Comparative Biochemistry and Physiology, Part A 187 (2015) 214–223

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

A nutrigenomic approach to detect nutritional stress from gene expression in blood samples drawn from Steller sea lions

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ARTICLE INFO

Article history:
Received 16 July 2014
Received in revised form 4 February 2015
Accepted 8 February 2015
Available online 18 February 2015

Keywords:
Genomics
Expression profile
q-PCR
Diet restriction
Biomarker
Monitoring

ABSTRACT

Gene expression profiles are increasingly being used as biomarkers to detect the physiological responses of a number of species to disease, nutrition, and other stressors. However, little attention has been given to using gene expression to assess the stressors and physiological status of marine mammals. We sought to develop and validate a nutrigenomic approach to quantify nutritional stress in Steller sea lions (*Eumetopias jubatus*). We subjected 4 female Steller sea lions to 3 feeding regimes over 70-day trials (unrestricted food intake, acute nutritional stress, and chronic nutritional stress), and drew blood samples from each animal at the end of each feeding regime. We then extracted the RNA of white blood cells and measured the response of 8 genes known to react to diet restriction in terrestrial mammals. Overall, we found that the genomic response of Steller sea lions experiencing nutritional stress was consistent with how terrestrial mammals respond to dietary restrictions. Our nutritionally stressed sea lions down-regulated some cellular processes involved in immune response and oxidative stress, and up-regulated pro-inflammatory responses and metabolic processes. Nutrigenomics appears to be a promising means to monitor nutritional status and contribute to mitigation measures needed to assist in the recovery of Steller sea lions and other at-risk species of marine mammals.

1. Introduction

Wildlife is increasingly being exposed to human-induced stressors such as habitat degradation, pollution, climate change, and reduced food supplies (Halpern et al., 2008). Such direct and indirect pressures can affect the fitness of individuals and ultimately lead to population declines (Österblom et al., 2008; Butchart et al., 2010). Measuring stress levels in free-ranging animals can be used to monitor populations, and identify and mitigate stressors. Unfortunately, there are few, if any, effective early warning indicators currently available (Dale and Beyeler, 2001; Müller et al., 2013). Hence, environmental and human pressures are frequently noted too late to prevent or contain population declines—particularly for long-lived, slow-reproducing, and wide-ranging species such as marine mammals (Trites and Donnelly, 2003).

Among marine mammals, seals and sea lions appear to be particularly sensitive to environmental changes because of their joint dependence on marine resources (for feeding) and terrestrial sites (for resting, breeding, and moulting). A number of these pinnipeds have declined significantly in recent decades due possibly to predation, disease, fisheries, and natural changes in the qualities and quantities of prey available to them (e.g., harbour seals *Phoca vitulina*, southern elephant seals *Mirounga leonina*, New Zealand sea lions *Phocarctos hookeri*, northern fur seals *Callorhinus ursinus*, and Steller sea lions *Eumetopias jubatus*) (Bowen et al., 2003; Trites and Donnelly, 2003; Mcmahon et al., 2005; Towell et al., 2006). Of these factors, nutritional stress is generally believed to be the most likely proximate cause of the population declines through reductions in fecundity and juvenile survival (Bowen et al., 2003; Trites and Donnelly, 2003).

Nutritional stress due to diet changes can arise from decreases in prey quantity (i.e., a reduction of ingested biomass) or a reduction in prey quality (i.e., a reduction of energy quantity per ingested unit mass) (Rosen, 2009). Such stress may be expressed in free-ranging animals as a decrease in blubber thickness (e.g., Koopman et al., 2002; Miller et al., 2011), an increase in circulating stress hormones (e.g., glucocorticoids), or by changes in metabolite concentrations in blood, urine, scats or hair (Hunt et al., 2004; Jeanniard du Dot et al., 2009; Sheriff et al., 2011; Müller et al., 2013). However, blubber thickness is not a reliable indicator of nutritional status because it also varies with season, reproductive status, and metabolic state. Similarly, the physiological causes of stress are not identifiable from expression of generalized stress responses. Thus, differentiating nutritional stress from other sources of stress or changes in body condition remains challenging. New approaches are needed to identify, quantify and efficiently monitor nutritional stress.

Changes in gene expression can provide explicit indicators of stress, and have an added advantage of being able to identify some specific physiological causes of stress (Steinberg et al., 2008; Veldhoen et al., 2012; Weirup et al., 2013). Among humans, gene

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http://dx.doi.org/10.1016/j.cbpa.2015.02.006
1095-6433/

expression profiles are commonly used as biomarkers of diseases or physiological stress (Strand et al., 2005; Abba et al., 2010; Zhou et al., 2012). Genomic responses to different stressors (such as thermal stress) have been reported in marine species (from coral to vertebrate species) under experimental and in situ conditions (Williams et al., 2003; Desalvo et al., 2008; Clark and Peck, 2009), while genes implicated in the immune response to pathogens or pollutants have also been studied in cell cultures as well as in whole organisms such as marine mammals (e.g., Fonfara et al., 2007; Mancia et al., 2007; Fonfara et al., 2008; Lehnert et al., 2014).

Nutrigenomics is a branch of the growing field of genomic expression studies that aims to identify molecular markers that respond to changes in nutritional status. To date, nutrigenomics has primarily focussed on human health and disease prevention (Müller and Kersten, 2003; Mutch et al., 2005). Studies have demonstrated how certain genes contained in tissues and white blood cells of humans and model species (e.g., mouse or pig) that control metabolism, immune response, cell growth or transcription regulation respond to diet restrictions (Weindruch et al., 2001; Byrne et al., 2005; Dhahbi et al., 2005; Han and Hickey, 2005). Such genomic responses may also apply to other mammalian species, and may be a means to identify changes in the nutritional status of wild populations. However, developing and validating molecular biomarkers of nutritional stress requires controlled studies.

To date, nutrigenomic studies have mostly looked at changes in gene expression in muscle or hepatic tissues, which requires invasive sampling and is often difficult to obtain for large wild animals. However, correlations between gene expression in hepatic, muscle or glandular tissues and gene expression in leukocyte cells means that blood samples can be used as a surrogate (Furukawa et al., 2004; Rudkowska et al., 2011) and effective biomarker (Liew et al., 2006) to detect genomic responses of animals to nutritional stress (Crujeiras et al., 2008b; Caimari et al., 2010; de Mello et al., 2012).

