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Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: Effect of haematophagous parasites on haematological parameters

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ABSTRACT

Evaluation of the health status of free-ranging populations is important for understanding the impact of disease on individuals and on population demography and viability. In this study, haematological reference intervals were developed for free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups within the context of endemic hookworm (*Uncinaria sanguinis*) infection and the effects of pathogen, host, and environment factors on the variability of haematological parameters were investigated. *Uncinaria sanguinis* was identified as an important agent of disease, with infection causing regenerative anaemia, hypoproteinaemia, and a predominantly lymphocytic–eosinophilic systemic inflammatory response. Conversely, the effects of sucking lice (*Antarctophthirus microchir*) were less apparent and infestation in pups appears unlikely to cause clinical impact. Overall, the effects of *U. sanguinis*, *A. microchir*, host factors (standard length, body condition, pup sex, moult status, and presence of lesions), and environment factors (capture-type and year of sampling) accounted for 26–65% of the total variance observed in haematological parameters. Importantly, this study demonstrated that anaemia in neonatal Australian sea lion pups is not solely a benign physiological response to host–environment changes, but largely reflects a significant pathological process. This impact of hookworm infection on pup health has potential implications for the development of foraging and diving behaviour, which would subsequently influence the independent survival of juveniles following weaning. The haematological reference intervals developed in this study can facilitate long-term health surveillance, which is critical for the early recognition of changes in disease impact and to inform conservation management.

1. Introduction

Evaluation of the health status of free-ranging populations is important for understanding the impact of disease on individuals and on population demography and viability (Deem et al., 2001; Smith et al., 2009; Thompson et al., 2010). Haematological analysis is a reasonably non-invasive and efficient tool used as part of routine health assessment, permitting repeated *in situ* sampling of live individuals with minimal impact on animal welfare and survival (Clark, 2004; Wimsatt et al., 2005). Changes in haematological values provide quantifiable measures of the impact of, and host-response to, disease. However, inherent host-specific differences and dynamic temporospatial adaptations to physiological stressors also influence haematological characteristics (Gray et al., 2005; Beldomenico et al., 2008; Hufschmid et al., 2014). For this reason, the establishment of species- and context-specific reference intervals is necessary to define and assess deviations from baseline health status (Sergent et al., 2004; Ceriotti et al., 2009). This would facilitate the implementation of long-term health surveillance, essential for both the early recognition of emerging disease and to inform species conservation management (Hall et al., 2007; Thompson et al., 2010).

As high trophic-level predators exploiting a variety of ecological niches, pinnipeds act as sentinels for marine ecosystem health (Bossart, 2011). In particular, the health status of maternally-dependent pinniped pups is sensitive to changes to pathogen–host–environment relationships such as shifts in prey abundance, major climatic events, the presence of environmental toxins and contaminants, the occurrence of infectious diseases, and increasing human-impacts (Beckmen et al., 2003; Soto et al., 2004; Greig et al., 2005; Castinel et al., 2007; Melin et al., 2010; Brock et al., 2013). Haematological reference intervals have been developed for pups of several pinniped species to facilitate health assessment and several studies have investigated haematological responses to physiological changes, identifying the influential role of host factors (for example age, body condition, and sex) and environment factors (including geographic location and capture-associated stress) (Bryden and Lim, 1969; Geraci, 1971; Lane et al., 1972; Banish and Gilmartin, 1988; Castellini et al., 1993, 1996; Horning and Trillmich, 1997; Hall, 1998; Rea et al., 1998; Sepúlveda

