matched scat by scat analysis included only those scats for which prey DNA had been successfully extracted. As opposed to the previous diet analyses, this data set included (to increase sample sizes) all additional scats collected in March 2012 east of 170°W, as well as those collected during summer 2012. The notably high occurrence (63%) of crustaceans identified using DNA detection methods alone were not included in these comparisons, as they are often considered secondary prey and consequently are not regularly reported using morphological hard part identification criteria.

Diet reconstruction of the numbers and size of prey consumed from prey hard parts

Bioenergetic reconstruction requires a number of iterative steps, which fall into two main types: (1) estimating the number, size, and energetic density of prey and (2) diet index modeling. The methods used in this study generally follow those outlined in Tollit et al. (2015), with supplementary information provided in Supplementary Tables S2 and S3.[1] A main source of prey length information was based on 903 Pacific IDentifications Inc. size-class estimates (from 2008 scats) using reference material. Median prey lengths were derived within each size-class type. Size-class types were typically 5–10 cm broad. Prey mass was subsequently calculated by applying prey length – prey mass allometric regressions (Tollit et al. 2015; Supplementary Tables S2 and S3[1]), 10 of which were Aleutian Islands specific regressions or newly derived based on NMML unpublished data. Prey size estimates were also supplemented by direct measurements of hard parts ($n = 165$), fish otoliths ($n = 51$), and cephalopod beaks ($n = 36$). As elements can undergo a reduction in size during digestion (Harvey 1989; Tollit et al. 2003), experimentally derived species (and where applicable) digestion grade specific DCFs were applied to hard part elements measured and graded in good or fair condition (Tollit et al. 2015; Supplementary Table S2[1]). Prey lengths were typically then calculated by applying a hard part length – prey length allometric regression to the DCF-corrected element length, followed by applying prey length – prey mass allometric regressions. Proxy species selections were sometimes necessary to estimate prey size for some "trace" species. Direct measurements of hard parts graded as good and fair condition were always used in preference to these species-specific mean sizes. Mean prey size estimates for each species type were calculated for 2008 scat data and applied to 2012 scat data in cases where no verified hard part measurements were available.

In addition to prey size estimates, Pacific Identification Inc. and NMML scientists attempted to determine the minimum number of individuals (MNI; Ringrose 1993) per species that was represented by all hard part structures within each scat. Where multiple individuals of the same species were found to be present within an individual scat, prey size was derived using only prey size estimates from that scat. All unique DNA occurrences were integrated with a MNI of 1. NCFs (Tollit et al. 2015) are applied to account for total digestion of hard parts, and where data were available, prey size specific NCFs were applied (e.g., Tollit et al. 2007, 2015; Supplementary Table S4[1]). Energetic density (ED; dry mass in kJ/g) data were collated to calculate representative energy content values of key prey type using data preferentially collected in spring and the Aleutians Islands where possible (R. Heinz, NMFS AFSC, Auke Bay Laboratory, unpublished data), as well as literature and gray literature sources (Perez 1994; Logerwell and Schaufler 2005; see Supplementary Table S4[1]). A mean dry mass energy content (21.85 kJ/g) was applied to remaining species.

Unique DNA occurrences were merged with hard part data to provide a dual-method composite diet. Mean mass estimates based on the size of hard parts from 2008 scat collections were used to derive mass estimates from unique DNA occurrences. Estimating the mass of these unique DNA occurrences was particularly problematic for giant Pacific octopus because of the general lack of measurable hard parts of this species isolated in scats and the presumption that the general lack of evidence for octopus was

Table 1. Diet contribution for top prey types (>2.5% composite SSFO) based on scats collected during the nonbreeding season of Steller sea lion (*Eumetopias jubatus*) in 2008 and 2012 using prey hard parts alone (number of scats = 598; individual prey identifications = 1547), prey DNA alone (number of scats = 480; individual prey identifications = 1019), and a composite of both methods (number of scats = 606; individual prey identifications = 2051).

	Hard parts		DNA		Composite	
Method rank	Prey type	% SSFO	Prey type	% SSFO	Prey type	% SSFO
1	Atka mackerel	20.0	Smooth lumpsucker	23.8	Atka mackerel	17.9
2	Rockfish spp.	14.1	Pacific cod	22.5	Pacific cod	12.9
3	Smooth lumpsucker	9.2	Cephalopod spp.	13.6	Smooth lumpsucker	12.3
4	Irish lord	8.1	Atka mackerel	13.2	Rockfish spp.	10.2
5	Walleye pollock	7.9	Rockfish spp.	7.9	Cephalopod spp.	8.5
6	Pacific cod	7.7	Walleye pollock	5.4	Walleye pollock	6.6
7	Greenling spp.	4.1	Salmon spp.	3.2	Irish lord	6.3
8	Salmon spp.	3.5	Greenling spp.	2.2	Greenling spp.	3.8
9	Rock sole	3.0	Irish lord	1.7	Salmon spp.	3.3
10	Cephalopod spp.	2.2	Rock sole	1.7	Rock sole	2.7
P		—		0.30*		0.65

Note: Spearman's rank-order correlation (*P*) tests were used to compare prey ranks of DNA and composite diets with hard parts; *, *P* was significant only for the hard part–DNA comparison.

due to the fact that larger beaks are retained in the stomach then egested in spews (Tollit et al. 1997; Gudmundson et al. 2006). NOAA fisheries scientists thus provided for giant Pacific octopus a hypothetical size frequency distribution based on an exponential distribution with $\lambda = 6$. The resulting mean mass estimate of giant Pacific octopus was 5656 g and was used in subsequent dietary estimates.

Two sensitivity scenarios were modeled to highlight the sensitivity of diet estimates to two key bioenergetic model assumptions. Firstly, we re-calculated diet using the predicted modal size of octopus (500 g) rather than mean size (5656 g) for all unique DNA octopus detections. Secondly, we substituted energetic density data for Atka mackerel (21.35 kJ/g) estimated for spring from unpublished data presented by Rosen and Trites (2013), rather than using NOAA data (29.47 kJ/g), which lacked relevant prey data from March and April.

Composite dietary index modeling

Custom R code was developed to provide two reconstructed composite diet model variants (variable (V) and fixed (F); sensu Laake et al. 2002). Proportion of prey biomass ($\hat{\pi}$) was first determined using the variable biomass (BR-V, where BR is biomass reconstruction) and fixed biomass (BR-F) models (Laake et al. 2002):

$$\text{BR-V:}\quad \hat{\pi}_i = \frac{\sum_{k=1}^{s} b_{i,k}}{\sum_{i=1}^{w}\sum_{k=1}^{s} b_{i,k}}$$

$$\text{BR-F:}\quad \hat{\pi}_i = \frac{\sum_{k=1}^{s}\left(b_{ik}\Big/\sum_{i=1}^{w} b_{ik}\right)}{s}$$

where b_i is the total biomass of prey type i, w is possible prey types, b_{ik} is the total biomass of prey type i in the kth scat, and s is the total number of fecal samples containing prey.

Overall dietary contribution was for comparison, re-calculated using percent frequency of occurrence, % SSFO methods (Olesiuk et al. 1990), and percent prey number. Energy density (ED) dietary estimates applied prey energy density data to BR estimates and variable energy density (ED-V) considered our best index to describe diet, based on the results of diet reconstruction captive feeding studies (e.g., Tollit et al. 2007; Phillips and Harvey 2009). Variable diet models allow for variability in foraging success (meal size) between animals. Fixed energy density (ED-F) models treat the biomass contribution of every scat equally, i.e., a fixed proportion of the total. Diet contribution was summarized across primary prey types or groupings. Diet was compared using a Spearman's rank-order correlation test (Wright 2010).

Percent ED-V estimates of diet were calculated for scat subsets of 2008 and 2012 separately and subsets of RCAs 1–3, 4, and 5 separately. An assessment of scat subsampling error levels was made using bootstrapping techniques in which median (500th sorted point estimate) diet estimates and nonparametric 95% confidence intervals (25th and 975th sorted point estimates) were derived by randomly subsampling scats with replacement and bootstrapping 1000 times.

