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Longitudinal evaluation of leukocyte transcripts in killer whales (*Orcinus Orca*)

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ABSTRACT

Early identification of illness and/or presence of environmental and/or social stressors in free-ranging and domestic cetaceans is a priority for marine mammal health care professionals. Incorporation of leukocyte gene transcript analysis into the diagnostic tool kit has the potential to augment classical diagnostics based upon ease of sample storage and shipment, inducible nature and well-defined roles of transcription and associated downstream actions. Development of biomarkers that could serve to identify "insults" and potentially differentiate disease etiology would be of great diagnostic value. To this end, a modest number of peripheral blood leukocyte gene transcripts were selected for application to a domestic killer whale population with a focus on broad representation of inducible immunologically relevant genes. Normalized leukocyte transcript values, longitudinally acquired from 232 blood samples derived from 26 clinically healthy whales, were not visibly influenced temporally nor by sex or the specific Park in which they resided. Stability in leukocyte transcript number during periods of health enhances their potential use in diagnostics through identification of outliers. Transcript levels of two cytokine genes, IL-4 and IL-17, were highly variable within the group as compared to the other transcripts. IL-4 transcripts were typically absent. Analysis of transcript levels on the other genes of interest, on an individual animal basis, identified more outliers than were visible when analyzed in the context of the entire population. The majority of outliers (9 samples) were low, though elevated transcripts were identified for IL-17 from 2 animals and one each for Cox-2 and IL-10. The low number of outliers was not unexpected as sample selection was intentionally directed towards animals that were clinically healthy at the time of collection. Outliers may reflect animals experiencing subclinical disease that is transient and self-limiting. The immunologic knowledge derived from longitudinal immunologic studies in killer whales, as was the target of the present study, has the potential to improve diagnostics and health related decision making for this and other domestic and free-ranging cetacean species.

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1. Introduction

Infectious disease, physical injury and stress can all contribute to morbidity and mortality in domestically-housed and free-ranging cetaceans. Resulting disease can be difficult, if not impossible, to identify in the early stages of compromised health. Timely treatment that can mitigate disease progression and development of any sequelae relies on early and accurate identification of the insult. As in all other medical and veterinary health models, the identification and diagnosis of infectious and other diseases encountered by cetaceans are routinely dependent upon diagnostics including hematology, serum biochemistry and microbiology. Interventional strategies, treatment for infectious disease, commonly involve the appropriate use of broad spectrum antimicrobial treatment. While treatment protocols are selected based upon the diagnosis, empirical therapies based on clinical indexes of suspicion may be initiated prior to the determination of a specific diagnosis. Leukocyte transcriptional biomarkers have potential to facilitate

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diagnostic and/or prognostic assessment of cetacean health, similar to those currently being pursued in human and veterinary medicine (Chaussabel, 2015; Irwin and Cole, 2011).

Cells of the immune system respond to stimuli by communicating with each other via differential expression of cell surface proteins (receptors and ligands) and secretion of soluble messengers. While many of these genes can be transcriptionally-induced by multiple and varied insults, the literature would support the hypothesis that insults could be differentiated based upon the "sum" of unique genes which are up- and/or down-regulated. The interest in applying real time quantitative PCR to measure leukocyte gene transcription for diagnostic purposes has resulted in the validation of techniques for both rapid fixation of mRNA in clinical blood samples and generation of reproducible quantitative data (Davis, 2005; Futh et al., 2004; Rainen et al., 2002; Stordeur et al., 2003). A multitude of published studies have described identification of unique leukocyte gene transcript/expression signatures in mammals associated with stress, physical trauma, microbial infection, vaccination and autoimmune disease (Cole et al., 2011; Ferrari et al., 2013; Fonfara et al., 2007; Kennerly et al., 2008; Muller et al., 2013; O'Loughlin et al., 2011; Powell et al., 2013; Sitt et al., 2010; Yahaya et al., 2013). Diagnostic utility has been demonstrated through application of modules of genes that have been enriched for activities related to biological functions including inflammation, viral response and signaling within B and T lymphocytes and neutrophils. Application of this approach to *Staphylococcus aureus (S. aureus)* infections in humans has demonstrated increased transcription of genes associated with neutrophils and inflammation and decreased transcripts associated with B lymphocytes and cytotoxic cells (Chaussabel et al., 2008). A parallel approach using analysis of human peripheral blood-derived cells with multiple axes, each containing probes specific for co-regulated gene transcripts, demonstrated utility in longitudinal characterization of clinical incidents and had potential to evaluate pre-clinical risk of disease (Preininger et al., 2013).

A modest number of gene transcripts were selected for application to the killer whale population in the current study with a focus on broad representation of inducible immunologically relevant genes. While many of the selected genes encode cytokines with varied and pleotropic actions, the general approach was to measure transcripts representing the following activities: i) pro-inflammatory features (TNFα and IL-17) (Coussens et al., 2004; Kolls and Linden, 2004; Sutherland et al., 2009; Pappu et al., 2011), ii) phagocyte and vasodilative activity (Cox-2) (Claria, 2003; Morita, 2002), iii) early T lymphocyte activity (CD69) (González-Amaro et al., 2013), iv) T_H1 activity (IFNγ) (Evaristo and Alegre, 2013), v) T_H2 activity (IL-4) (Gadani et al., 2012), vi) regulatory/anti-inflammatory features (IL-10) (Trifunović et al., 2015), vii) apoptosis (FADD) (Curtin and Cotter, 2003) and viii) antiviral activity (MX-1) (Haller and Kochs, 2011). The gene encoding the ribosomal subunit S9 was selected as a house-keeping marker to establish normalized transcript values (Bowen et al., 2007; Sitt et al., 2008). Similar and related approaches and applications of leukocyte transcript analyses in whales and dolphins has been reviewed (Beineke et al., 2010), and has also been employed in other wildlife species (Bowen et al., 2015, 2012)

The current study represents the first longitudinal leukocyte transcript analysis in cetaceans; individual animals were repeatedly collected and analyzed over an extended period of time. Such an approach has the added attribute of allowing an animal to serve as its' own baseline, thus potentially increasing the ability to identify outlier events. The data collected from this study in killer whales may provide a foundation for the application of transcriptional biomarkers as diagnostic tools for this and other domestically housed and free-ranging cetacean species.