The objective of our study was to determine whether RNA expression in peripheral blood cells is a viable means to detect nutritional stress in Steller sea lions. We specifically set out to assess the feasibility of measuring gene expression in blood samples from captive sea lion — under the expectation that the genomic responses of sea lions to nutritional stress would be consistent with human nutrigenomic studies and with changes in other physiological variables (body mass and blood parameters). To do that, we selected a set of candidate genes involved in different biological processes (Table 1) for which expression was known to respond to diet restriction in terrestrial mammals—including humans (Weindruch et al., 2001; Byrne et al., 2005; Dhahbi et al., 2005; Han and Hickey, 2005).

2. Material and methods

2.1. Captive experimental design and sample collection

Experiments were conducted at the Vancouver Aquarium on four trained, female Steller sea lions (9 year old at the start of the experiment). All holding, experimental facilities and procedures met international standards and were accredited by the International Association of Zoos and Aquariums, and the University of British Columbia Animal Care Committee.

Our experimental design consisted of 70-day trials from May to July 2012 in which we subjected four Steller sea lions (respectively, F03AS, F03IZ, F03RO and F03WI) to diets of contrasting qualities and quantities. During all trials, the sea lions were weighed (±0.2 kg) each day on a platform scale prior to eating. Each animal acted as its own control and was maintained on a normal diet consisting primarily of herring (*Clupea pallasii*) at ad libitum levels for at least one month before any experimental diet changes (Fig. 1). During this preliminary phase (T_0), animals were deemed to be in good nutritional status without being overweight. We took blood samples at the end of this preliminary phase to establish reference levels of gene expression. During the second phase, the food intake (mass) of the animals was reduced by 35%—sufficient to cause the sea lions to lose ~10% of their body mass over this 14-day period (acute nutritional stress). An ~10% reduction of body mass in captive Steller sea lions induced by nutritional stress have been demonstrated to invoke changes in animal physiology (Jeanniard du Dot et al., 2009; Rosen, 2009). We took a second set of blood samples at the end of this phase of acute mass loss (T_1)—and adjusted food levels during the third and final phase to maintain their lower body masses for an additional 28-day period (chronic nutritional stress)—after which a final set of blood samples was taken (T_2). This resulted in 12 blood samples being taken from 4 different animals.

We drew the blood samples from a caudal gluteal vein directly into tubes—beginning with EDTA tubes for haematology and finishing with PAXgene™ Blood RNA tubes (2.5 ml) specially designed for collecting and stabilizing cellular RNA from whole blood (®QIAgen). Samples in PAXgene™ Blood RNA tubes were incubated overnight to ensure complete lysis of blood cells and to increase RNA yields, then stored at −70 °C until RNA extraction.

2.2. RNA extraction and cDNA synthesis

Whole-blood RNA was extracted using the PAXgene™ Blood RNA Kit. Blood sampling and RNA extraction strictly followed manufacturer protocol (®QIAgen). Because the DNase treatment included in the kit was not fully efficient (DNA contamination), we performed a second treatment with DNase I (SIGMA). RNA quality ($A_{260}/A_{280} > 1.9$) and quantity was evaluated using a NanoDrop spectrophotometer. We then verified RNA integrity on gel electrophoresis.

Complementary DNA (cDNA) was synthesised with the Verso cDNA Synthesis kit protocol (Thermo Scientific) and no template controls (no RT). Reverse transcription reaction was performed in a 20-μl final volume with 100 to 300 ng of RNA, using oligoDT, for 30 min at 42 °C followed by 2 min at 95 °C.

2.3. Candidate genes and primer design

The set of candidate genes (Table 1) we considered included one gene implicated in oxidative stress (CYP3A34), two genes implicated in immune response (TGFβ and IL1), one gene implicated in generalized stress response (HSP70), and three genes implicated in metabolism

Table 1
Genes amplified and sequenced in Steller sea lions, their length, and their expected expression (pathways) under nutritional stress.

Gene	Gene symbol	Pathways: biological processes, molecular functions	Length
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	(House-keeping gene)	137 bp
Cytochrome P450 3A34	CYP3A34	Oxidative stress: cell protection, oxidation–reduction process	256 bp
Transforming growth factor beta	TGFβ	Immune reponse: proliferation control, cellular differention	341 bp
Interleukin-1	IL1	Immune reponse: inflammatory response	283 bp
Heat shock 70 kD protein	HSP70	Stress response: molecular chaperone	233 bp
NAD-dependent deacetylase sirtuin-2	SIRT2	Cell cycle: lipid accumulation, cellular differentiation	302 bp
Acetyl-CoA acetyltransferase 2	ACAT2	Lipid metabolism: cholesteryl ester synthesis	282 bp
Thyroid hormone (T3) receptor alpha	TRα	Lipid/protein metabolism: lipolysis, protein synthesis	150 bp

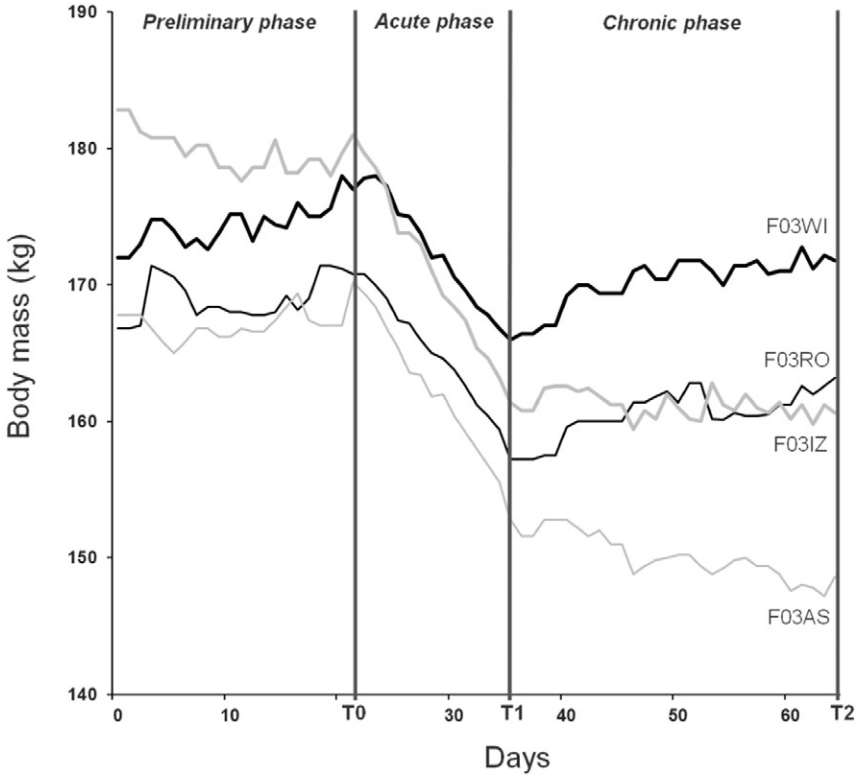

Fig. 1. Change in individual body mass of four female Steller sea lions (F03AS, F03IZ, F03RO, F03WI) over 64 days. Also shown are the three points in time following the preliminary phase (T_0), the acute phase (T_1) and the chronic phase (T_2) when blood samples were drawn from the animals.