Results

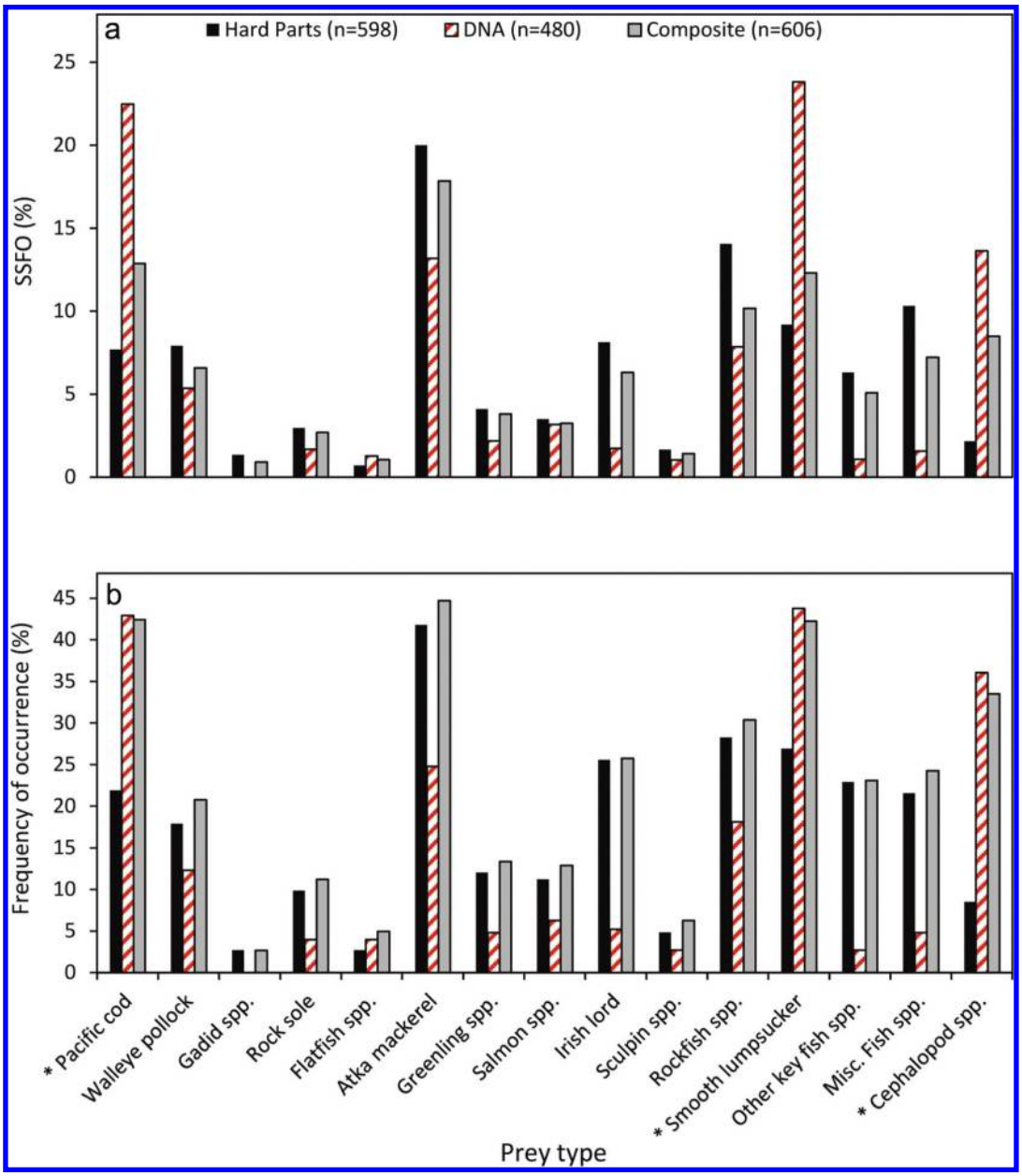
Prey hard part, prey DNA, and composite nonbreeding season diet by occurrence

The composite nonbreeding season diet (hard parts and unique DNA detections) was based on 2051 prey occurrences (3.4/scat, $n = 606$ scats), which combined 1547 prey occurrences (2.6/scat, $n = 598$ scats) identified by hard parts and 1019 prey occurrences (2.1/scat, $n = 480$) identified by DNA methods. The majority of scats ($n = 341$; 56%) were collected in RCA 4, with 145 collected across RCAs 1–3 combined and 120 collected in RCA 5. The overall composite % SSFO diet contained seven species types contributing more than 5%, with Atka mackerel (18%), Pacific cod (13%), and smooth lumpsucker (12%) being the most dominant, and rockfish spp. (10%), cephalopod spp. (~8%), walleye pollock (7%), and Irish lord spp. (6%) were also featured (Table 1; for comprehensive species listing see Fritz et al. 2016).

Hard parts and DNA identified an identical suite of the top 10 most prevalent species types; however, ranking varied significantly (Table 1). Percent SSFO diet composition based on hard parts was dominated by Atka mackerel (20%) and rockfish spp. (14%), followed by smooth lumpsucker, Irish lord, walleye pollock, and Pacific cod (all 8%–9%), whereas diet based on DNA was dominated by smooth lumpsucker (24%) and Pacific cod (23%), then contributions from cephalopod spp. (14%) and Atka mackerel (13%), followed by rockfish spp. (8%) and walleye pollock (5%) (Table 1).

The composite diet incorporated 504 new DNA-based prey occurrences (33% increase) compared with hard parts alone, but the addition of DNA occurrences did not significantly alter primary prey type rankings when comparing hard parts alone against the composite diet (Table 1; Figs. 2*a*, 2*b*). New DNA occurrences were dominated by cephalopod spp. ($n = 152$, 30% of total new occurrences), Pacific cod ($n = 126$, 25%), and smooth lumpsucker ($n = 95$, 19%), resulting in significant increases in these three primary in-

Fig. 2. Diet contribution measured as (*a*) percent split-sample frequency of occurrence (% SSFO) and (*b*) percent frequency of occurrence of key prey types based on scats collected during the nonbreeding season of Steller sea lion (*Eumetopias jubatus*) in 2008 and 2012, as determined by prey hard parts, DNA, and a composite of both methods (*n* = number of scats). An asterisk associated with a prey type indicates a significant increase in dietary contribution using composite methods compared with hard parts alone (all $P < 0.001$). Color online.



dividual prey types, when comparing hard parts alone with the composite diet (Table 2). DNA identifications thus did clearly impact the nonbreeding season composite % SSFO diet, most notably with ~3-fold increases in the contribution by cephalopod spp. (2%–9% SSFO or 34% frequency of occurrence from 9%), but also a two-thirds increased contribution by Pacific cod (8%–13% SSFO or 42% frequency of occurrence from 22%) and a third increased contribution by smooth lumpsucker (9%–12% SSFO or 42% frequency of occurrence from 27%; Figs. 2*a*, 2*b*) compared with using hard parts alone. These conclusions were also consistent when only matched scats were compared. The occurrence of most individual prey types differed between prey hard parts and prey DNA detection (Table 2). For example, DNA notably identified significantly fewer Irish lord, skate spp., Atka mackerel, greenling spp., salmon spp., myctophid spp., rockfish spp., and rock sole spp. than hard parts alone (Table 2), reducing their importance overall (Figs. 2*a*, 2*b*) compared with the overall composite diet estimate.

Percent SSFO diet composition varied regionally, with RCAs 1–3 dominated equally by Hexagrammidae (a combination of both Atka mackerel and greenling spp.) and smooth lumpsucker. In contrast, RCA 4 diet included a strong contribution from Pacific cod and walleye pollock, as well rockfish spp., Hexagrammidae, cephalopod spp., and smooth lumpsucker, whereas RCA 5 was dominated by Atka mackerel, with smaller contributions of cephalopod spp. and smooth lumpsucker. Consequently, the overall RCA nonbreeding season diet diversity index (DDI) was considered comparatively high, with the most diverse diet found in RCA 4 and the least found in RCAs 1–3 (Table 4). Shannon's diversity index (*H*) based on 20 species types was also highest in RCA 4, but was lower in RCA 5 and RCAs 1–3 (Table 4). The inclusion of

Table 2. Statistical comparison of the frequency of occurrence of main prey species types using different prey identification methods (sensu Wright 2010).