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et al., 1999; Trumble and Castellini, 2002; Lander et al., 2003, 2014; Richmond et al., 2005; Boily et al., 2006; Clark et al., 2007; Trillmich et al., 2008; Greig et al., 2010; Brock et al., 2013). Yet, despite the widespread host distribution of haematophagous hookworm and lice species (Leonardi and Palma, 2013; Nadler et al., 2013), the effects of these parasites on the haematological values of pups and their implications for the assessment of health status remain unresolved. For example, although hookworm and lice can cause anaemia (Olsen, 1958; Dailey, 2001; Lyons et al., 2001), the population-wide occurrence of anaemia in neonates of many pinniped species has generally been attributed to a physiological host-response to the increased oxygen availability compared to the environment *in utero* and the expansion of plasma volume with growth (Richmond et al., 2005; Clark et al., 2007; Trillmich et al., 2008). A notable exception to the occurrence of neonatal anaemia is observed in land-bound northern elephant seal (*Mirounga angustirostris*) pups at Año Nuevo State Reserve (Castellini et al., 1990; Thorson and Le Boeuf, 1994) in which hookworm infection has not been detected (Lyons et al., 2012). Critically, few studies have considered parasitosis as a cause of anaemia in pinniped pups and there are no reports that characterise this anaemia by the presence or absence of reticulocytosis; classifying the erythroid response to anaemia as regenerative or non-regenerative in this way is fundamental to differentiating between pathological and physiological mechanisms (Stockham and Scott, 2008).

The impact of disease on the health status and population demography of the endangered Australian sea lion (*Neophoca cinerea*) is considered a key knowledge gap for understanding the impediments to population recovery in this species and for informing conservation management to mitigate the risks of population extinction (Goldsworthy et al., 2009). Whilst haematological reference intervals for free-ranging Australian sea lions older than six months of age have been reported (Needham et al., 1980; Fowler et al., 2007), data from neonatal pups is lacking. Additionally, the effects of disease on haematological values in this species have not been reported. Hookworm (*Uncinaria sanguinis*) endemically infects 100% of neonatal Australian sea lion pups at Seal Bay and Dangerous Reef in South Australia, two of the largest breeding colonies of this species, and is hypothesised to be an important agent of disease and cause of pup mortality across the species' range (Marcus et al., 2014a,b). Pups are infected *via* the transmammary route shortly after birth and demonstrate patent infection from 11–14 days of age for approximately 2–3 months (Marcus et al., 2014a; see Fig. 1). The extended breeding season of the Australian sea lion (approximately 7–9 month duration; Goldsworthy et al., 2012; McIntosh et al., 2012) facilitates the high prevalence of hookworm infection in pups by increasing the period of time in which breeding females can acquire infective free-living hookworm larvae (Marcus et al., 2014a). Additionally, the extended breeding cycle of approximately 18 months results in alternate 'summer' and 'winter' breeding seasons, occurring asynchronously between colonies (Higgins, 1993; McIntosh et al., 2012). The magnitude of colony pup mortality is associated with fluctuations in the intensity of hookworm infection, mediated by seasonal and colony-specific factors; an oscillating pattern of high-pup-mortality-with-high-infection-intensity and low-pup-mortality-with-low-infection-intensity has been observed at Seal Bay for summer and winter breeding seasons, respectively, and the opposite seasonal association has been described for Dangerous Reef, reflecting the different environmental attributes of the two colonies (Goldsworthy et al., 2012, 2013; Marcus et al., 2014a). Infestation with sucking lice (*Antarctophthirus microchir*) is also reported in Australian sea lion pups (McIntosh and Murray, 2007), although the epidemiology and clinical impact of this parasite have not been investigated in this host.

The aim of this study is to develop haematological reference intervals for free-ranging neonatal Australian sea lion pups within the context of endemic hookworm infection. In addition, this study will investigate the impact of *U. sanguinis* and *A. microchir* on pup health by estimating their effects on the variability of haematological parameters whilst considering the concurrent influence of host and environment factors. In particular, by characterising the erythroid changes in anaemia, this study will assess the hypothesis that neonatal anaemia is non-pathological, caused predominantly by physiological responses to host–environment changes.