(SIRT2, ACAT2 and TRα). We selected GAPDH as our house-keeping gene to normalize candidate gene expression in our study because it has been previously identified to be the best normalizing gene for cetaceans (Spinsanti et al., 2006) and has been used in gene expression studies for seals and fur seals (Beineke et al., 2004; Sharp et al., 2006; Fonfara et al., 2007). Given the lack of genomic resources for Steller sea lions, we first used existing mammal species sequences available in Genbank to design primers that would detect GAPDH and candidate genes in higher conserved regions (from otariids, phocids, canine, bovine, or mice). We then sequenced the single PCR-products obtained with these primers (GATC Biotech, Germany) to obtain specific partial sequences for Steller sea lions (Annex 1), which we used to design specific primers using Primer3 (Rozen and Skaletsky, 2000). We optimized PCR conditions for each target gene — and controlled the specificity of primers by electrophoresis, and measured the resulting efficiencies (Table 2).

2.4. Quantitative real-time PCR

We quantified relative mRNA levels by real time RT-PCR using the MX3005P™ Real-Time PCR System (Agilent technologies, Stratagene) and the Brilliant II Fast SYBR® Green QPCR Master Mix (Agilent technologies, Stratagene) according to the manufacturer's instructions. These quantitative PCR reactions were performed in a 20 μl reaction with cDNA template, 12.5 μl SYBR Green Master Mix (which included SYBR Green, buffer, Taq polymerase and dNTPs), and 0.4 nM of forward and reverse primers. Real-time PCR amplification settings consisted of 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and

Table 2
Specific primers used in quantitative PCR analysis designed from Steller sea lion sequences (Annex 1).

Gene	Primer sequence (5′ → 3′)		Product size	Tm	Efficiency
GAPDH	Forward	GCCAGAGGAGCCAAGCAGTT	89 bp	62.8 °C	84%
	Reverse	GTGATGGGTGTGAACCACGA		60.3 °C	
CYP3A34	Forward	TGGACATCAGGGTGAGTGG	52 bp	58.6 °C	83%
	Reverse	GCACTTCTCTTTCCTTCCTTGTG		59.8 °C	
TGFβ	Forward	CGGAGAAGAATTGCTGCGTG	99 bp	59.9 °C	81%
	Reverse	AGAAGTTGGCGTGGTAGCC		60.0 °C	
IL1	Forward	ATCTTTACCTTGCTCACAGCC	91 bp	58.3 °C	79%
	Reverse	CACTTCACAGACGATGGGTTC		58.9 °C	
HSP70	Forward	CACCTTCGACGTGTCCATC	156 bp	60.1 °C	74%
	Reverse	TGGCTGATGTCCTTCTTGTG		59.8 °C	
SIRT2	Forward	CACTACTTCATCCGCCTGCT	70 bp	59.8 °C	94%
	Reverse	GCGTGTCTATGTTCTGCGTG		59.6 °C	
ACAT2	Forward	TGCTAATCGAGGACTCACACC	68 bp	60.3 °C	116%
	Reverse	TGGAAGGCTCCACACCTACT		59.7 °C	
TRα	Forward	CATCGCAGCACGAATGC	97 bp	61.2 °C	100%
	Reverse	CATCCGGCATAGAGACGA		58.2 °C	

62 °C for 20 s. We ran each blood sample (biological replicates) in triplicate (technical replicates), and ran two controls (no-RT and no template) for each replicate and each gene to confirm the absence of contamination. In addition, we performed the analyses of the dissociation curves at each quantitative PCR reaction (and found a sharp peak for each PCR product indicating high specificity of the primers). We also controlled the efficiency of amplification for each primer pair in a quantitative PCR reaction performed for dilution series of cDNA samples from two different animals.

2.5. Relative quantification

We determined the relative quantity of each target gene transcript using GAPDH transcript as the reference gene following the Pfaffl method—Relative Expression Software Tool REST© (Pfaffl, 2001; Pfaffl et al., 2002). The relative gene expression is a ratio computed from a mathematical algorithm (that needs no calibration curve) and is based on real-time PCR efficiency and crossing point (C_t) values as follows:

$$\text{Ratio} = \frac{\left(E_{\text{target}}\right)^{DC_t}_{\text{target(control-sample)}}}{\left(E_{\text{GAPDH}}\right)^{DC_t}_{\text{GAPDH(control-sample)}}}$$

where C_t value corresponds to the number of cycles at which a fluorescence emission monitored in real time exceeds the threshold limit; ΔC_t is the deviation value between control and diet restricted samples; and E is PCR efficiently determined by a standard curve using serial dilution of cDNA. We calculated E as $E = 10^{(-1/\text{slope})}$. The expression level at the control (normal diet) has a designated value of "1", such that the expression ratio of samples under diet restriction is expressed in relation to the control. Data from each animal are presented as means ± SE, and were computed from the REST® 2009 software that used a randomisation test with a pair-wise reallocation to assess the statistical significance of the differences in expression between control and diet restricted samples. *P*-values <0.05 were considered statistically significant. Relative quantification analyses were done independently for each animal and as well as by combining all animals to investigate the common response.

2.6. Body mass and haematology

We simultaneously measured individual body mass and some blood parameters (Table 3) to explore whether changes in gene expression could be related to changes in body mass or blood biochemistry and haematology. Blood biochemistry and haematology measurements were performed at IDEXX Laboratories (Langley, BC, Canada) following standard procedures. As was done for gene expression, body mass and blood parameters under control conditions (normal diet) were designated with a value "1", and subsequent measurements were expressed in relation to the control. We used the "*corr.test*" function (Pearson method and Holm adjustment) included in the R package "*psych*" (Revelle, 2014) to assess the correlation between gene expression of target genes and relative body mass, and relative values of blood parameters. *P*-values <0.05 were considered statistically significant. Correlation coefficients

Table 3
Body mass and blood parameters for four Steller sea lions following the preliminary phase (T_0), the acute phase (T_1) and the chronic phase (T_2) of food restrictions.