	Hard parts vs. DNA			Hard parts vs. composite		
Prey type	Φ	χ^2	*P*	Φ	χ^2	*P*
Pacific cod	**0.23**	**54.7**	**<0.0001**	**0.22**	**57.93**	**<0.0001**
Walleye pollock	–0.08	6.41	0.0113	0.04	1.62	0.2031
Gadid spp.	**–0.11**	**13.04**	**0.0003**	0	0	1
Rock sole	**–0.11**	**13.85**	**0.0002**	0.02	0.59	0.4424
Flatfish spp.	0.04	1.39	0.238	0.06	4.24	0.0395
Atka mackerel	**–0.18**	**34.24**	**<0.0001**	0.03	1.04	0.31
Greenling spp.	**–0.13**	**17.41**	**<0.0001**	0.04	2.21	0.1371
Salmon spp.	**–0.13**	**17.41**	**<0.0001**	0.04	2.21	0.1371
Irish lord	**–0.27**	**80.2**	**<0.0001**	0	0	1
Sculpin spp.	–0.05	3.36	0.0710	0.03	1.16	0.2815
Rockfish spp.	**–0.12**	**15.11**	**<0.0001**	0.02	0.64	0.4237
Smooth lumpsucker	**0.18**	**33.4**	**<0.0001**	**0.16**	**31.21**	**<0.0001**
Sandfish spp.	–0.09	8.83	0.0030	0	0	1
Snailfish spp.	–0.08	6.61	0.0101	0.01	0.1	0.7518
Skate spp.	**–0.2**	**44.74**	**<0.0001**	0	0.01	0.9203
Myctophid spp.	**–0.17**	**29.89**	**<0.0001**	0	0	1
Misc. fish	**0.24**	**61.9**	**<0.0001**	0.03	1.23	0.2674
Cephalopod spp.	**0.36**	**142.55**	**<0.0001**	**0.33**	**132.21**	**<0.0001**

Note: Comparisons (2 × 2 contingency tests) are based on scats collected during the nonbreeding seasons of Steller sea lion (*Eumetopias jubatus*) in 2008 and 2012, as determined by hard parts, DNA analysis, and a composite method. Significant results ($P < 0.05$) are set in boldface type (Bonferroni correction, $P = 0.00139$). A negative Phi (Φ) indicates higher hard part occurrences.

DNA prey occurrences increased RCAs 1–5 DDI estimates by 10%–18%, on average, compared with hard parts alone, with the largest diversity increase observed for RCAs 1–3 (Table 4).

DNA methods were useful in resolving species (or species groups) within prey types typically identifiable only to the family level or found lacking hard part remains. Analysis of nonbreeding season DNA data categorized 37 Salmonidae identifications as 60% sockeye salmon (*Oncorhynchus nerka* (Walbaum, 1792)) or pink salmon (*Oncorhynchus gorbuscha* (Walbaum, 1792)), 24% chum salmon (*Oncorhynchus keta* (Walbaum, 1792)), and 11% chinook salmon (*Oncorhynchus tshawytscha* (Walbaum,1792)). The large majority (93%) of 89 Sebastidae identified were grouped into a type containing dark-blotched rockfish (*Sebastes crameri* (Jordan, 1897)), northern rockfish (*Sebastes polyspinis* (Taranetz and Moiseev, 1933)), or dusky rockfish (*Sebastes variabilis* (Pallas, 1814)), whereas more than 83% of Hexagrammidae (greenling) spp. were considered rock greenling (*Hexagrammos lagocephalus* (Pallas, 1810)) and most (92%) Irish lords (*Hemilepidotus* spp.) were either red Irish lord (*Hemilepidotus hemilepidotus* (Tilesius, 1811)) or yellow Irish lord (*Hemilepidotus jordani* Bean, 1881). The majority (68%) of the fish grouped into Pleuronectidae (flatfish) type were arrowtooth flounder (*Atheresthes stomias* (Jordan and Gilbert, 1880)). Hard parts from the nonbreeding season were used to identify 39 cephalopods to order, of which most (37) were simply categorized as squid spp. DNA provided additional genus or species resolution for 92 cephalopod identifications, with a surprising 54% as giant Pacific octopus (given hard parts had identified 2/39 as octopus spp.). The remaining identifications were all squid, with various species of the genus *Gonatus* Gray, 1849 (24%) being the most dominant, followed by species of the genera *Gonatopsis* Sasaki, 1920 (12%) and *Berryteuthis* Naef, 1921 (9%).

Prey hard parts and prey DNA occurrence – matched scat comparison

DNA–DGGE sequencing techniques using seminested 16S primer sets (Tollit et al. 2009) were applied in this project to a total of 639 homogenized soft scat matrix subsamples (nonbreeding and breeding 2000s, RCAs 1–6), of which 572 (90%) successfully amplified fish or cephalopod prey DNA from 35+ species. Most scats (87%) that did not amplify prey DNA were from 2008 and likely reflect that these samples were stored frozen without a preservative for 5 years prior to analysis. Crustacean PCR positives amplified across 63% of samples. PCR positive amplifications for cephalopods were three times more successful in 2012 relative to 2008.

A matched scat by scat comparison of 572 scats highlighted that overall DNA identified fewer prey occurrences ($n = 1263$, 2.2 prey/scat) than hard parts ($n = 1526$, 2.7 prey/scat). Notably, 612 prey occurrences were unique to DNA identifications, resulting in a composite prey occurrence ($n = 2138$, 3.7 prey/scat) estimate 40% higher (or 1 prey/scat) than using hard parts alone. Up to a third of these new DNA-based occurrences were cephalopod spp., 22% were Pacific cod, and 16% were smooth lumpsucker. Within a scat, DNA and hard parts had identical species detections in only 662 cases (43%). Direct matches were mainly Atka mackerel (25%), smooth lumpsucker (19%), rockfish spp. (13%), and Pacific cod (13%), reflecting the four top prey species overall (Fig. 3). Hard parts detected 864 prey occurrences undetected by DNA methods, highlighting the necessity of using both techniques. The major species frequently not detected by DNA methods were Irish lord (15%; $n = 129$), Atka mackerel (9%), rockfish spp. (9%), other greenling spp. (6%), and skate spp. (6%). Although detected in relatively low numbers by hard parts, DNA did not detect any myctophid spp., sandfish spp., or sandlance (Fig. 3).

Bioenergetic composite nonbreeding season diet (prey hard parts and DNA)

Prey hard parts were enumerated, sizes were reconstructed, and prey DNA was identified from a subset of 606 Steller sea lion scats collected in March–April 2008 and 2012 in the Aleutian Islands. A total of 2353 prey items were estimated to represent a biomass total of 2248 kg (Supplementary Table S4[1]). The bioenergetic contribution of key prey types was determined while attempting to account for differential digestion of hard parts (using DCFs and NCFs) and sensitivity to two key assumptions. Spearman's rank-order correlation tests highlighted no significant differences (all $P > 0.05$) in main prey type ranking across sensitivity scenarios (varying the size of DNA-based octopus detections and variability in the energetic density of Atka mackerel), NCF application, scat mass scaling (variable versus fixed contribution per scat) assumptions, nor indeed diet based on different indices (% BR and % SSFO). Nevertheless, relatively large (2+-fold) differences in percent contribution were apparent for individual species as a result of these different assessment methods, especially for Pacific cod and cephalopod spp. (Table 3, Fig. 4), both higher using bioenergetic indices.

The primary index considered to best describe the overall diet and to make methodological comparisons was percent bioenergy with variable contribution by scat (% ED-V). Two prey species were clearly identified as primary in importance across all scenarios (Table 3): Atka mackerel, which contributed 22% of the energy overall (minimum–maximum scenario range: 17%–27%), and Pacific cod, which contributed 20% (20%–24%). The bioenergetic contribution of cephalopods was dominated by the giant Pacific octopus (Table 5). Cephalopods contributed 19% when using the octopus mean mass assumption (species type ranked 3rd, and considered an upper level limit based on scenario sensitivity assumptions), but contributed only 2% when a median octopus mass was modeled (species type ranked 11th, and considered a lower level limit; Fig. 4). Thus, the potential importance of octopus and cephalopods overall ranges from considerable to minor, depending on the size assumption when this species was detected by DNA. All other species were of course proportionally up-weighted to maximum scenario ranges when the far lower median octopus mass was modeled.