2. Materials and methods

2.1. Sample collection

Samples were collected from Australian sea lion pups (n = 295) during consecutive winter and summer breeding seasons at two South Australian colonies, Seal Bay, Kangaroo Island (35.994°S, 137.317°E) in

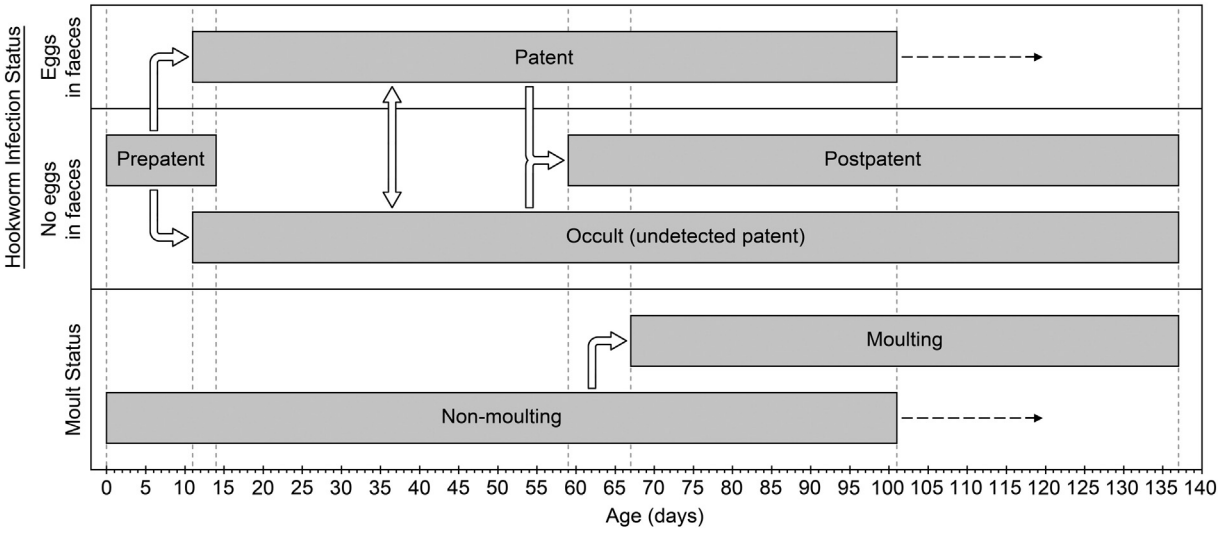

Fig. 1. Flowchart displaying the timing of hookworm (*Uncinaria sanguinis*) infection and moult status in neonatal Australian sea lion pups (*Neophoca cinerea*) from birth to 137 days of age. Grey boxes indicate the occurrence of each category with respect to pup age. White arrows indicate possible directions of change between categories. Dashed black arrows demonstrate the uncertainty in the upper limit for the occurrence of patent hookworm infection and non-moulting pups.
Adapted from Marcus et al. (2014a).

2010 and 2012, and Dangerous Reef, Spencer Gulf (34.815°S, 136.212°E) in 2011 and 2013. During 2010, pups ≥10 kg were sampled on one occasion only, whilst in other years, pups including those <10 kg body weight were captured for sample collection on up to three occasions at least 14 days apart. Based on the estimated or observed starting dates for pupping during each breeding season (Goldsworthy et al., 2012, 2013), the maximum possible age of sampled pups was approximately seven months. During the 2012 breeding season at Seal Bay, samples were also collected from a cohort of known-age pups (n = 41; aged 10–137 days).

Pups were captured by hand or net during maternal absence and restrained manually within canvas bags. Capture-type was categorised as sleeping, awake (pup alert, minimal pup exertion), or mobile (pup alert, captured after a short period of pup exertion). Standard length (measured to the nearest 0.5 cm), body weight (measured to the nearest 0.1 kg; Salter hanging scale, Avery Weigh-Tronix, West Midlands, UK), body condition (subjectively scored poor/fair/good/excellent based on the palpable prominence of the vertebral spinous processes, pelvic bones, and skeletal muscle and adipose tissues), pup sex, and moult status (non-moulting/moulting) were recorded. Pups were examined for the presence of clinically significant lesions (including dermatitis, cutaneous ulceration, and subcutaneous abscesses; absent/present) and the dorsal and ventral pelage of the thorax and abdomen was examined for the presence of ectoparasites (lice; categorised as negative/positive). Faecal samples were collected per rectum using rayon-tipped dry swabs (Copan Diagnostics, Murrieta, USA) within a lubricated open-ended polyethylene sheath (modified 1–3 mL transfer pipette, Livingstone International, Sydney, Australia) or from the ground if pups were observed to defecate.