		F03AS		F03IZ			F03RO			F03WI		
		T_0	T_1	T_0	T_1	T_2	T_0	T_1		T_0	T_1	T_2
BM	kg	169.4	151.6	179.6	160.8	161.4	170.8	157.2	161.2	177.8	166.4	170.6
WBC	10.12/l	5.6	4.5	5.6	4.5	3.9	5.1	4.9	5.3	5.6	4.7	5
RBC	g/l	3.9	4.5	3.9	3.9	4.3	4.4	4.5	4.2	4.6	4.5	4.3
HGB	l/l	137	171	137	138	145	170	170	165	166	166	157
HCT	fl	0.4	0.51	0.4	0.4	0.44	0.49	0.5	0.45	0.5	0.48	0.46
MCV	pg	103.1	112.5	103.1	102.1	102.3	111.4	112	108.7	108.6	106.9	105.6
MCH	g/l	35.6	38.1	35.6	35.5	33.8	38.9	37.9	39.8	36.4	36.7	36.3
MCHC	%CV	345	339	345	348	330	349	338	366	335	344	344
RDW	10.9/l	14.7	14.5	14.7	14.4	14.1	14.4	13.8	14	14	13.4	13.7
Platelets	fl	288	250	288	264	300	204	179	168	206	154	166
MPV	abs	10.7	11.2	10.7	11	10.7	11.4	11.6	11.4	12.4	13	13.1
Neutrophils	abs	4.458	2.619	4.458	3.465	3.011	2.912	3.381	3.376	2.895	2.256	3.45
Lymphocytes	abs	1.092	1.814	1.092	0.887	0.749	2.106	1.078	1.754	1.596	1.88	1.25
Monocytes	abs	0.05	0.059	0.05	0.149	0.14	0.082	0.441	0.17	1.075	0.376	0.3
Eosinophils	abs	0	0	0	0	0	0	0	0	0.022	0.188	0
Basophils	abs	0	0.009	0	0	0	0	0	0	0.011	0	0
Glucose	m m ol/l	6.7	7.3	6.7	6.8	6.2	6.6	5.9	6.4	7.7	6.8	6.9
Urea	m m ol/l	7.8	7.1	7.9	6.9	8.9	9.5	7.3	8	8.7	4.9	6.5
Creatinine	um ol/l	77	78	86	91	66	97	99	95	101	107	101
Sodium	m m ol/l	148	148	146	148	147	147	148	147	146	145	146
Potassium	m m ol/l	3.8	3.9	3.8	4	4.1	4	4.3	3.9	4.3	3.7	3.9
Chloride	m m ol/l	109	108	107	108	107	108	106	107	110	108	109
Bicarbonate	m m ol/l	25	26	26	24	25	22	27	27	20	22	23
Calcium	m m ol/l	2.28	2.23	2.36	2.34	2.39	2.28	2.32	2.35	2.27	2.34	2.33
Phosphorus	m m ol/l	2.05	1.82	2.03	1.97	2.17	1.74	1.6	1.68	1.93	1.68	1.78
Total Protein	g/l	77	76	74	74	78	76	76	83	73	73	76
Albumin	g/l	39	38	40	39	43	40	42	42	40	40	40
Globulin	g/l	38	38	34	35	35	36	34	41	33	33	36
Total bilirubin	um ol/l	3	7	2	4	4	4	3	4	3	4	3
ALP	iu/l	51	46	41	39	47	58	53	49	47	45	42
ALT	iu/l	51	60	174	93	278	83	83	117	69	60	60
AST	iu/l	24	50	27	16	63	22	16	18	18	17	14
GGT	iu/l	79	109	57	70	107	63	66	79	51	48	49
CK	iu/l	256	1427	78	139	126	258	104	138	191	226	98
Calc osmolality	m m ol/kg	297	297	293	296	296	297	296	295	296	288	292

BM: body mass, WBC: white blood cells, RBC: red blood cells, HGB: haemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration, RDW: red cell distribution width, MPV: mean platelets volume, ALP: alkaline phosphatase, ALT: alanine transferase, AST: aspartate aminotransferase, GGT: aspartate aminotransferase, CK: creatine phosphokinase.

(*r*-values) between 0.7 and 1.0 (−0.7 and −1.0) indicated a strong positive (negative) linear relationship via a firm linear rule.

3. Results

The gross energy intake of the Steller sea lions averaged 4833 ± 455 MJ d^{-1} during the preliminary phase (normal diet, control), and was 1940 ± 347 MJ d^{-1} during the acute restricted phase and 4632 ± 765 MJ d^{-1} during the chronic phase of diet restriction. Resultant mass loss was 1.1 ± 0.7 kg d^{-1} during the 14 days of acute restricted phase, which represented an average final loss of 6.8 ± 3.5% of the initial (control) body mass (Fig. 1).

All of the 8 target nuclear genes (Table 2) were expressed in the peripheral blood cells of each of the four Steller sea lions following the bouts of nutritional stress (Fig. 2). However, RNA integrity of one sample was too poor to measure gene expression at T_2 (F03AS)—and resulted in us excluding this sample from further analyses. At the individual level, the variability observed within technical replicates was low to moderate (mean s.e. values were 0.21 and maximal s.e. values were <0.7)—thereby allowing the control and diet restricted samples to be statistically compared (Fig. 2).

Expression of all target genes was significantly ($P < 0.05$) modulated when the sea lions experienced dietary restrictions (T_1, T_2), although there were differences between individual animals. Some genes were up-regulated (IL1, HSP70, SIRT2, ACAT2) while others were down-regulated (CYP3A34, TGFβ; Table 1). Only TRα showed an inconsistent response among animals (i.e., no regulation in F03WI, down-regulated in F03AS, and up-regulated in F03IZ and F03RO at T_1). Overall, the observed changes in gene expression caused by nutritional stress were low to moderate. The largest changes occurred for TRα (which was up-regulated 2.99 ± 0.13 times relative to control in F03IZ at T_2) and CYP3A34 (which was down-regulated 0.30 ± 0.09 times relative to control in F03AS at T_1).