Table 1
Animal samples.

Animal	Sex	Age range of animal[a] (years)	N[b]	Date range of samples
1	F	1–2	8	2006–2008
2	F	1–4	4	2006–2009
3	F	1–3	2	2006–2007
4	F	2	1	2006
5	F	2	2	2008
6	M	2–7	13	2003–2008
7	M	2–4	5	2004–2006
8	F	2	1	2009
9	M	3–5	6	2003–2006
10	F	3	2	2005
11	M	4–10	18	2003–2009
12	M	5–10	9	2003–2008
13	F	7–12	6	2004–2008
14	M	8–11	7	2003–2006
15	M	10–14	8	2004–2007
16	M	12–16	15	2004–2009
17	M	12–18	16	2003–2009
18	F	12–17	8	2003–2008
19	F	14–19	14	2003–2008
20	F	15–21	18	2003–2009
21	F	16–21	10	2003–2009
22	F	19–21	5	2004–2006
23	M	24–27	14	2004–2008
24	M	25–30	11	2003–2009
25	F	29–34	13	2004–2009
26	F	29–34	16	2004–2009

[a] Ages of the animals during the sampling period.
[b] N = number of total samples per animal.

2. Materials and methods

2.1. Animals

Twenty six domestic killer whales (*Orcinus orca*), ranging in age from one to 34 years and including a mix of females (15) and males (11), were housed in three different marine parks with similar husbandry practices (Table 1). Varying numbers of aliquots (1–18) were made available for experimental leukocyte transcript analysis at different intervals (single time point – six years) (Table 1). As sample acquisition was opportunistic (dependent upon when veterinarians sampled the animals and whether FedX shipment for transcript analysis was feasible), experimental design, from the perspective of establishing optimal animal numbers and frequency of analysis for the study, was not incorporated into this pilot program.

Blood samples were collected by Park veterinarians as part of their routine veterinary care program (i.e. routine CBC and chemistry); thus an IACUC protocol was not required. The population of killer whales described here (largest collection in the world) was particularly well suited for this study as all animals are trained to voluntarily present their flukes at pool-side for blood collection. This conditioned behavior is part of the overall animal health and wellness program. As such, it not only facilitates acquisition of peripheral blood from whales presenting with potential illness, but also minimizes the impact of stress on the animals and on the associated data derived from routine blood sampling.

2.2. Sample collection

Blood samples were collected in PAXgene™ Blood RNA tubes (PreAnalytiX, Switzerland) containing a proprietary additive which stabilizes RNA and prevents its' degradation. Samples were opportunistically taken (as described in Section 2.1) from 2003 through 2009 (Table 1). Following collection, blood tubes were shipped the same day to the laboratory overnight where they were frozen at −80 °C until extraction of RNA.

Samples used in the current analysis were restricted to those defined as being from "clinically healthy" killer whales. "Clinically healthy" was defined as any animal that had normal hematology/chemistry as determined by the attending veterinarian. Samples were additionally retrospectively screened by excluding collections from animals that experienced urgent blood collections due to potential health concerns, either the month before, the month after, or during the month in which the sample in question was obtained. In total, 232 individual samples from a total of 26 animals were analyzed. As such, there was a wide range of samples numbers per animal and varying sampling intervals.

2.3. RNA extraction and cDNA synthesis

Samples collected in PAXgene™ tubes are stable for up to 8 years when kept frozen (PreAnalytiX, Switzerland). RNA was typically extracted within a month of arrival to the laboratory. Leukocyte RNA was isolated using the PAXgene™ blood RNA kit (PreAnalytiX, Switzerland), according to manufacturer's standard protocols, which included an on-column DNase treatment to remove contaminating genomic DNA (silica-based microspin technology) and stored at −80 °C until cDNA synthesis. cDNA was synthesized as previously described using a QuantiTect Reverse Transcription® Kit (Qiagen, Valencia, CA), diluted 1:5, and stored at −20 °C until further analysis (Sitt et al., 2010).

2.4. Real-time PCR

Real-time PCR analysis was as previously described (Sitt et al., 2010, 2008). Genes analyzed were chosen based upon their immunologic relevance and the primer sequences were as previously described (Lee, 2010) (Table 2). Briefly, 5 μl of diluted (1:5) cDNA was added to individual master mixes containing 12.5 μl of QuantiTect SYBR Green® Master Mix [5 mM Mg2+] (Qiagen, Valencia, CA), 1 μl each of forward and reverse sequence specific primers, 0.5 μl of Uracil-N-Glycosylase (Invitrogen, Carlsbad, CA), and 5.0 μl of RNase-free water; total reaction mixture was 25 μl. Samples were loaded into 96 well plates in duplicate and sealed with optical sealing tape (Applied Biosystems, Foster City, CA). Reaction mixtures containing water, but no cDNA, were used as negative controls. Amplifications were conducted on a 7300 Real-time Thermal Cycler (Applied Biosystems, Foster City, CA). Reaction conditions were as follows: 50 °C for 2 min, 95 °C for 15 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 31 s, an extended elongation phase at 72 °C for 10 min, followed by a dissociation step. Product specificity was monitored by analysis of melting curves and periodically confirmed by sequencing the amplified products. Threshold crossing (CT) values for "immunologic" gene transcripts of interest were normalized to the S-9 ribosomal gene.