Differences in energetic density selection of Atka mackerel led to a 5% difference in ED-V contribution (22% versus 17%). Only

Fig. 3. Diet contribution (percent frequency of occurrence) comparison of all prey types using 572 matched scats collected during the nonbreeding season of Steller sea lion (*Eumetopias jubatus*) in 2008 and 2012 for prey hard parts (HP) alone (black bars), only those identified using DNA (red (gray in print) hatched bars), and those species identified identically (gray bars) or matched by both techniques within the same scat (n = total prey occurrences for each bar color). Color online.

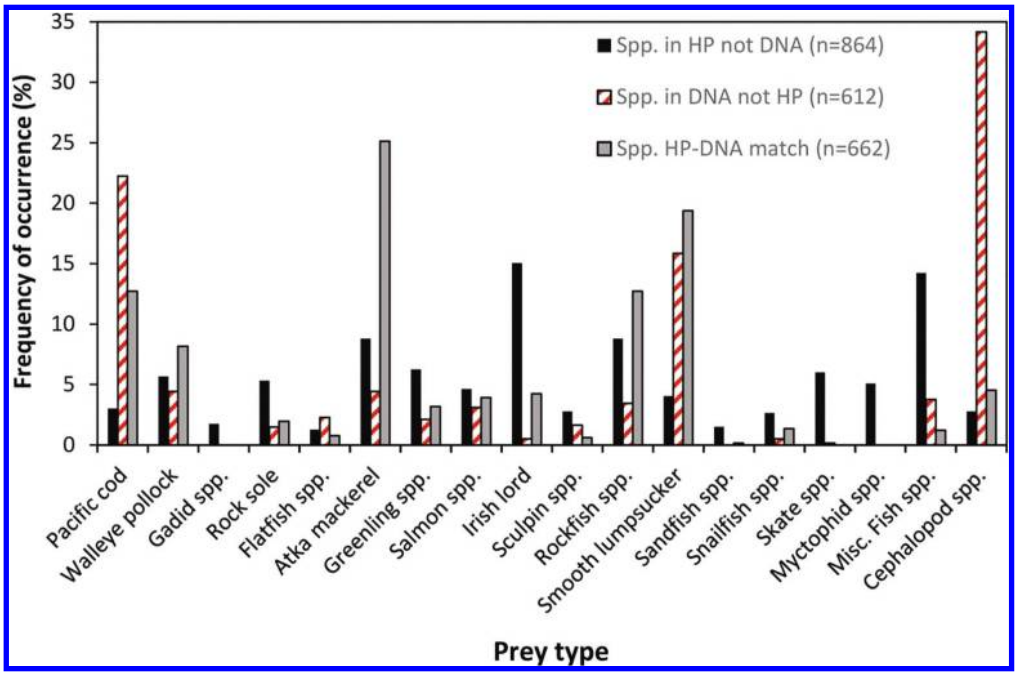


Table 3. Composite diet contribution of main species types based on scats collected during the nonbreeding season of Steller sea lion (*Eumetopias jubatus*) in 2008 and 2012, comparing sensitivity scenarios and key diet indices (% ED is the mean bioenergetic diet estimated by reconstructing prey number with numerical correction factors (NCF), prey biomass, and prey energy density (ED); % BR is the biomass diet estimated reconstructing prey number and prey biomass; NCF are numerical correction factors that aim to account for interspecies differential digestion of hard parts; % SSFO is the diet estimated using split-sample frequency of occurrence of prey; V is the diet estimated with each scat contributing variable proportion of mass; F is the diet estimated with each scat contributing a fixed proportion of mass).

	% ED-V			% BR-V					
Species category	Primary diet index	Modal octopus mass	Minimum Atka ED	With NCF	No NCF	% ED-F	% SSFO	% Number	Rank by % ED-V
Pacific cod	**20.1**	**24.3**	**21.4**	**21.7**	**35.5**	**20.1**	**12.5**	**11.6**	**2**
Walleye pollock	1.5	1.8	1.6	1.5	2.1	3.5	6.4	6.3	12
Gadid spp.	0.4	0.5	0.4	0.4	0.4	0.6	0.9	0.7	14
Rock sole	0.4	0.5	0.4	0.4	0.5	0.8	2.7	3.1	15
Flatfish spp.	0.5	0.6	0.6	0.5	0.6	0.5	1.1	1.4	13
Atka mackerel	**22.0**	**26.7**	**17.0**	**15.5**	**10.4**	**21.3**	**18.3**	**18.6**	**1**
Greenling spp.	2.1	2.6	2.3	2.6	1.8	2.5	3.8	3.7	10
Salmon spp.	3.4	4.2	3.7	3.5	3.0	3.8	3.3	3.8	8
Irish lord	8.4	10.2	9.0	9.0	6.2	7.7	6.4	6.9	5
Sculpin spp.	1.9	2.3	2.0	2.0	1.2	1.8	1.4	1.7	11
Rockfish spp.	4.2	5.1	4.5	3.6	4.4	7.5	10.7	8.7	6
Smooth lumpsucker	9.2	**11.1**	9.7	11.2	6.8	**11.2**	**12.2**	**11.3**	4
Other key fish	2.6	3.2	2.8	2.3	2.0	2.1	5.1	6.8	9
Misc. fish	4.0	4.8	4.2	3.8	4.0	5.4	7.2	6.5	7
Cephalopod spp.	**19.3**	2.1	**20.5**	**21.9**	**21.1**	**11.2**	8.0	9.0	**3**

Note: The top three species categories are set in boldface type.

three other species types potentially contributed ≥5% of the energy consumed: smooth lumpsucker contributed 9% (9%–11%), Irish lords contributed 8% (8%–10%), and rockfish contributed 4% (4%–5%). Salmon, walleye pollock, greenlings, and sculpins made smaller contributions to energy consumed (2%–4%; Table 5). Percent ED-V (median and 95% confidence intervals) are provided for the top 20 key species types (Table 5; for finer scale species subgrouping contributions see Fritz et al. 2016), with composite diet using alternate indices (% ED-F, % SSFO, and by % number) detailed in Table 3.

Estimates of fish fork length averaged 32 cm with coarse mean size estimates of 59 cm for Pacific cod (range 38–79 cm, mode 60 cm), 33 cm for Atka mackerel (range 21–48 cm, bimodal 21 and 41–48 cm), 34 cm for walleye pollock (range 28–45, mode 45 cm), 19 cm for smooth lumpsucker, 31 cm for rockfish, 29 cm for Irish lord, and 43 cm for salmon (see Fritz et al. 2016).

Incorporating prey numbers and mass to occurrence-derived indices increased the importance of larger prey, such as Pacific cod and octopus, as well as multiple prey of a single species occurring within each scat, often encountered for Atka mackerel.

Table 4. Diet diversity indices (DDI) by rookery cluster area (RCA) based on scats (*n*) collected during the nonbreeding season of Steller sea lion (*Eumetopias jubatus*) in 2008 and 2012, as determined using percent split-sample frequency of occurrence (% SSFO) on both composite and prey hard parts (HP) alone.

			DDI based on 7 species groupings		DDI based on 8 species grouping	
RCA	*n*	*H* based on 20 species categories; composite	HP	Composite	HP	Composite
1–3	145	2.19	2.84	3.68	3.58	4.42
4	341	2.42	4.41	4.90	5.88	6.34
5	120	2.05	3.65	4.24	4.14	4.69
All	606	2.48	4.02	4.76	5.57	6.14

Note: A Shannon's diversity index (*H*) was calculated based on the primary 20 prey categories. A DDI for seven species groupings are comparable with methods described by Merrick et al. (1997), whereas a DDI for eight species groupings are comparable with methods described by Trites et al. (2007).