Combining all animals (using each animal as a biological replicate) provides relative changes in expression of the 8 targeted genes and the first profile of genomic response in Steller sea lions experiencing nutritional stress (Fig. 3). This analysis shows that the sea lions significantly down-regulated CYP3A34 (0.72 ± 0.06 times relative to control) and TGFβ (0.74 ± 0.17×) at the end of the chronic phase of diet restriction

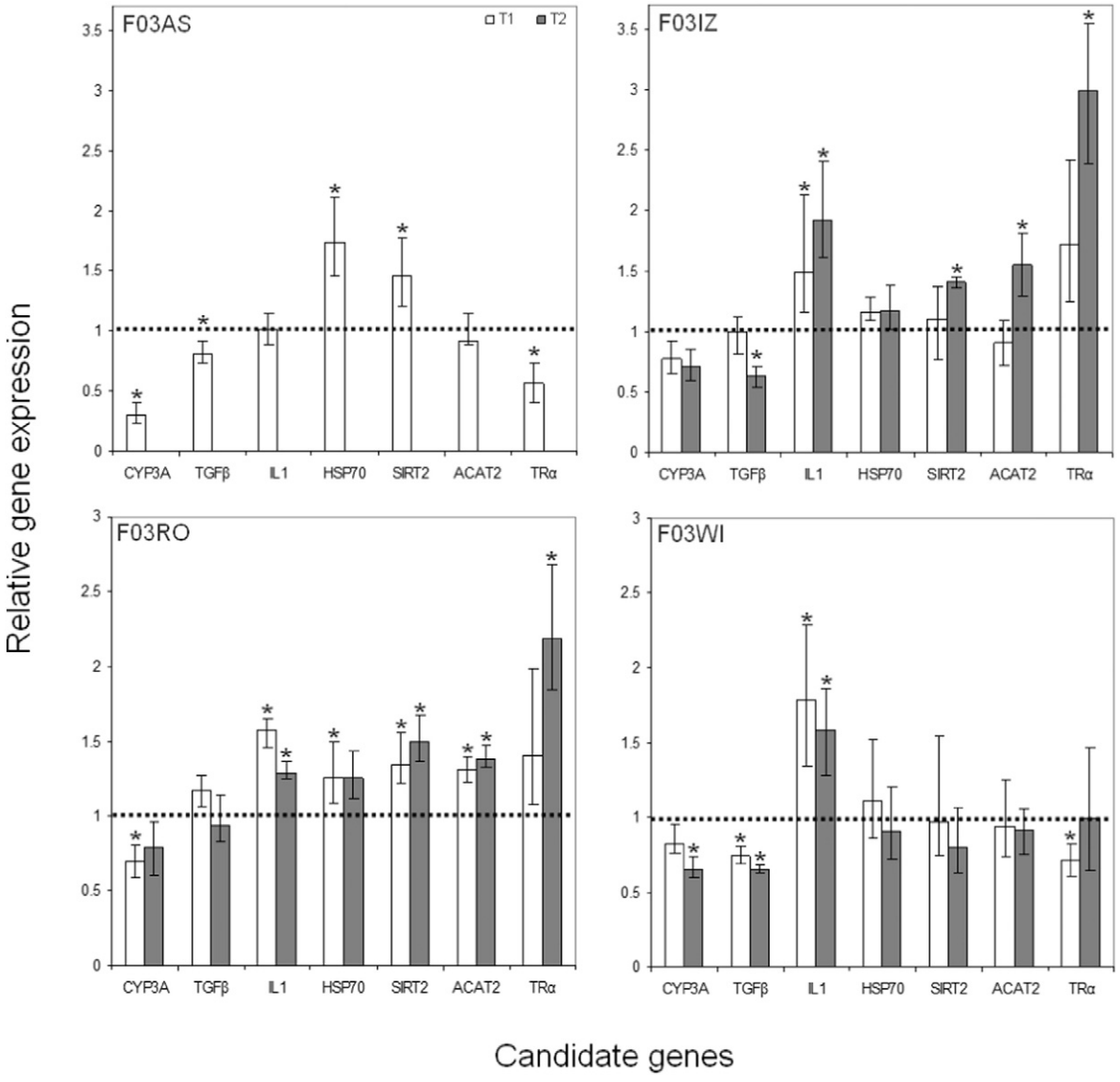

Fig. 2. Relative expression of candidate genes in each of four captive Steller sea lions (F03AS, F03IZ, F03RO, F03WI) during acute diet restriction (T_1) and chronic diet restriction (T_2). Values are mean ± s.e. after permutation tests on 3 replicates for each animal; and asterisks indicate significant (*P*-values < 0.05) differences between a sample and its respective control (T_0). The dashed line denotes the expression level for the control (i.e. relative gene expression is set to "1" during normal diet). RNA integrity of F03AS was too poor to measure gene expression at T_2.

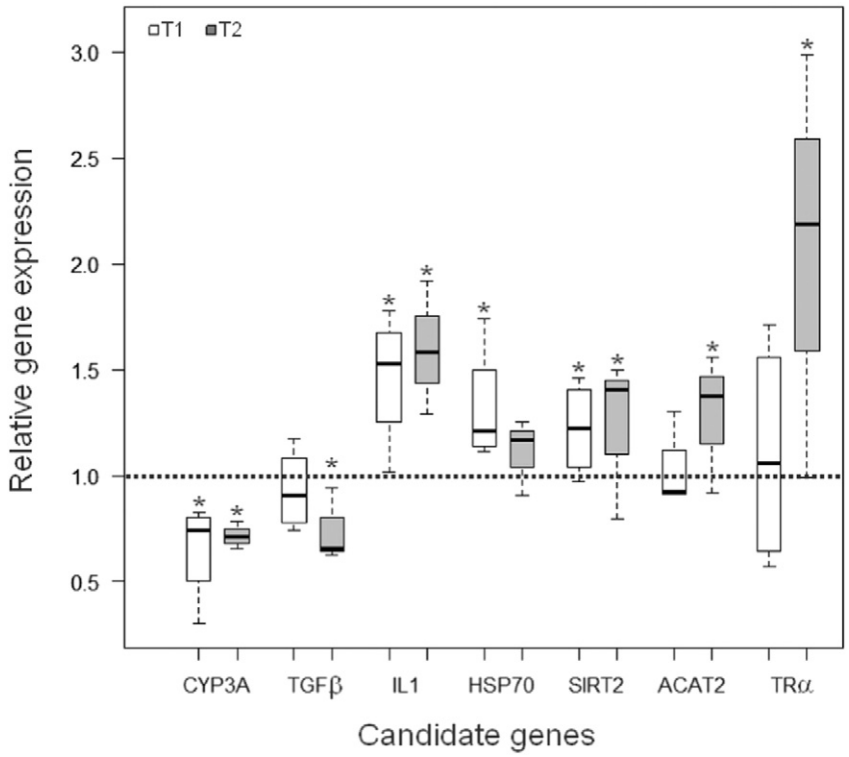

Fig. 3. Boxplots showing the profile of relative expression of candidate genes in captive Steller sea lions following acute diet restriction (T_1, n = 4) and chronic diet restriction (T_2, n = 3). The bold solid line within each box is the median of biological replicates; the bottom and top of each box represent respectively the 25th and 75th percentiles; and the whiskers represent the 10th and 90th percentiles. The asterisks indicate significant (*P*-values < 0.05) differences between samples and control—while the dashed line denotes the expression level at the control (i.e., relative gene expression is set to "1" during normal diet).