2.5. Real-time PCR analysis

Analysis of real-time quantitative PCR data was conducted using normalized values (NV); housekeeping gene threshold-crossing (in real-time quantitative PCR, the point at which amplification is exponential) subtracted from the gene of interest threshold-crossing for each animal (McLoughlin et al., 2006; Sitt et al., 2010).

2.6. Statistical analysis

Mixed effects statistical models were used to simultaneously estimate and account for multiple influences on gene transcription levels, which make them appropriate for the multiple uncontrolled factors that occurred as a result of opportunistic samples acquired for this study (i.e. vastly different numbers of samples per individual, different sample intervals, unequal geographic representation).

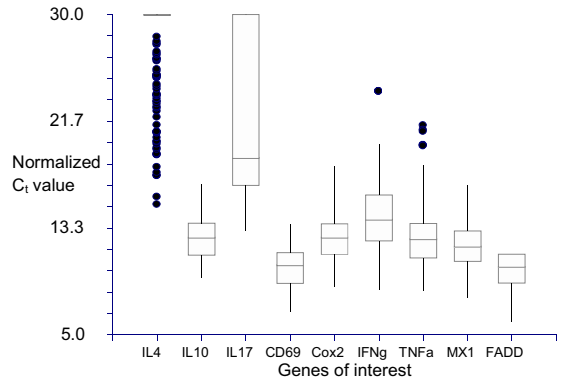

Fig. 1. Distribution of average cycle threshold (C_T) values across genes targeted by the panel of nine primer sets. Blood was sampled from 26 captive killer whales. Bars range from the 10th to the 90th percentile of values for each gene; lines represent maximum and minimum values for each gene, normalized to the S9 housekeeping gene for each animal. Circles represent 5th and 95th percentile outliers (NCSS, Statistical and Power Analysis Software, 2007, Kaysville, UT, USA).

Distribution of normalized values was examined across genes targeted by the panel of nine primer sets (Table 2). Bars range from the 10th to the 90th percentile of values for each gene, normalized to the S9 housekeeping gene for each animal (Figs. 1 and 2). Circles represent 5th and 95th percentile outliers. We used linear mixed effects models to analyze the influence of park, sex, age, and year on each gene transcription level. Park, sex and age were treated as categorical fixed effects while animal ID and year were treated as random intercept effects. Parameter estimates for all model effects were calculated using the *lme4* package in R 2.8.1 (R Development Core Team, 2012). We then used principal component analysis (PCA) to identify orthogonal factors among the gene transcription levels and which of the gene transcription variables loaded highly in each factor (R Development Core Team, 2012). We used Spearman correlations (NCSS, Statistical and Power Analysis Software, 2007, Kaysville, UT, USA) to identify relationships between gene transcript and hematology parameters. Additionally, data were subjected to hierarchical clustering with complete linkage disequilibrium (Genesis, Graz, Switzerland) to generate a heat map of gene transcripts (Fig. 3). Due to the high number of samples with values exceeding the threshold limit of 40 cycles, IL-4 and IL-17 were omitted from the heat map analysis.

3. Results

3.1. Box plot analysis

Box plots were used to illustrate relative levels of individual gene transcripts within the population (Fig. 1) (NCSS, Statistical and Power Analysis Software, 2007, Kaysville, UT, USA). The 95th percentiles appeared to be independent of the sampling time span, indicating that the dynamic ranges did not depend upon the duration of sampling (Table 1, Fig. 1). The number of samples taken per individual did affect the 95th percentiles, but only in those animals from which only a single sample was obtained. Modest variation in transcript levels was apparent for IL-10, CD69, Cox-2, IFNγ, TNFα, Mx-1 and FADD. In contrast transcript levels for IL-4 and IL-17 tended to be much more variable and with higher NVs. Aside from the many outliers recorded for IL-4 (relatively high transcript levels) within the entire population, the only other transcript outliers were high NVs (relatively low transcript levels) for IFNγ and TNFα.

Box plots illustrating transcript levels on an individual animal basis are represented in Fig. 2; IL-4 and IL-17 were not included. Animals are arranged on the X axis as a function of age with the youngest being #1 and the oldest being #26. A few outliers were

Table 2
Orcinus orca specific primers.[1]