Relatively small prey (including walleye pollock, all squid species) or those prey with lower energetic density (e.g., smooth lumpsucker, greenling) were reduced in importance. Conversely, NCFs reduced the importance of gadids, particularly Pacific cod, and to lesser extent cephalopods (Table 3), reflecting the scientific findings of numerous captive feeding studies and highlighting the hard part robustness of these species. Prior to the application of NCFs, Pacific cod contributed 36% by mass compared with only 13% by occurrence, largely reflecting the largest mean mass (2.9 kg) of fish estimated in this study. In contrast, walleye pollock contributed 6% by occurrence and only ~2% by mass, reflecting relatively small prey sizes, averaging approximately 0.3 kg.

Uncertainty was assessed in how scat mass was proportioned (variable and fixed variants) and effects of subsampling errors through bootstrap analysis. Conceptually, variable and fixed variants can perhaps be best viewed as coarse point estimate diet bookends, in which the variable variant allows for variability in the biomass contribution of each scat, whereas the fixed variant standardizes the biomass each individual scat represents. Variable variants, in theory, better allow for differences in meal size and (or) foraging success. In this study, differences in model variants were negligible for the two primary prey, but were notable for three species (Table 3, Fig. 4). Octopus importance was 2-fold higher using variable models (reflecting its above average mean mass within multiple scats), whereas walleye pollock and rockfish were close to half as important using variable models (reflecting not only smaller prey size, but also that these prey species were also found relatively more often as single species in scats). Subsampling error analyses are useful in minimizing the impact of "outlier" scats, especially in cases where a few scats have a very large biomass of prey. They are therefore typically largest for variable model variants and upper intervals are higher than lower. Subsampling errors (% ED-V; Table 5) ranged from ±9% to 12% for frequently occurring species, but were considerably higher for less abundant species (from ±20% to 50%).

Steller sea lion diet (percent with scat mass contribution variable, % ED-V) was collapsed by RCA (Table 5) and by year (Table 5), but the documented variability in each influenced the overall diet reported in this study. The bioenergetic diet of RCAs 1–3 combined was relatively high in smooth lumpsucker (18%) and octopus (21%, using mean mass), and also had high–moderate levels of Atka mackerel (15%) and Pacific cod (13%). RCA 4 bioenergetic diet was dominated by Pacific cod (30%), with moderate contributions from Atka mackerel (12%) and octopus (13%). RCA 5 bioenergetic diet was highly dominated by Atka mackerel (48%), with moderate contributions of octopus (16%) and Irish lord (10%). Rock and kelp greenlings were mainly found in RCAs 1–3 and rockfish were mainly found in RCA 4. Irish lord contributed 8%–10% across all RCA clusters (Table 5). Variability in diet across years was most apparent for two species. Atka mackerel contributed 28% in 2008 and only 16% in 2012, whereas conversely smooth lumpsucker contributed only 6% in 2008 and 12% in 2012. Differences partly reflect that scats were not collected in RCAs 1–3 in 2008.

Discussion

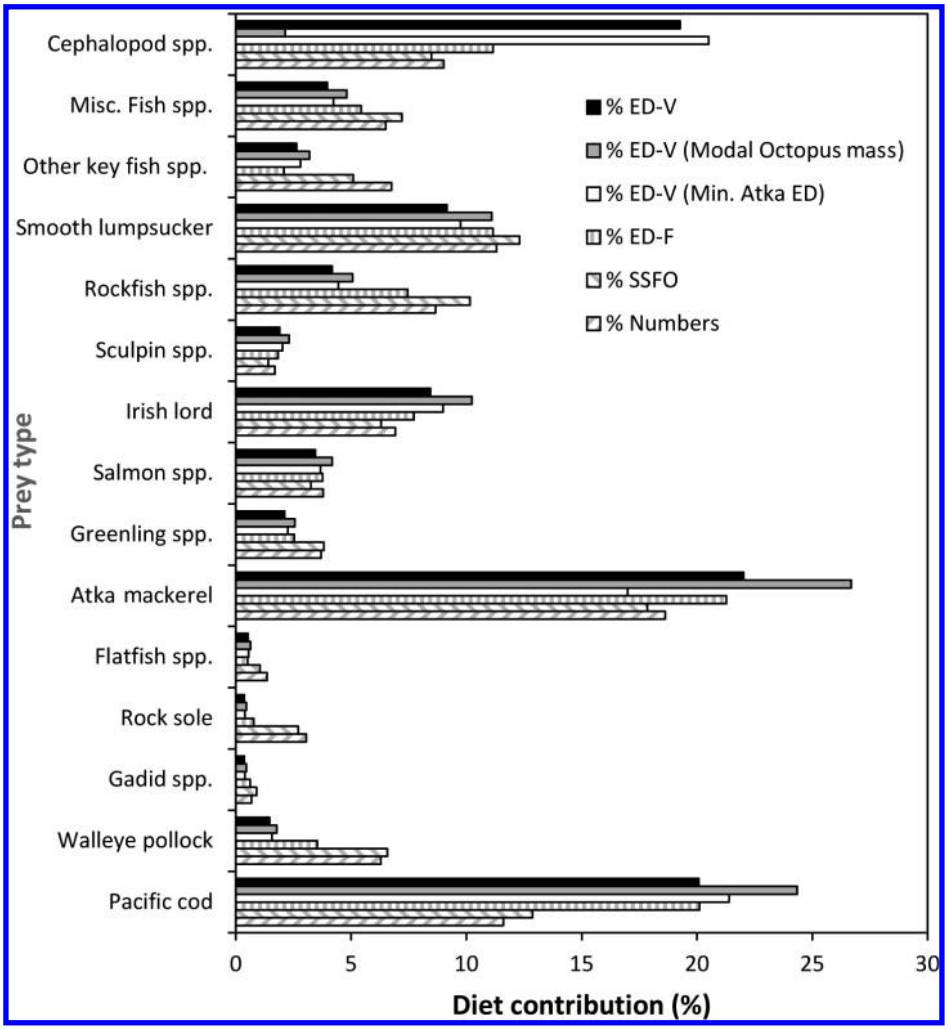
Building upon previous Steller sea lion dietary studies (Tollit et al. 2009, 2015), we used both prey hard part and prey DNA (PCR–DGGE) identifications from over 600 scat samples to determine the composite bioenergetic contribution of prey consumed by endangered Steller sea lions in the Aleutian Islands during the nonbreeding season. This is the first bioenergetic diet reconstruction that integrates prey species, number, size, and energetic density for Steller sea lions in the Aleutian Islands, a region that continues to see population declines. This paper is partitioned into two parts: (1) we test hypothesis one, i.e., whether diet determined using traditional techniques (prey hard part identification) is the same as that determined using DNA prey detection methods alone or determined using a composite approach of both methods (including an exploration of why differences may occur between methods); (2) we describe the overall nonbreeding season composite diet based on a bioenergetic dietary reconstruction, testing our second hypothesis (that diet based on frequency of occurrence methods is the same as that based on biomass reconstruction), and exploring the insights gained from using a dual-method approach.

In summary, this study confirmed the primary importance of adult and subadult Atka mackerel in the nonbreeding season overall. In contrast to previous occurrence-based diet assessments (Sinclair and Zeppelin 2002; Sinclair et al. 2013), we found that the bioenergetic contributions of adult Pacific cod and potentially giant Pacific octopus had been considerably underestimated (Table 3). This key result reflects the relatively large size of Pacific cod and size predictions of octopus, coupled with both being more frequently identified by DNA techniques compared with prey hard parts. Young adult smooth lumpsucker, small Irish lords, and rockfish also contributed energetically. Atka mackerel contributed strongly in 2008 and RCA 5, whereas smooth lumpsucker was more dominant in 2012 and RCAs 1–3. Pacific cod contributed in both collection years, particularly in RCA 4. Cephalopods were common in both years and all study areas. As a result, the overall nonbreeding season Aleutian Island diet in the 2000s was found to be more diverse than in the breeding season (Sinclair and Zeppelin 2002; Sinclair et al. 2013). Fritz et al. (2016) describe a full comparative analysis of diet diversity and sea lion population trends in the Aleutian Islands.