(T_2), while up-regulating IL1 (1.6 ± 0.31×), ACAT2 (1.28 ± 0.33×), SIRT2 (1.23 ± 0.38×), and TRα (2.06 ± 1.00×). Only HSP70 mRNA did not appear to change significantly between control and samples at T_2. Some of these responses to diet restriction noted at T_2 were already established during the acute phase (T_1) and did not change further during the chronic phase (i.e., mRNA levels were similar between T_1 and T_2 in CYP3A34, SIRT2 and IL1). Conversely, acute nutritional stress at T_1 did not regulate TGFβ, ACAT2 and TRα, but chronic nutritional stress at T_2 did affect the expression of these genes. In contrast, HSP70 mRNA was regulated during acute stress at T_1, but returned to its control level while the sea lions experienced chronic nutritional stress at T_2.

Overall, the blood parameters we measured did not exhibit dramatic changes during the diet restriction—and only a few enzymes (mainly ALT, GGT and CK) appeared to vary in response to the dietary restrictions (Table 3). There were some significant positive and negative correlations between gene expressions and changes in body mass or blood parameters (Table 4). Most notably, the relative expressions of 4 of the 7 candidate genes (CYP3A34, IL1, HSP70 and SIRT2) were significantly correlated to body mass loss. Similarly, the relative expression of all genes involved in immune response (CYP3A34, TGFβ and IL1) were significantly correlated with the quantity of white blood cells—whereas genes involved in metabolic pathways (SIRT2, ACAT2, TRα) were significantly correlated with plasma concentrations of total protein, albumin and liver enzymes (alanine transferase and aspartate aminotransferase). The other noteworthy correlation occurred between the relative expression of genes involved in cell protection (CYP3A34 and HSP70) and the plasma concentrations of total bilirubin and creatine phosphokinase.

4. Discussion

Testing the efficacy of changes in gene expression to assess the nutritional status of Steller sea lions required developing a methodology specific to sea lions. We thus measured the response of 8 genes extracted from the RNA of white blood cells of Steller sea lions subjected to periods of chronic and acute nutritional stress—and found

Table 4
Coefficient correlations (*r*) and *P*-values between relative gene expression of target genes and relative body mass or relative values of blood parameters (ALT: alanine transferase, GGT: aspartate aminotransferase, CK: creatine phosphokinase); ns: non significant.

	CYP3A34		TGFβ		IL1		HSP70		SIRT2		ACAT2		TRα	
	P-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*
Body mass	0.01	0.79	ns		0.04	−0.6	0.02	−0.7	0.04	−0.6	ns		ns	
White blood cells	0.05	0.59	0.02	0.67	0.03	−0.7	ns		ns		ns		ns	
Total protein	ns		ns		ns		ns		ns		0.03	0.67	0.01	0.71
Albumin	ns		ns		ns		ns		ns		<0.01	0.97	<0.01	0.78
Total bilirubin	0.03	−0.6	ns		ns		0.03	0.65	ns		ns		ns	
ALT	ns		ns		ns		ns		0.03	0.66	<0.01	0.79	ns	
GGT	ns		ns		ns		ns		0.02	0.7	0.03	0.66	0.01	0.77
CK	0.01	−0.7	ns		ns		<0.01	0.84	ns		ns		ns	

that nutritionally stressed individuals up-regulated pro-inflammatory responses and metabolic processes, and down-regulated some cellular processes involved in immune response and oxidative stress. Our findings are consistent with similar studies of terrestrial mammals subjected to nutritional stress (e.g. Byrne et al., 2005; Crujeiras et al., 2008a,b; Martinez et al., 2013), and shows that nutrigenomics can be used to differentiate between nutritional stress and a generalized stress response commonly revealed via corticosteroid levels. The gene expression profile of nutritional stress we describe for Steller sea lions can help to resolve whether declining populations of sea lions in Russia and Alaska are nutritionally stressed, and is a potentially useful tool to monitor and assess the nutritional status of other species of marine mammals.

4.1. Methodological validation

Obtaining reliable measures of RNA can be a real challenge in environmental or wildlife studies. In our case, we followed recommended procedures to reduce technical biases (Bustin et al., 2009; Rieu and Powers, 2009; Bustin et al., 2010; Taylor et al., 2010) and believe our measurements of gene expression are robust. We used the PAXgene™ blood RNA collection system in which RNA is stabilized during phlebotomy and storage because it is more efficient than the standard erythrocyte lyses method to isolate and preserve RNA quality and quantity (Wang et al., 2004; Chai et al., 2005). We also used two different DNAse treatments and checked the absence of PCR amplification on control samples prior to reverse-transcription (no-RT control) to avoid the possibility of having DNA contamination affect the accuracy of our qPCR results.

We paid particular attention to how primer specificity varied within our study animals while designing our primer and optimizing our qPCR. Constructing the dissociation curve for each qPCR run to check for a single sharp peak (indicating high specificity for each replicate) led us to eliminate two target genes (ACAA1: acetyl-CoA acyltransferase 1 and IGFR: insulin-like growth factor 1 receptor) for which specificity was variable among individuals.

In addition to having good primer specificity, it is also important to have a high PCR efficiently to accurately quantify mRNA. Although, the ΔC_q value is probably the most popular method for determining normalized differences in gene expression between control and samples using a reference gene, this method is often misemployed because the target and reference genes must have similar efficiently to allow an accurate comparison of gene expression. We thus used a quantitative model to correct for differences in amplification efficiency between target and reference genes (Pfaffl, 2001; Pfaffl et al., 2002). However, the absence of very large changes observed in gene expression between control and samples meant that the impact of differences in efficiency was limited in our study.