Gene	Primer sequence (5′–3′)	Direction	Amplicon length (bp)	Annealing temperature (°C)	Melting temperature (°C)
IL-4	CATGGACACAAGTGCGATGTTAC	AS	127	55	62.8
	TATGAAGAAGCACAGTCCC	S			62.3
IL-10	GACTTTAAGGGTTACCTGGGTTGC	AS	158	55	57.8
	TCCACCGCCTTGCTCTTGTTTTC	S			60.2
IL-17	CCACCTCACCTTGGACTCTCCA	AS	126	55	66.4
	GCTCCCTTCGCAGGACCAG	S			66.64
Cox-2	GGGAGGAAAGAGCTTCCTGATTCAA	AS	146	55	58.6
	GTCCACCCCATGGCTCTGTCC	S			62.4
TNFα	GAGGGAGAGTTCCCAACTGGCTA	AS	78	55	60.2
	CTGAGTACTGAGGTTGGCTACAAC	S			57
CD69	GGTCCATTCAAGTTCCCATCCCAT	AS	45	55	64.6
	TACTGGCCCACTGATAAAGCAACG	S			64.6
IFNγ	GTGGGCCTCTTTTCTTAGAA	AS	158	55	58.4
	TCATCCAGTTTCTCAGAGCT	S			58.4
Mx-1	CAGCCAGCCGACATTGAATATCA	AS	138	55	62.8
	CCACCTCCTGAGCCATGCG	S			66.6
Fadd	GGAGCAGAACGAGATGAGCCG	AS	152	55	66.5
	AACGCTGCCCGCAGGTCTC	S			66.6
S-9	GAGGATTTCTTGGAGAGACGCCTGC	AS	129	55	61.3
	GAGAAGTCGATGTGCTTCTGGGAGTC	S			60.6

[1] From (Lee, 2010).

observed, but with the exception of elevated transcripts of Cox-2 for #25 and IL-10 for #6, the remaining outliers were relatively low transcripts. A cursory inspection of transcript variability across the population, not including younger animals with few sample collections, identified relatively tight clustering of most transcripts for most animals with the exception of variable TNFα levels in animal #19 (Fig. 2).

3.2. Mixed effects model analysis

The mixed effects models identified significant effects (age, year) on gene transcript levels (Table 3). The effects of park and sex on gene transcription levels were not significant. Killer whale age and year of sampling influenced transcript levels of IL-10, Cox2 and IFNγ. Transcript levels of MX-1 were only significantly influenced by year of sampling, while transcript levels of FADD were only significantly influenced by killer whale age at time of sampling.

3.3. Principal components analysis (PCA)

PCA revealed two principal components with eigenvalues greater than unity that together explained 83.0% of the total variance within our dataset. The first principal component explained 68.7% of the total variance and had all negative loadings, therefore representing an overall magnitude of gene expression that was nearly evenly loaded by all of the gene transcription variables (32.2–39.4%) with the exception of IFNγ which had greater weight (53.4%). The second principal component explained another 14.3% of the total variance and had only one negative but strong loading by IFNγ (80.6%) in contrast with smaller all positive loadings by the other gene transcription variables (4.4–46.7%). The second principal component measures the degree to which the IFNγ gene transcription contrasts with, i.e. exceeds beyond, the other gene transcripts (Fig. 3).

3.4. Correlation matrix

Although correlation matrices identified many strong correlations within hematological and gene transcription parameters, few weak to moderate correlations were found between the two sets of variables. IFNγ was moderately well-correlated with creatine kinase ($r = 0.53$, $p = 0.00$), ceratine ($r = 0.42$, $p = 0.00$), total protein ($r = 0.45$, $p = 0.00$), globulin ($r = 0.42$, $p = 0.00$) and alkaline phosphatase ($r = 0.40$, $p = 0.00$). IL-10 was weakly correlated with creatine kinase ($r = 0.34$, $p = 0.00$).

3.5. Heat map analysis

Hierarchical cluster analysis was conducted using individual killer whale transcript data (Fig. 3). Green represents relatively high, red indicates relatively low and black represents relatively neutral transcript levels. Differences were identified both among individuals and within individuals between time points, yielding three primary groups of killer whales clustered according to transcript profile; primarily high, low and mixed transcript levels. A large proportion (43.5%: 101/232 samples) of samples were in the mixed category; 28.0% (65/232 samples) fell into the high transcription profile and 28.4% (66/232 samples) fell into the low transcription profile clusters (Fig. 3). Heat map analysis also identified clusters among genes. IL-10 and Cox-2 appeared to be tightly linked as did CD69 and FADD. In general, clusters contained groupings of multiple individuals, parks, ages and sampling years. However, one cluster contained a single individual killer whale (#19). This animal presented with relatively high IFNγ and Cox-2 and low TNFα, IL-10, MX-1 and CD69.

Varying amounts of high, low and mixed transcripts were identified in animals from parks 1, 3 and 4. Seventeen percent of park 1 transcripts fell into the high transcript cluster, while 59% were mixed and 24% were low. Thirty one percent of park 3 transcripts fell into the high transcription cluster while 37% were mixed and 32% were low. Thirty two percent of park 4 transcripts fell into the high transcription cluster while 41% were mixed and 27% were low. Five killer whales were housed in more than one location with samples taken accordingly. Killer whales #9, #11, #15 and #20 were sampled in parks 3 and 4 while killer whale #18 was sampled in parks 1 and 3. Individuals clustered irrespective of which park they were housed in at the time of sampling.

Age classes were not spread equally across all clusters. Only 5% of transcripts from sexually immature animals (animals below 6 years of age) fell into the high transcript cluster, while 65% fell into the low cluster and 30% were identified in the mixed transcript cluster. Conversely, 33% of transcripts from sexually mature individuals were categorized in the high transcript cluster, 20% in the low transcript cluster and 47% in the mixed transcript cluster.