Blood samples (n = 387) were collected from the brachial vein (Barnes et al., 2008) using 21-gauge × 1-inch needles attached to 5 or 10 mL plastic syringes, transferred to 1.3 mL EDTA, lithium heparin, and plain serum tubes (Sarstedt, Nümbrecht, Germany), and stored at approximately 4 °C prior to processing. To facilitate individual pup identification for recapture, pups were uniquely identified by one or more of the following methods: a temporary bleach mark on their lumbosacral pelage (Schwarzkopf Nordic Blonde, Henkel Australia, Melbourne, Australia), a subcutaneous passive integrated transponder (23 mm microchip, Allflex Australia, Brisbane, Australia), and/or tags applied to the trailing edge of both fore-flippers (Supertag Size 1 Small, Dalton ID, Oxfordshire, UK).

2.2. Haematological analysis

EDTA anti-coagulated whole blood samples were processed for haematological analysis within 10 h of collection. Packed cell volume (PCV; L/L) was measured in duplicate in microhaematocrit tubes (IRIS Sample Processing, Westwood, USA) following centrifugation at 15,800 rpm for 120 s (StatSpin MP, StatSpin Technologies, Norwood, USA); mean values were utilised for statistical analysis. Total plasma protein (TPP; g/L) was measured using a hand-held refractometer (Reichert TS Meter, Cambridge Instruments, Buffalo, USA). Air-dried blood smears were fixed with 100% methanol and treated with a Romanowsky-type rapid stain (Diff Quik, Lab Aids, Sydney, Australia; or Rapid Diff, Australian Biostain, Traralgon, Australia) for examination using an Olympus BH-2 microscope (Olympus, Australia). Absolute reticulocyte counts (RET; $\times 10^9$/L) were performed by estimating the number of reticulocytes per 1000 erythrocytes on air-dried smears prepared by incubation of 50 μL blood 1:1 with citrated 1% brilliant cresyl blue (Sigma Chemical, St. Louis, USA) for 20 min. Smears from samples collected at Seal Bay were examined immediately whilst those collected from Dangerous Reef were fixed with methanol for 20 s and examined at a later time. For blood samples collected from Seal Bay, total erythrocyte and leucocyte counts were performed using Ery-TIC and Leuko-TIC kits (Bioanalytic, Freiburg, Germany), respectively, using a Neubauer improved haemocytometer (Glaswarenfabrik Karl Hecht, Sondheim, Germany). Erythrocytes in the five diagonal central group squares and leucocytes in all nine large squares of the haemocytometer were enumerated in duplicate at 400× magnification; mean values were utilised for statistical analysis. As field conditions at Dangerous Reef precluded use of the haemocytometer method for erythrocyte and leucocyte estimations, 100–200 μL aliquots of EDTA anti-coagulated whole blood samples collected at Dangerous Reef were mixed 1:1 with Streck Cell Preservative (Streck, Omaha, USA) and stored at approximately 4 °C. Preserved samples were analysed using a Sysmex XT-2000*i*V automated haematology analyser (Sysmex, Kobe, Japan) at the Veterinary Pathology Diagnostic Service, Faculty of Veterinary Science, The University of Sydney within nine days of blood collection. Leucocyte counts (WBC; $\times 10^9$/L) were calculated by manually gating the WBC/BASO scattergram using the manufacturer's software (version 00-10; Sysmex) and one profile was applied to all analysed samples. Automated erythrocyte counts (RBC; $\times 10^{12}$/L) were calculated by the impedance method using default parameters. Values obtained were doubled to correct for dilution with cell preservative. To facilitate comparison of haematological parameters between Seal Bay and Dangerous Reef, manual haemocytometer counts were converted to Sysmex-equivalent values (Supplementary Material Appendix S1). Finally, differential leucocyte counts were performed on Romanowsky-type stained blood smears to determine the proportion of neutrophils, lymphocytes, monocytes, eosinophils, and basophils; one hundred leucocytes were identified for every 10×10^9/L WBC. The proportion of nucleated erythrocytes (mainly late normoblasts) to leucocytes was also recorded to estimate absolute nucleated erythrocyte counts (nRBC; $\times 10^6$/L), corrected leucocyte counts (cWBC; $\times 10^9$/L), and absolute neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts ($\times 10^9$/L). The equations used to calculate haematological values are presented in Supplementary Material Appendix S1. Due to the availability of test kits and occasional samples where an insufficient volume of blood was collected to permit complete haematological analysis, values for all haematological parameters could not be determined for every individual.