We selected GAPDH as our reference gene because its expression has been shown to be highly stable during normal and physiologically stressful situations in mammalian species, including cetaceans (Stürzenbaum and Kille, 2001; Beineke et al., 2004; Spinsanti et al., 2006). GAPDH has often been used as a normaliser in gene expression studies of seals and fur seals (Sharp et al., 2006; Fonfara et al., 2007, 2008), and has been shown to be stable in elephant seals experiencing prolonged fasts (Soñanez-Organis et al., 2013; Suzuki et al., 2013). Nevertheless, the expression of reference genes can sometimes be influenced by stress or applied treatments, and GAPDH is part of the glycolytic pathway. We are thus unable to confirm that GAPDH was the best normaliser, even though our results appear congruent. It is noteworthy, however, that the GAPDH in our study had a low deviation threshold cycle (CTmean = 21.31; s.d. ± 1.06; number of assay = 104), which compares favourably to deviations reported for other studies that have concluded GAPDH to be a stable and suitable housekeeping gene (e.g. Silver et al., 2006). Additional work is warranted to test different house-keeping genes for their stability and resulting guarantee of valid normalization (Vandesompele et al., 2002). Further investigation of the expression of GAPDH during diet restriction and the use of other reference genes in addition to GAPDH should also be undertaken in the development of accurate molecular markers of nutritional stress (e.g., beta-actin, 18s ribosomal RNA, and tyrosine 3-monooxygenase) to select the most stable house-keeping gene.

Finally, qPCR experiments are often limited by a low number of samples. Ideally, an experiment should encompass at least three independent biological replicates and at least two technical replicates for each biological replicate (Rieu and Powers, 2009). Here, our experimental and statistical design included four biological replicates and three technical replicates for each of the biological replicates—and our blood samples were taken during the experiment at similar times of day to avoid the potential influence of circadian rhythms. Nevertheless, it is possible that factors associated with captivity could have influenced our results, and that the strength of the regulations we observed might differ during other seasons and for other sex and age groupings. Thus, our measures of gene expression specifically reflect those of four young adult females during the summer after two weeks of acute diet restriction and one month of chronic diet restriction.

None of the above limitations alters the fact that changes in RNA levels (as measured by quantitative PCR) consistently occurred in the blood of captive Steller sea lions experiencing diet restriction—and that these changes occurred in 8 nuclear genes (GAPDH, CYP3A34, TGFβ, IL1, SIRT2, ACAT2 and TRα) that represent different biological pathways of physiological responses and gene expression. The number of available nuclear sequences is generally low for non-model species and especially for marine mammals. Thus, the partial sequences of 10 genes we provided (see Supplementary Materials) increases the number of available nuclear gene sequences for Steller sea lions.

4.2. Physiological validation

To validate the genomic response of Steller sea lions to nutritional stress, we first selected a set of candidate genes based on previous stress and nutrigenomic studies conducted on human or model species—under the expectation that we would observe similar patterns in our Steller sea lions. Nevertheless, studies conducted on humans that examined the effect of caloric nutrition on obese people demonstrated that body mass loss in obese individuals may be physiologically different from that of starved individuals. Past methods to investigate the physiological mechanisms involved would have relied on biopsy sampling. As biopsies are also often difficult to obtain, human blood cells are often used as surrogate tissue to investigate molecular mechanisms underlying human diseases or stress including diet restriction (Liew et al., 2006). We therefore hypothesized that genomic responses to nutritional stress would show similar expression patterns in peripheral white blood cells compared to muscle, hepatic or glandular tissues (de Mello et al., 2012).

Cytochrome P450 enzymes (CYP) constitute a super family of enzymes involved in detoxification and in the metabolism of lipophilic substrates. These enzymes play a major role in the oxidative stress response. Infections and inflammations lead to the down-regulation of CYP and limit the production of ROS (Reactive Oxygen Species) (Morgan, 1997). Interleukins are important mediators of the immune system, whereby IL1 induces pro-inflammatory cytokines to regulate the inflammatory response. Transforming Growth Factor-β (TGFB1) also plays an important role in cell growth and cell differentiation regulation, in extracellular matrix production and in immune response.

The down-regulation of both CYP and TGFβ expressions in the Steller sea lions were congruent with some previous studies on humans and rodents experiencing diet restriction (Okuda et al., 1991; Manjgaladze et al., 1993; George et al., 1996). Similarly, our finding that interleukins were up-regulated is also consistent with analyses on other human blood and adipose tissue samples during diet restriction (Clement et al., 2004; Crujeiras et al., 2008b). Moreover, IL1 is directly involved in the regulation of CYP3A—and a down-regulation of CYP3A induced by an increase IL1 has been

previously demonstrated (Sunman et al., 2004). Cytokine expression has also been previously investigated in blood samples from harbour seal pups in rehabilitation facilities, which showed higher RNA levels of IL1beta, IL6, IL8 and IL12 in starved seal pups at admission than after rehabilitation (Fonfara et al., 2008; Weirup et al., 2013). Regulation of CYP expression was also associated with pollutant exposure in wild seals (Kim et al., 2005; Hirakawa et al., 2007).

Heat shock proteins (HSP) are molecular chaperones involved in the stress response. HSP and particularly HSP70 have been intensely investigated in studies of the effects of environmental stress, especially thermal stress (Clark and Peck, 2009). In our case, our sea lions only regulated HSP70 during the acute phase of diet restriction—and showed no significant difference between controls and the chronic phases of diet restriction. Relevant HSP70 regulations have been previously observed in marine mammals that have been stressed (Fonfara et al., 2008; Weirup et al., 2013), but HSP70 is probably not a gene to systematically use as a stress response biomarker in marine ecosystems because it has a high plasticity of induction and expression response (Clark and Peck, 2009). This is further supported by our finding that this gene did not appear to be a relevant biomarker of nutritional stress in Steller sea lions.

Genes involved in metabolism showed an overall up-regulation during nutritional stress. Acetyl-Coenzyme A acetyltransferase 2 (ACAT2) is an enzyme involved in the synthesis of cholesterol esters. Interestingly, both a down- and an up-regulation of ACAT2 gene expression have previously been respectively reported in rat and human blood samples during diet restriction (Crujeiras et al., 2008b; Caimari et al., 2010). Differences in study design associated with food composition and exposition time could explain some of the variability in ACAT2 expression.