Sexes were spread relatively similarly across all transcript clusters. 25% of females fell into the high transcript cluster, while 28%

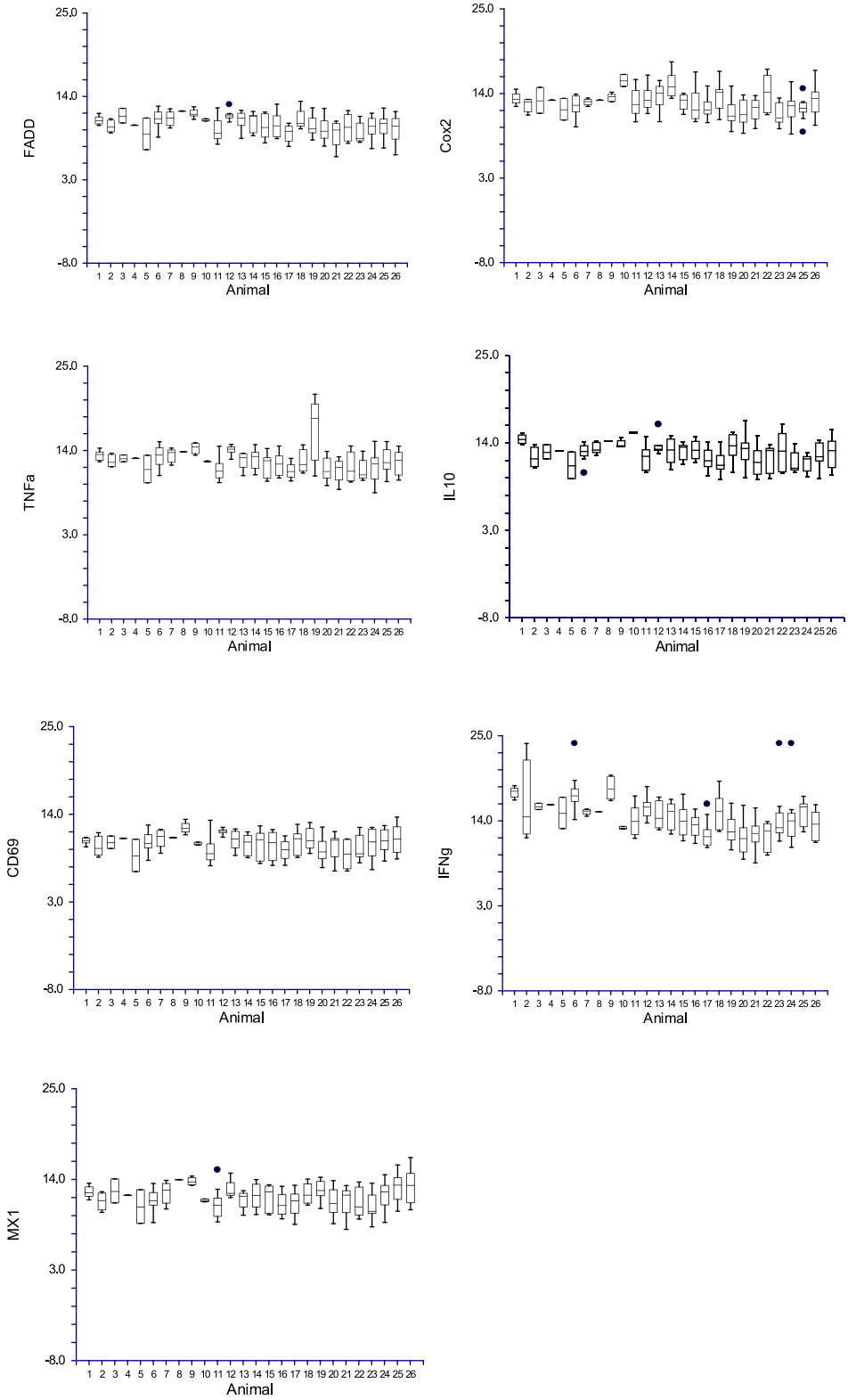

Fig. 2. Distribution of average cycle threshold (C_T) values across genes targeted by the panel of nine primer sets, per animal, labelled 1–26. Blood was sampled from 26 captive killer whales. Bars range from the 10th to the 90th percentile of values for each gene; lines represent maximum and minimum values for each gene, normalized to the S9 housekeeping gene for each animal. Circles represent 5th and 95th percentile outliers (NCSS, Statistical and Power Analysis Software, 2007, Kaysville, UT, USA).

fell into the low transcript cluster and 46% were identified in the mixed transcript cluster. Similarly, 30% of males fell into the high transcript cluster, while 29% fell into the low transcript cluster and 41% were identified in the mixed transcript cluster.

4. Discussion

Killer whales (*Orcinus orca*) are members of the dolphin (*Delphinidae*) family and are the most widely distributed cetacean species in the world (NOAA, 2015). Killer whales can be long-