2.3. Hookworm infection status

Hookworm eggs were detected using a modified McMaster flotation with saturated NaCl solution or, for small faecal samples, a direct smear. Where eggs were evident in faecal samples, pups were classified as 'patent' (n = 213 pups; n = 256 time points). Where eggs were not evident, hookworm infection status was inferred based on pup age, moult status, and the timing of infection, or using repeated faecal samples (Marcus et al., 2014a; see Fig. 1): pups ≤14 days of age or with subsequent patent faecal samples were classified as 'prepatent' (n = 6 pups; n = 7 time points) and pups ≥59 days of age, showing signs of moult, or with previous patent faecal samples were classified as 'postpatent' (n = 83 pups; n = 91 time points). As such, the prepatent and postpatent groups could have included pups with occult (undetected patent) hookworm infection (Fig. 1). Hookworm infection status was considered to be unknown (n = 33) for non-moulting pups (or pups with no recorded moult status) of undetermined age with negative repeat faecal samples (or no repeat sample) and for those pups sampled for blood collection but with no faecal sample.

2.4. Statistical analysis

2.4.1. Haematological reference intervals

Nonparametric 95% reference intervals were calculated for haematological parameters (PCV, RBC, mean corpuscular volume, RET, nRBC, TPP, cWBC, and differential leucocyte counts), partitioned by hookworm infection status as a proxy for age and because haematological values were expected to significantly differ between groups. Outliers, values more than 1.5 times the interquartile range above or below the interquartile range (Tukey, 1977), were excluded from each group

prior to the development of reference intervals to improve the accuracy of the reference interval limits (Horn et al., 2001). To adjust for repeated measures from pups sampled on more than one occasion, reference intervals were developed for each parameter using bootstrap estimation (1000 replicates) with observations drawn randomly from the dataset with replacement and weighting to ensure equal probability of selection from individual pups, based on methodology described by Taylor et al. (1996) and Alatzas et al. (2014). For each bootstrap replicate, the reference interval (2.5th and 97.5th percentiles) and median value (50th percentile) were determined. Final estimates of the reference interval and median value for each parameter were calculated as the median of the replicated bootstrap percentile values. Approximate 95% confidence intervals (CI) were calculated around each estimate as the 2.5th and 97.5th percentiles of the replicated bootstrap percentile values (Efron, 1982). For pups with prepatent hookworm infection, minimum and maximum values are reported due to insufficient sample size for the calculation of nonparametric 95% reference intervals (Ceriotti et al., 2009) and median values were calculated as non-bootstrapped weighted values as bootstrapping may under-represent the true biological variability with small sample sizes (Chernick, 2011). Haematological data from pups with unknown hookworm infection status were excluded from reference interval development.

The validity of partitioning reference intervals based on hookworm infection status for pups with patent and postpatent hookworm infection was determined by observing the proportion of values in each group that fell outside of common reference limits (Lahti et al., 2004). The combined reference interval was developed by combining the outlier-removed datasets for these two groups and using bootstrap estimation, with observations weighted to adjust for both repeated measures and unequal numbers of reference values between the groups. Following the recommendations of Lahti et al. (2004), the combined reference interval for each parameter was considered invalid if $\geq$4.1% or $\leq$0.9% of reference values from either group, adjusted for repeated measures, were outside either the upper or the lower combined reference limits. Additionally, the underlying distributions of reference values for each parameter for these two groups were considered significantly different ($P < 0.01$) if their median CI did not overlap (Cumming, 2009).

The erythroid response to anaemia was classified as regenerative or non-regenerative based on the absolute reticulocyte count; a reticulocytosis greater than 65.0×10^9/L was considered evidence of a regenerative erythroid response (Hodges and Christopher, 2011).