Comparing occurrence of prey hard parts and DNA

This project successfully improved and applied group-specific nested PCR primers, high-resolution DGGE, and BLAST program sequence matching for recovering and analyzing prey DNA from scat material collected from wild Steller sea lions. Prey DNA degradation during digestion and low concentration of prey DNA in scats can be a concern (Symondson 2002), but our extraction success rates were high (90%), despite the 5-year frozen storage of one-third of all samples processed. The DNA of more than 35 species of prey was identified from 572 matched scat soft part matrix subsamples, averaging 2.2 prey species types per scat (compared with hard parts that averaged 2.6/scat). New DNA-based identifications increased the overall composite prey occurrence total to 2138 (an increase of 1+ new prey species per scat), and the taxonomic resolution for seven key prey types. However, only 662 (43%) of 2138 composite prey occurrences across matched scats were identical in the two methods. High numbers of unique (method-specific) prey occurrences identified by both hard parts (n = 864) and DNA (n = 612) reconfirms that noninvasive DNA methods can provide valuable comparative and complementary

Fig. 4. Composite diet based on scats collected during the nonbreeding season of Steller sea lion (*Eumetopias jubatus*) in 2008 and 2012 using percent bioenergetic prey type contribution (with scat mass contribution variable, % ED-V), including scenarios highlighting assumptions around predicting octopus sizes detected by DNA and variability observed in using a minimum energy density of Atka mackerel (*Pleurogrammus monopterygius*), as well as percent bioenergetic contribution with scat mass contribution fixed (% ED-F), using percent split-sample frequency of occurrence of prey (% SSFO) and by percent number.



prey occurrence data for pinnipeds and that resulting composite diet estimates are likely to be optimal.

Overall, we found no evidence from our DNA analyses for hard part identification having substantially missed major dietary components. Atka mackerel was the top-ranked nonbreeding season (RCAs 1–5) prey based on both hard parts occurrence alone (20%) and composite methods (18%). Pacific cod, smooth lumpsucker, and rockfish spp. all contributed >10% to the composite diet, with cephalopod spp., walleye pollock, and Irish lord contributing >5%. The top 10 most prevalent species were identical across both identification techniques (Table 1). Generally, this is a reassuring result for past Steller sea lion diet studies based solely on hard part identification of scats (Sinclair and Zeppelin 2002; Trites et al. 2007; McKenzie and Wynne 2008; Sinclair et al. 2013).

However, in testing hypothesis one, we found prevalent prey rankings were significantly different between hard parts and DNA analyses, and in particular, three prey types were significantly underestimated by using hard parts alone. Most notably, the importance of cephalopods and to a lesser degree Pacific cod and smooth lumpsucker were underestimated (Figs. 2*a*, 2*b*, 3). For example, across the nonbreeding season, cephalopod spp. was ranked 10th based on hard parts (2% SSFO), ranked 3rd by DNA (14%), and ranked 5th in the resulting composite diet estimate. This 3+-fold increase reflected the large proportion of unique cephalopod DNA-based identifications found. Pacific cod was ranked 6th by hard parts (8%), 2nd by DNA (22%), and 2nd in the composite diet (13%) (Table 1). Smooth lumpsucker was ranked 3rd by hard parts (9%), was the top ranked by DNA (24%), and also ranked 3rd in the composite diet. Atka mackerel and rockfish spp. were ranked 1st and 2nd by hard parts, 4th and 5th by DNA, and 1st and 4th by composite methods, respectively (Table 1; Figs. 2*a*, 2*b*).

It is not possible to determine the exact cause(s) of the differences observed between the two identification methods, nor whether they are due to ecological or methodological factors (or both). Examining ecological factors first, the DNA-based results clearly provide support to well-documented scat biases (Pierce and Boyle 1991; Tollit et al. 2010; Bowen and Iverson 2013). Firstly, cephalopods are generally under-represented in scats due to the retention and (or) regurgitation of beaks (Bigg and Fawcett 1985; Kiyota et al. 1999; Gudmundson et al. 2006). In our study, this was particularly true for giant Pacific octopus and various squid species of the genus *Gonatus*. Higher rates of regurgitation of larger cephalopods (with larger beaks) have also been observed in captive Steller sea lion feeding and diet reconstruction studies (Tollit et al. 2003). DNA results could simply reflect detection of the consumption of larger sized cephalopods, such as octopus, whose

Table 5. Composite diet contribution based on scats collected during the nonbreeding season of Steller sea lion (*Eumetopias jubatus*) in 2008 and 2012, using the primary bioenergetic diet reconstruction method (percentage with scat mass contribution variable (% ED-V)) overall, by year, and by rookery cluster area (RCA) compared for all 20 candidate prey species types determined using 1000 bootstrap reconstructions to describe median (50th percentile (50%)) and 95% confidence intervals (2.5th percentile (2.5%) and 97.5th percentile (97.5%)).

										Years 2008 and 2012								
	Years 2008 and 2012; RCAs 1–5			Year 2008; RCAs 1–5			Year 2012; RCAs 1–5			RCAs 1–3			RCA 4			RCA 5		
Species type	50%	2.5%	97.5%	50%	2.5%	97.5%	50%	2.5%	97.5%	50%	2.5%	97.5%	50%	2.5%	97.5%	50%	2.5%	97.5%
Pacific cod	**20.14**	18.16	22.18	**21.48**	18.14	24.81	**18.76**	16.28	21.34	**13.43**	9.95	17.19	**30.34**	27.32	33.63	5.69	3.45	7.96
Walleye pollock	1.47	1.21	1.73	2.06	1.66	2.51	0.87	0.63	1.14	0.33	0.10	0.61	2.55	2.11	3.00	0.30	0.10	0.54
Gadid spp.	0.38	0.18	0.61	0.18	0.01	0.44	0.57	0.24	0.98	0.36	0.00	0.88	0.49	0.21	0.85	0.14	0.00	0.51
Rock sole	0.38	0.29	0.47	0.35	0.23	0.49	0.40	0.28	0.55	0.09	0.02	0.19	0.65	0.48	0.82	0.09	0.02	0.19
Flatfish spp.	0.53	0.32	0.78	0.49	0.22	0.82	0.56	0.28	0.91	0.26	0.05	0.61	0.31	0.12	0.58	1.11	0.56	1.83
Atka mackerel	**21.89**	19.48	24.81	**28.38**	24.15	33.07	**15.54**	12.74	18.56	**14.94**	10.90	20.26	**11.75**	8.97	14.40	**48.47**	42.22	55.53
Greenling spp.	2.11	1.58	2.71	1.10	0.61	1.71	3.11	2.21	4.11	5.96	4.39	7.99	0.99	0.56	1.63	0.78	0.27	1.41
Salmon spp.	3.39	2.52	4.42	2.94	2.02	4.09	3.84	2.56	5.79	4.39	2.05	7.99	3.98	2.92	5.18	1.24	0.43	2.44
Irish lord	8.45	7.29	9.73	8.46	6.76	10.24	8.49	6.88	10.02	8.72	6.25	11.27	7.56	5.83	9.28	**9.95**	7.50	12.49
Sculpin spp.	1.90	1.26	2.64	1.33	0.55	2.44	2.41	1.52	3.47	2.51	1.09	4.28	1.30	0.62	2.06	2.45	0.94	4.69
Rockfish spp.	4.13	3.35	5.36	4.62	3.26	6.87	3.64	3.01	4.35	0.98	0.50	1.60	6.66	5.37	8.81	1.79	1.17	2.43
Smooth lumpsucker	9.14	8.31	10.13	6.25	5.05	7.57	12.07	10.72	13.58	18.30	16.29	20.63	8.14	6.80	9.55	2.95	1.85	4.22
Other key fish																		
Sandfish spp.	0.09	0.06	0.13	0.09	0.04	0.15	0.10	0.06	0.14	0.03	0.01	0.08	0.10	0.05	0.17	0.13	0.07	0.19
Snailfish spp.	0.01	0.00	0.01	0.00	0.00	0.02	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.00	0.02	0.00	0.00	0.00
Skate spp.	2.39	1.78	3.03	2.37	1.64	3.27	2.35	1.58	3.21	0.60	0.01	1.38	2.54	1.70	3.43	3.62	2.27	4.97
Myctophid spp.	0.16	0.09	0.23	0.19	0.08	0.32	0.12	0.06	0.19	0.13	0.05	0.23	0.18	0.09	0.31	0.11	0.00	0.29
Misc. fish	3.97	3.35	4.63	2.39	1.66	3.15	5.56	4.59	6.58	6.50	5.08	8.15	4.11	3.29	5.02	1.39	0.73	2.08
Cephalopod spp.																		
Octopus	**15.26**	12.27	18.19	**14.38**	10.25	18.37	**15.99**	11.89	20.12	**20.67**	14.31	26.80	**12.72**	8.93	16.62	**15.50**	9.71	21.16
Squid	0.11	0.07	0.16	0.03	0.01	0.05	0.19	0.13	0.29	0.08	0.03	0.14	0.12	0.06	0.20	0.13	0.07	0.21
Unidentified	3.85	2.88	5.03	2.59	1.22	4.18	5.16	4.01	6.50	1.27	0.40	2.46	5.16	3.55	6.97	3.69	1.98	5.72