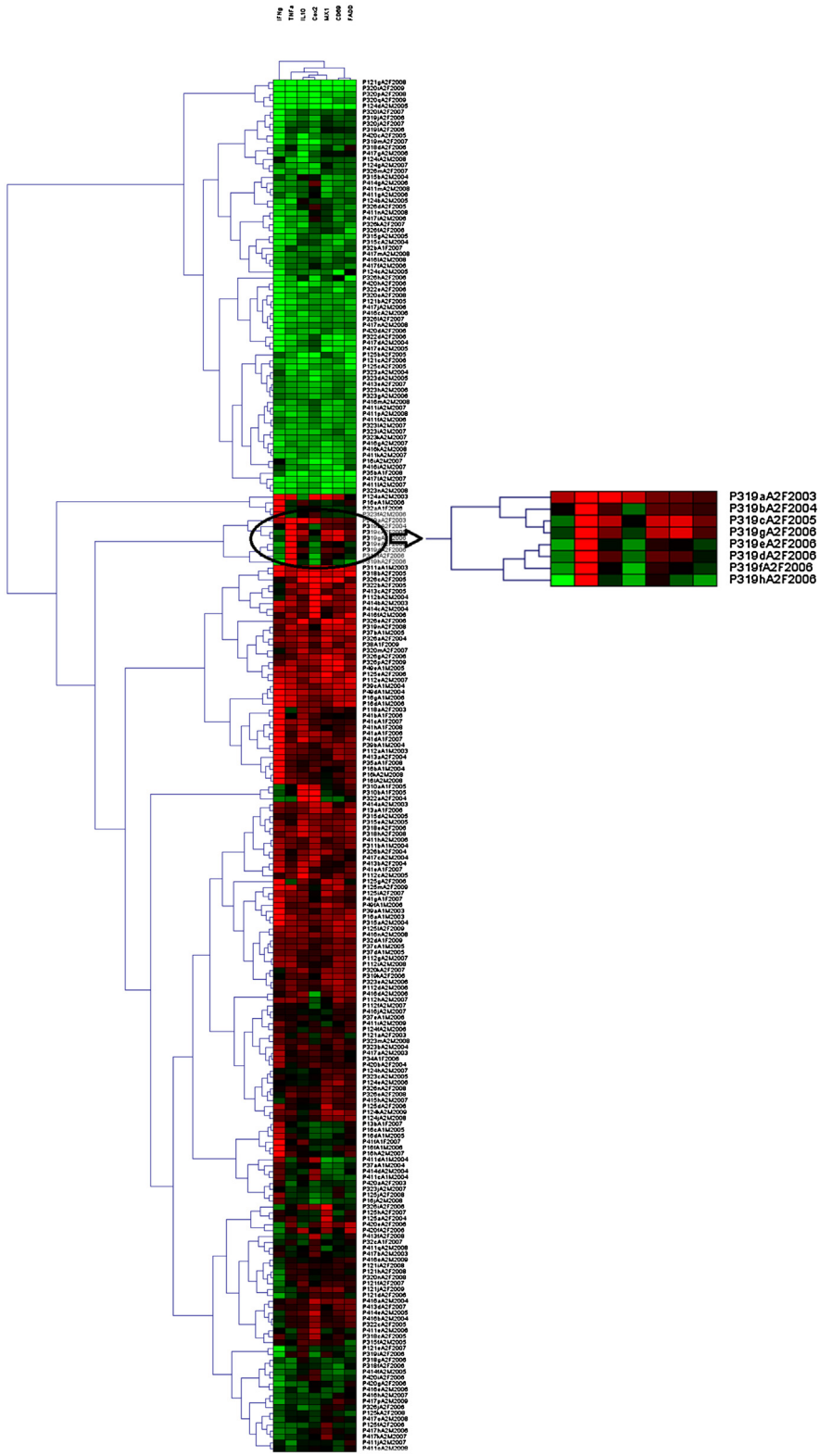

Fig. 3. Gene profiling: transcription matrix of nine target genes in killer whales samples at three marine parks between 2003 and 2009 (Hierarchical clustering with complete linkage disequilibrium; Genesis, Graz, Switzerland). Green indicates higher relative transcription levels and red indicates lower relative transcription levels. Expanded area shows cluster comprised solely of animal 19 (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lived with life spans of some free-ranging female animals even approaching 100 (NOAA, 2015). Domestically-housed killer whale populations are largely maintained through natural and artificial breeding programs and maintained in a unique aquatic environment that is characterized by high water quality standards. Maintenance of high water purity facilitates the maintenance of visual clarity and minimizes animal exposure to water-borne microbes. The impact of such management on development and maintenance of the immune system is of special interest to animal health care professionals.

The current study was conducted to further the understanding of the cetacean immune system and ultimately attempt to expand the diagnostic tool kit being applied to free-ranging animals and routine monthly monitoring programs of domestically-housed populations. The ability to rapidly establish species-specific leukocyte gene primers/probes, ease of sample acquisition, storage (does not require immediate analysis of fresh blood) and shipment to distant diagnostic laboratories, are attributes that favor their diagnostic utility, especially when working with free-ranging animals in remote locations. As the killer whales in this study are trained to voluntarily present their flukes at pool- side for blood collection, potential stress associated with sample collection is decreased, decreasing the likelihood of seeing "capture- associated" changes in transcription patterns, as have been described in wild dolphins (Mancia et al., 2008). Access to baseline leukocyte gene transcripts in domestic counterparts would be of value in better interpreting studies of free-ranging marine mammals. Correlation matrices were used to compare cytokine transcript numbers with standard hematologic and serum chemistry values derived from the samples collected in this study. Although many strong correlations were visible within hematological and gene transcription parameters, few weak to moderate correlations could be found between the two sets of variables. IFNγ was moderately well-correlated with creatine kinase ($r = 0.53$, $p = 0.00$), creatine ($r = 0.42$, $p = 0.00$), total protein ($r = 0.45$, $p = 0.00$), serum globulins ($r = 0.42$, $p = 0.00$) and alkaline phosphatase ($r = 0.40$, $p = 0.00$). IL-10 was weakly correlated with creatine kinase ($r = 0.34$, $p = 0.00$). Attempts to interpret these modest correlations between IFNγ and serum chemistry values should probably be avoided as they would be highly speculative at best. In the absence of any clinical disease in the population from which the samples were acquired, modest correlations and biologically irrelevant changes in transcript number would be expected.