Sirtuins (SIRT1 through SIRT7) are involved in a wide range of biological processes including transcription, apoptosis and cellular differentiation. Sirtuins appear to play an important role in the regulation of physiological responses to diet restriction in human (Li and Kazgan, 2011). An up-regulation of sirtuins including SIRT2 has been regularly observed during diet restriction in human blood samples (Crujeiras et al., 2008a,b). Hence, the genomic response of the seven sirtuins may provide pertinent biomarkers of nutritional stress in marine mammals and should be further investigated.

Finally, thyroid hormones (T_3 and T_4) and THRs (Thyroid Hormone Receptors) play a crucial role in the regulation of metabolism. To our knowledge, our study is the first to investigate changes in the gene expression of the thyroid hormone receptor α (TRα) during diet restriction. TRα was up-regulated during chronic diet restriction in our experiment. An up-regulation of the thyroid hormone receptor β was previously observed in mammal muscle and liver after prolonged food deprivation (Martinez et al., 2013) confirming that THRs activity are up-regulated when sea lions experience food restriction. During a nutritional stress, circulating T_3 levels are generally low. A high level of empty THRs (without T_3) in mammal tissues leads to the activation of the generally negative-regulated gene coding for TSH (Thyroid Stimulating Hormone) to drop T_3 levels (Oppenheimer and Samuels, 1983).

Changes in thyroid hormones have been previously investigated in Steller sea lions during diet restriction. Although concentrations of total T_3 were significantly lower during diet restriction in summer, these changes in thyroid hormones (total T_3 and total T_4) were overall less pronounced than expected for nutritionally stressed animals, particularly in winter (Jeanniard du Dot et al., 2009). This could be explained by the regulation mechanism induced by an over-expression of TRα. Assuming that genomic response in white blood cells and glandular tissues follows a similar pattern, it would be particularly interesting to further quantify the blood concentrations of free T_3, free T_4, and TSH, as well as gene expression in blood cells for these hormones and their receptors to better investigate the role of thyroid hormones in Steller sea lions during nutritional stress.

In addition to changes in thyroid hormone concentrations, changes in blood concentrations of glucocorticoids and insulin-like growth factor-I (IGF-I) have also been identified as relevant biomarkers of nutritional stress in Steller sea lions (Jeanniard du Dot et al., 2009). Thus, differential gene expression of these hormones and their receptors should also be investigated in the further development of molecular markers of nutritional stress.

Differential gene expression observed in blood cells can be also related to changes in haematology and blood chemistry (Bonaterra et al., 2007). Our results suggest that changes in blood parameters are associated with changes in gene expression during nutritional stress. Given the importance of white blood cells to the immune response, it is not surprising that all of the genes involved in immune response (IL1, TGFβ and CYP3A34) correlated significantly with the white blood cell counts.

Elevated serum bilirubin concentrations have been associated with the down-regulation of some CYP proteins in human livers, although the mechanisms for this relationship are unclear (George et al., 1996). We also observed a similar pattern with HSP70 expression. The liver plays a crucial role in metabolism and especially in lipid regulation and protein synthesis. Interestingly, we observed significant correlations between all target genes involved in metabolic pathways (SIRT2, ACAT2, TRα) and changes in plasma concentrations of liver enzymes (alanine transferase and aspartate aminotransferase). Such results reinforce the reliability of blood cells as a surrogate tissue to investigate molecular mechanisms underlying nutritional stress.

In summary, previous marine mammal studies of stress have mainly focussed on gene expression of immune responses (e.g., Fonfara et al., 2008; Müller et al., 2013; Weirup et al., 2013; Lehnert et al., 2014). Our study of gene expression is the first to investigate multiple pathways (including immune responses, stress responses, oxidative stress, and lipid and protein metabolism) in marine mammals subjected to nutritional express under controlled experimental conditions. The differential gene expressions we measured in our Steller sea lions are consistent with patterns of diet restriction regulation observed in genomic studies on human and model species.

Our study is the first step towards developing a molecular indicator of nutritional stress in marine mammals based on changes in gene expression. Changes in gene expression should provide pertinent biomarkers to detect early signs of animals experiencing nutritional stress. Further steps should be taken to validate the stability of the house-keeping gene under nutritional stress, as well as to sample healthy Steller sea lions in captivity and in the wild to establish the normal expression range of targeted genes. Finally, additional samples from captive and wild animals suffering from nutritional stress should also be analysed to confirm the profile of genomic response we propose be used as biomarkers of nutritional stress. We recognize that age, sex, reproductive or immune status and environmental conditions (e.g., temperature, season, lighting) may influence gene expression (Peyon et al., 1999; Roth et al., 2002; Swanson et al., 2009), and recommend that these factors be further tested along with individual variability of gene expression (Whitney et al., 2003). In the meanwhile, we believe that our findings show the potential for using biomarkers to monitor the nutritional status of free-ranging wildlife.

5. Conclusions

Nutrigenomic approaches have been largely adopted to study physiological responses of humans to diet restrictions, but its application in animal biology and ecology remains relatively unexplored. We methodologically and physiologically demonstrated that differential gene expressions can be measured in the blood of Steller sea lions that have experienced nutritional stress. Our study supports the contention that gene expression profiling of blood cells is a promising biomarker to identify pinnipeds that are experiencing nutritional stress. The differential expression of target genes we investigated appears to be consistent with previous nutrigenomic studies of terrestrial mammals. Thus, we conclude that gene coding for sirtuins, thyroid hormones and their

receptors, cytochrome enzymes, interleukins, and transforming growth factor are probably among the most suitable set of genes to build a molecular biomarker of nutritional stress in Steller sea lions and potentially other marine mammals. However, we recognize that free-ranging animals can accumulate different environmental stresses and that some factors influencing gene expression can be difficult to assess in field samples (e.g., reproductive status, infections, age, etc.). Thus, identifying nutritional status of pinnipeds from wild population through a reliable gene expression profile will require further refinement. In the meanwhile, our captive study has laid a foundation for applying nutrigenomic approaches to resolving questions about the nutritional statuses of top marine predators.

Acknowledgments

We are particularly grateful to the training and veterinarian staff of the Vancouver Aquarium for their role in training the sea lions and collecting the data and blood samples. We also thank the Molecular Core Facility at the University of La Rochelle, and the reviewers for their constructive comments and suggestions. Financial support was provided by an NSERC (Natural Sciences and Engineering Research Council of Canada) discovery grant awarded to Andrew Trites with additional support from the U.S. National Oceanic and Atmospheric Administration (NOAA NA11NMF4390124).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2015.02.006.

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