Note: The top three species types in each grouping are set in boldface type.

beaks are infrequently retained and deposited in scats. Secondly, hard parts often underestimate prey with fragile skeletal elements (Jobling and Breiby 1986). Smooth lumpsucker have almost tissue-like bones and very small otoliths. Additional DNA detections in scats are thus not surprising given their overall frequency in the diet in some regions in both years. The additional DNA occurrences of Pacific cod may also be caused by a number of theorized ecological explanations. Perhaps, most compelling is the preferential regurgitation of hard parts of large fish observed in captivity in Steller sea lions (Tollit et al. 2003), but also evidenced within the samples collected during scat collection surveys. All five regurgitations found during scat collections for this study contained the vertebrae of very large gadids, of which the majority were Pacific cod. Removal of the head of large fish has been well documented (Pierce and Boyle 1991) and the heads of Pacific cod are relatively large and bony. Cranial structures (such as gill rakers) are frequently used to identify Pacific cod and the noningestion of the large cod head and the regurgitation of vertebrae may plausibly explain the differences between our DNA and hard part Pacific cod results.

Potentially, observed differences between the two identification methods reflect differences in transit time through the digestive gut. Prey DNA present in scat soft part matrix represents only the most recent feeding events, estimated to be diet over 1–2 days by both Deagle et al. (2005) and Casper et al. (2007*b*). In contrast, hard parts found in scats are from a composite of many past meals (typically 1–3 days, but up to 7 days when eating gadids and even longer if cephalopods are consumed; Tollit et al. 2003). Thus, hard parts can reflect prey consumed on longer or more distant foraging trips. DNA methods detected far fewer sculpins, Hexagrammidae, rockfish spp., and skate spp. compared with hard parts (Figs. 2*a*, 2*b*) and did not detect any myctophid, sandfish spp., or sandlance, though these last groups were only found in only a few scats. Hard part identification is potentially very sensitive and detections can be made based on a single scale, tooth, or gill raker. Lower rates of detection by DNA analysis may thus highlight method sensitivity differences, evidence of secondary prey ingestion (where a small prey is eaten first by a predatory fish and then itself consumed by a sea lion), or reflect interspecific prey digestibility. Certainly, the consumption of small amounts of a prey species may be beyond the detection limit of PCR, especially considering our use of "in bag" homogenizing and subsampling of scats. Deagle et al. (2005) reliably detected prey fed at 6% (by mass), sampling "blended" scats after overnight soaking and stirring (a preferable homogenizing technique), but found meals were not consistently distributed within pre-blended scats. In a similar, smaller comparison of species detected by DNA and not by hard parts, Tollit et al. (2009) highlighted salmon, and to a lesser degree, flatfish, elasmobranchs, and cephalopods as new prey detection. This may reflect regional differences in diet, foraging, and prey sizes consumed, but also highlights conclusions from one region cannot necessarily be carried over to another.

King et al. (2008) reviewed the pros and cons of different DNA-based approaches to molecular analysis of predation. In addition to sensitivity issues, it is important to recognize issues involving primer specificity and binding efficiency, e.g., due to mismatches (von Wintzingerode et al. 1997) and biases towards low guanine–cytosine (GC) content templates (Dutton et al. 1993). Potential problems associated with haplotype diversity, allelic variation, PCR artifacts, and cryptic bands highlight the need for continued validation of prey standards, good primer design, and assay optimization. For example, some positive cephalopod samples were neither visualized nor well separated on the primary DGGE gels. New DGGE conditions were developed and low numbers of false positives and negatives were subsequently identified in this secondary optimization process. Further optimization for cephalopods is recommended, as well as testing primer efficiency for those species consistently under-represented by DNA methods compared with hard parts.

DNA identification methods did not always resolve every prey to an individual species, yet for certain prey families and genera (particularly Salmonidae, *Sebastes* spp., Cephalopoda, *Hemilepidotus* spp., and Hexagrammidae), it was able to consistently increase taxonomic resolution compared with hard part identification. The cephalopod species consumed by Steller sea lions in the Aleutians during the nonbreeding season were mainly giant Pacific octopus and squid species of the genus *Gonatus*, whereas the relatively few salmon species consumed were mainly sockeye or pink or chum. Rock greenling was the predominant Hexagrammidae consumed (other than Atka mackerel), red and yellow Irish lords were the dominant *Hemilepidotus* spp. found, and most *Sebastes* spp. identified were darkblotched rockfish, northern rockfish, or dusky rockfish, not the more abundant Pacific Ocean perch (*Sebastes alutus* (Gilbert, 1890)) (Spencer and Ianelli 2015*a*, 2015*b*).

The nonbreeding season diets of Steller sea lions in the Aleutians are more diverse and have more temporal and spatial variation than the breeding season diet (Sinclair and Zeppelin 2002; Sinclair et al. 2013; Fritz et al. 2016). Atka mackerel is the most prevalent prey species consumed in both seasons, but the frequency of occurrence was lower during the nonbreeding season than during the breeding season, along with that of salmon and squid. In contrast to the breeding season in which only 5 prey taxa had a frequency of occurrence >5%, there were 12 species during the nonbreeding season (Fritz et al. 2016). In addition, this study showed 12 prey species were significantly more prevalent in the nonbreeding than breeding season, including pollock, Pacific cod, and smooth lumpsucker that aggregate to spawn in winter or spring, which likely makes them more attractive targets for sea lions (Yoshida and Yamaguchi 1985; Sinclair and Zeppelin 2002; Sinclair et al. 2013; Barbeaux et al. 2015; Thompson 2015; Thompson and Palsson 2015).

Diet composition varied spatially across the Aleutian Islands, though cautious interpretations are necessary when numbers of scats used in comparisons are small (Trites and Joy 2005). Compared with past hard part only nonbreeding season diet descriptions (Sinclair and Zeppelin 2002; Sinclair et al. 2013), this dual-method study (noting the majority of scats (56%) were collected in RCA 4) highlights a lower dominance of Atka mackerel, with Steller sea lions consuming a more diverse mix of prey, most notably higher occurrences of smooth lumpsucker, Pacific cod, and rockfish (both notably high in RCA 4), as well as cephalopods.

The majority of the prevalent prey identified during the nonbreeding season are considered benthic or semidemersal in nature, suggesting Steller sea lions are often feeding on or close to the sea bed. However, the two dominant prey species during March–April, Atka mackerel and Pacific cod, are generally aggregated (Pacific cod to spawn and Atka mackerel to feed), which would make them attractive targets for foraging sea lions. Atka mackerel undergo diurnal migrations throughout the year, spending nights on the bottom and entering the water column to feed during the day (Nichol and Somerton 2002). Atka mackerel energy density varies considerably through the year, reaching its lowest value in March and April (Rosen and Trites 2013) when most scats for this study were collected. Energetically, they still remain higher in density than both Pacific cod and smooth lumpsucker. Pacific cod prevalence in Steller sea lion diets is generally higher in the nonbreeding season throughout its range in Alaska, which makes them better foraging targets for sea lions when they are aggregated in late-winter to spawn (Sinclair and Zeppelin 2002). Smooth lumpsucker are poor swimmers, relying on camouflage to avoid predators, and form inshore spawning concentrations. The peak spawning period appears to be spring (Yoshida and Yamaguchi 1985; range December to June), coincident with this study's scat collection time period, suggesting that Steller sea lions may take advantage of such local concentrations. They were documented as notably abundant during walleye pollock surveys in the Aleutian basin and are found as a major diet item of Pacific cod (NOAA North Pacific Groundfish Diet Data, 1981–2007).