Relative transcript levels of TNFα, IL-17, Cox-2, CD69, IFNγ, IL-4, IL-10, FADD and MX-1 were analyzed on both a population basis and an individual basis. The number of samples taken from an individual animal did not appear to visibly impact the variability within transcript values between animals. This latter feature was expected as samples were selected in a manner that favored healthy donors as opposed to animals in poor health. Principal components analysis (Fig. 4) identified two components related to IFNγ that together explained the majority of the total variance in the data set. IFNγ can be produced by a number of leukocyte subsets including NK, NKT, T_H1 and CTLs; these variable sources could contribute to its' variance. Mixed effects model analysis was used to identify potential effect(s) of park, sex, animal age and year of sample acquisition. Neither the park of animal origin nor animal sex significantly influenced gene transcript values. In contrast, transcript values were influenced by age and year of sample acquisition. Relative to age, younger animals had relatively fewer transcripts of IFNγ, IL-10 and FADD, as compared to their older cohort (Table 3). Increasing age has been associated with increasing percentages of peripheral blood lymphocytes with an effector/memory phenotype as compared to percentages of naïve T lymphocytes numbers; naïve T lymphocyte production begins to fall off with the onset of thymic involution at puberty. Effector/memory lymphocytes have been reported to maintain cytokine-encoding mRNA due to a combination of increased transcription coupled with increased mRNA stability (Marçais et al., 2006). Transcript values of IL-10, Cox2, IFNγ and MX-1 were also influenced by the sampling year. The basis of transcript variation as a function of year is unknown and we cannot offer any substantive hypotheses as to an underlying cause. Absence of park-associated differences in transcript levels in the current study make it difficult to envision an environmental component being responsible for the observation. The three Parks from which the killer whales resided have substantially different environments (California, Texas & Florida) but yet presented with undistinguishable leukocyte transcript numbers.

Table 3
Genes, effects, F values, and P values for ANOVA style general linear mixed models used to analyze for influence of effects (park, sex, age, year) on gene transcription levels. Park, sex and age were treated as fixed effects while animal ID and year were treated as random effects. Parameter estimates for all model effects were conducted using the lme4 package in R 2.8.1 (R Development Core Team, 2012).

Gene	Effects	F value	P-value
IL-10	Park	1.31	0.28
	Sex	4.17	0.06
	Age	12.07	0.00
	Year	10.15	0.00
CD69	Park	1.82	0.18
	Sex	0.00	0.94
	Age	1.87	0.19
	Year	1.66	0.20
Cox2	Park	0.34	0.72
	Sex	1.07	0.31
	Age	4.56	0.04
	Year	22.24	0.00
IFNg	Park	3.00	0.06
	Sex	0.03	0.86
	Age	12.52	0.00
	Year	11.36	0.00
TNF alpha	Park	0.46	0.63
	Sex	0.57	0.46
	Age	2.87	0.10
	Year	3.69	0.06
MX1	Park	1.12	0.34
	Sex	2.18	0.16
	Age	0.05	0.82
	Year	9.63	0.00
FADD	Park	1.26	0.30
	Sex	0.07	0.79
	Age	11.41	0.00
	Year	0.92	0.34

Transcript levels of two cytokine genes, IL-4 and IL-17, were highly variable within the group as compared to the other transcripts. IL-4 transcripts were typically absent (CT>40) with the outliers ranging between 15 and 28 NV. An evidence-based explanation for the absence of IL-4 in the majority of samples is lacking. Given IL-4's understood function in mammalian species, it is possible that relative absence of IL-4 transcripts could be genetically influenced and/or due in part to minimal T_H2 anti-parasitic activity. The fact that samples with measurable IL-4 transcripts appeared in at least one sample from each of 15 different animals suggests minimal genetic influence. Interestingly, killer whale #19 had detectable IL-4 transcription in half of its samples. Five of the six highest transcript levels of IL-4 were in two animals, #11 and #16. By way of speculation, the animals presenting with measurable IL-4 transcript levels might either be experiencing allergies, responding to unidentified environmental parasites or merely the result of "innocent bystander" stimulation. The authors find the hypothesis that the higher IL-4 levels might be due to atopy an interesting possibility, given their hygienic environment, anti-helminthic treatment protocols and high quality food. Variability in transcript numbers encoded by the IL-17 gene was also evident and somewhat resembled IL-4 in that several samples had no detectable transcripts (data not shown). Similarly, we would speculate that the relative absence and/or variability of IL-17 transcription could be genetically influenced, a reflection of the relatively clean environment in which the animals live and/or high quality food. Transcript levels of the remaining seven biomarkers were relatively stable within the population as a whole (Fig. 1).

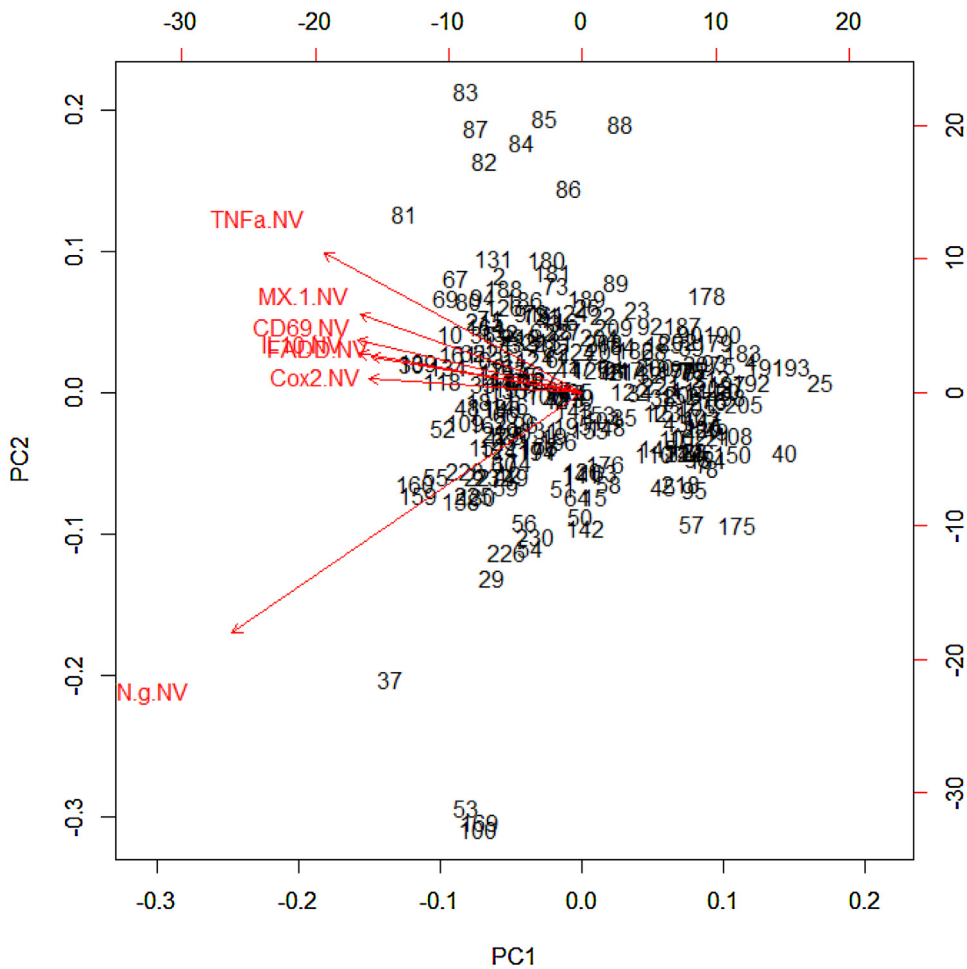

Fig. 4. Principal component analysis (PCA) using the normalized transcription values for seven genes of interest. PCA revealed two principal components with eigenvalues greater than unity that together explained 83.0% of the total variance within our dataset. Both principal components were related to IFNγ (PC1loading = −0.53, PC2 loading = −0.81).

Analysis of transcript levels on an individual basis (excluding IL-4 and IL-17 due to their extensive variability on a population basis) identified both high and low outliers that were not visible when analyzed in the context of the entire population (Fig. 2). The majority of outliers (9 samples) were relatively low in transcripts (high NVs) and included 4 samples from 4 different animals for IFNγ, and one each for Cox2, IL-17, FADD, IL-10 and MX-1. Four additional outliers (excluding IL-4) had relatively elevated transcript levels including IL-17 for 2 animals and one each for Cox-2 and IL-10. The low number of outliers was not unexpected as sample selection was intentionally directed towards animals that were clinically healthy at the time of collection. The presence of elevated outliers (low NVs) could be indicative of early subclinical disease that could then be further described when each animal is analyzed as a function of their own baseline. Animal #21 had the highest transcript level of IFNγ at the same time we recorded the highest level of MX-1; this could be suggestive of an immune response to a subclinical infection with an intracellular pathogen, potentially a virus (Haller et al., 1998; Sadler and Williams, 2008).

An unusual profile of highly variable TNFα transcript levels was visible for whale #19 with a median number for all samples being lower than those recorded for the other 25 whales (Fig. 2). Interestingly, this animal was also remarkable as a unique cluster in the heat map analysis of all data (Fig. 3). Based upon the clinical records there is no clear evidence that this animal was experiencing infectious or inflammatory disease. However, the relatively unusual transcript profile could possibly be attributed to mild, "subclinical" disease. Alternatively, the changes might not be disease-related at all but rather represent a normal response to social stressors in the environment. Studies described by Powell et al. (2013) identified associations between social stress and unique patterns of leukocyte gene transcripts which were highlighted by upregulation of inflammatory gene expression. While #19's leukocyte transcript profile does not parallel these human studies (i.e. elevated TNFα transcripts), this whale's heat map profile does identify relatively high levels of Cox-2 and IFNγ.

Within the longitudinal transcript analyses, minimal variation within transcript patterns would suggest more consistent health whereas those animals with extensive variation within the biomarkers (either individual or multiple biomarkers) would be consistent with infectious or inflammatory events and/or environmental/social stressors; the authors would propose that this type of analysis offers the potential to serve as 'early indicators' of disease.

To summarize, this longitudinal study was designed as a preliminary inquiry as to the potential for immune-gene transcript analysis to serve as a tool for assessing immediate and/or longitudinal health of domestic killer whales. Sampling was opportunistic and thus developing associations between transcript levels and animal health were not possible.

5. Conclusion

Application of blood leukocyte transcript analysis to mammalian wildlife species is a viable approach to augment diagnostics. Leukocyte gene transcripts encoding cell-surface and secreted messengers are under tight transcriptional control and exhibit short half-lives, and thus can serve as sensitive and real-time measures of immunologic perturbations. The present data provides the basis for developing preliminary baseline leukocyte transcript levels for the targeted killer whale genes and justifies expansion of studies to develop diagnostically useful associations with both immediate and long-term health.

Conflict of interest

The authors have no conflict of interest.

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