Diet diversity (sensu Merrick et al. 1997; Trites et al. 2007) estimated for RCAs 1–5 across the nonbreeding season was found to be higher than the rookery (breeding season) diversity across the Alaskan range (1990–1994) and also higher than the 1990–1994 haul-out (nonbreeding) data from Southeast Alaska (Trites et al. 2007; Fritz et al. 2016). Inclusion of DNA methods increased diet diversity based on hard parts alone by an additional 10%–18%. In our study, diet diversity was generally lowest in RCAs 1–3, where Hexagrammidae and smooth lumpsucker dominated the diet and where population trajectories are all negative (Fritz et al. 2016). However, there are two issues to consider regarding this result: (1) RCA 5 had the lowest Shannon diversity index (*H*) based on 20 species types, yet population trends in this area have been generally stable or increasing slightly in the 2000s (Fritz et al. 2016); (2) diet diversity indices in RCAs 1–3 in the nonbreeding season were higher than indices from other areas with increasing population trends. These results indicate that the relationship between diet diversity and population trend may not be as simple and direct as indicated by Merrick et al. (1997) and Trites et al. (2007) when applied across large geographic ranges with different prey communities. Diet diversity indices that collapse prey of a generalist predator into just seven to eight prey groupings may provide a coarse proxy for energy content or some measure of nutrition (Trites et al. 2007), but diet choice by Steller sea lions (and subsequent diversity) is considered more a function of abundant and accessible intermediate-sized prey, taking advantage of seasonal prey concentrations, especially if they are nutritious (Sigler et al. 2009). Indices based on a wider suite of prey (Sinclair et al. 2005) and using a bioenergetics approach are recommended for assessing dietary effects on populations (e.g., Sigler et al. 2009; Bowen 2000).

Overall, the unique DNA detections and increased species resolution achieved highlight the benefits of using an integrated approach, whereas the resulting differences between techniques afford a much needed assessment of potential biases, current limitations, and merits of each. DGGE approaches, like all DNA-based prey identification methods, require further validations to maximize accuracy and assess sensitivity. Ultimately, less time consuming, mass-target DNA prey detection systems (e.g., next-generation multisequencing or Fluidigm techniques; King et al. 2008; Deagle et al. 2010; Thomas et al. 2014, 2016), which could potentially also concurrently assess health or demographic information (e.g., Reed et al. 1997; Ream 2001), are considered the next steps to understanding ecosystem and anthropogenic interactions.

Composite diet using bioenergetic reconstruction

Bioenergetic diet reconstructions require multiple steps. Many of the key limitations are now well understood and can be addressed by the application of DCFs and by combining with DNA analyses (Tollit et al. 2010, 2015). Although we have attempted to draw upon best available data for multipliers such as DCFs, NCFs, and energy density, there is still considerable uncertainty and variability in many of these metrics, especially in the accuracy of NCFs applied (Bowen 2000). It is also important to recognize that the mass of prey identified using DGGE methods cannot reliably be estimated and consequently approximately half of the 2353 individual prey items in this study had no direct size estimates. This was particularly pertinent when assessing the importance of giant Pacific octopus (Fig. 4). Consequently, diet reconstructions have been presented with and without NCFs to provide clarity on the overall consequence of applying NCFs. In addition, two sensitivity scenarios were also developed to provide clarity on the effects of our assumptions of mass for octopus identified by DNA and notable monthly variability in the energy density of Atka mackerel (Fig. 4). Notably, in testing hypothesis two, we found no

statistical difference in main prey species overall ranking across energetic, biomass, and occurrence-based indices or between our three % ED-V scenarios. This suggests previous occurrence-based temporospatial comparisons of main prey rankings for Steller sea lions remain valid. Nevertheless, for many prey, 2+-fold differences in percent diet estimates occurred between bioenergetically reconstructed and occurrence-based diet methods (Fig. 4). Both Pacific cod and cephalopods were found to be more important bioenergetically, reflecting the large size of these prey and many unique DNA detections. Our octopus size predictions were based on the fact that small cephalopods are typically well represented in scats, whereas the beaks of larger cephalopods are often retained in the stomach and subsequently regurgitated (Bigg and Fawcett 1985; Tollit et al. 1997; Gudmundson et al. 2006). Of all our sensitivity assessments, octopus size prediction assumptions resulted in near 10-fold differences in diet contribution among cephalopods (19% versus 2%), highlighting high levels of uncertainty in this taxa's importance and also limitations of DGGE-DNA methods which can only identify prey presence. The bioenergetic importance of walleye pollock, rockfish, and rock sole were all far lower than that estimated by occurrence methods (Fig. 4). Differences among indices largely reflect the interplay between prey size, energetic density, and number of species and individuals within each scat.

This study aimed to account for additional uncertainty due to scat sampling, by generating nonparametric 95% confidence intervals. Confidence intervals ranged from ±9% to 12% for frequently occurring species, but were considerably higher for less abundant species (from ±20% to 50%). Given the potential biases and low precision highlighted in these analyses, point values of prey proportions presented here should be treated with appropriate caution, especially if they are subsequently used for calculating prey consumption estimates. Overall, analyses such as these are used optimally to understand which prey species may be primary prey to Steller sea lions, especially species that may have been previously underestimated or overestimated in describing diet using methods that do not account for number, size, or energy content. One final recognition is one of understanding that the 600 scats assessed in this diet study reflect combining two relatively short temporal "dietary-time windows" across a significant spatial scale.

Overall, we assessed, using an energetic (ED-V) approach, that three prey species types dominated the nonbreeding season diet of Steller sea lions in the Aleutian Islands in the 2000s: Atka mackerel, Pacific cod, and cephalopods (largely giant Pacific octopus). Of secondary importance were smooth lumpsucker and Irish lord. Rockfish, salmon, greenling, sculpin, and walleye pollock contributed lesser amounts (Table 5). Overall, this diverse nonbreeding season diet also reflects clear RCA-derived variability, with smooth lumpsucker being a primary prey in RCAs 1–3, Pacific cod dominating in RCA 4, and Atka mackerel being very dominant in RCA 5 (Table 5). Compared with the 1990s, the same range of species comprise the diet of Steller sea lions, but diversity was higher, with less Atka mackerel and salmon and larger contributions from Pacific cod, smooth lumpsucker, Irish lord, rockfish, and potentially (assuming our size estimates are reasonable) giant Pacific octopus (Sinclair and Zeppelin 2002).

This diet reconstruction study highlights three key conclusions. Firstly, there are clear advantages of a dual composite approach that uses both traditional prey hard part identifications and DNA-based ones. DNA detections increased the number of prey occurrences by 33% compared with hard parts alone, with significant individual species increases for cephalopods (3-fold increase), Pacific cod, and smooth lumpsucker. Secondly, size matters for two reasons. Firstly, many prey missed by hard parts and detected by DNA in this study are hypothesized to be relatively large in size. Based on direct measurements, the modal size of Pacific cod found as hard parts in this study is 60 cm, noting *Gadus macrocephalus* translates as "large-headed cod". We theorize that Steller sea lions may discard the heads of large prey like Pacific cod before consuming them or regurgitate hard parts if they ingest them, thus reasonably explaining the amount of DNA-only detections observed for this species. Similarly, large cephalopod beaks are known to be preferentially regurgitated, whereas small beaks pass through the digestive system. Secondly, mass-based diet reconstruction models are unsurprisingly strongly influenced by prey with above average mass (Laake et al. 2002; Tollit et al. 2015). Lastly, bioenergetic diet reconstructions of this type are time consuming and reliant on multiple assumptions. Considerable progress in mass-target DNA prey detection systems and DNA-based diet quantification techniques has been made and these likely represent the future direction of diet assessments for pinnipeds.

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