2.4.2. Factors explaining haematological parameter variability

Correlational analysis was performed to characterise the pattern of haematological changes associated with hookworm infection. Pairwise Spearman's rank correlations (ρ), partitioned by hookworm infection status, were calculated for all haematological parameters reported except for mean corpuscular volume (MCV) and cWBC as variability in these parameters is explained by the variability in PCV and RBC, and the differential leucocyte counts, respectively. To adjust for repeated measures from pups sampled on more than one occasion, median estimates of ρ with 95% CI were calculated using bootstrap replication as previously described. Correlations were categorised as 'weak' for $\rho < 0.35$, 'moderate' for $0.35 \leq \rho < 0.75$, and 'strong' for $\rho \geq 0.75$ (Shi and Conrad, 2009), and were considered statistically significant ($P < 0.05$) if their CI excluded zero. Haematological data from pups with prepatent hookworm infection were excluded from correlational analysis due to small sample size.

The effects of *U. sanguinis* and *A. microchir* on haematological parameters were investigated using linear mixed modelling with REML estimation. Terms prospectively included in the models as fixed factors were pathogen factors (hookworm infection status and presence of lice), host factors (standard length, body weight, body condition, pup sex, moult status, and presence of lesions), and environment factors (capture-type and year of sampling). Standard length, body weight, and moult status were included as proxies for growth and pup age (Marcus et al., 2014a) as known-age pup data was only available for one breeding season. Presence of lesions was included as a factor to account for the variance related to the occurrence of other disease processes whilst the physiological effects of capture-associated stress were investigated by including capture-type as a factor. Year of sampling was included as a factor to represent the interaction between colony (Seal Bay/Dangerous Reef) and season (summer/winter). Pup-identity was specified as the random factor to account for repeated measures and an appropriate correlation structure was chosen using the change in model deviance. The assumptions of homogeneity of residual variance and normality were checked by visually assessing the fitted-value plots and histograms of residuals and, where necessary, the response variate was power or log-transformed. Models were constructed by the backwards stepwise removal of factors with the lowest explanatory power (highest Wald F-test P-value) to arrive at the final models that included only significant predictors ($P < 0.05$). The amount of variance explained by the final models was estimated using the marginal coefficient of determination (R^2_m; fixed factors only) and the conditional coefficient of determination (R^2_c; fixed and random factors) (Nakagawa and Schielzeth, 2013). The predicted effects of factors included in the final models are reported as the regression coefficient $\pm$ standard error. For factors with more than two levels (body condition, capture-type, hookworm infection status, and year of sampling), the predicted level effects were considered significantly different ($P < 0.05$) if the 95% CI for their difference excluded zero. Model construction was undertaken for all haematological parameters except for MCV and cWBC as previously outlined. Haematological data from all sampled pups were prospectively included in model construction with listwise deletion employed to exclude cases with missing factor data for each model. All statistical analyses were performed using GenStat 16.1 (VSN International, Hemel Hempstead, UK) and statistical significance was considered at $P < 0.05$.

3. Results

3.1. Haematological reference intervals

Haematological reference intervals for Australian sea lion pups, partitioned by hookworm infection status, are presented in Table 1. The proportion of values identified as outliers for each hookworm infection status group are presented in Supplementary Table S1. Combined reference intervals for pups with patent and postpatent hookworm infection did not adequately represent the true distributions of reference values for any of the measured haematological parameters (Supplementary Table S2). When compared to pups with patent hookworm infection, postpatent pups had significantly higher median values ($P < 0.01$) and reference interval limits for PCV, RBC, and TPP; and significantly lower median values ($P < 0.01$) and reference interval limits for MCV, RET, nRBC, and all leucocyte parameters. Pups with prepatent hookworm infection had the highest median values for PCV, RBC, neutrophil, and monocyte counts; median values intermediate to the other hookworm infection status groups for RET, nRBC, TPP, cWBC, and lymphocyte counts; and the lowest median value for MCV. Median eosinophil counts were invariant between pups with prepatent and patent hookworm infection. Basophils were not identified in any of the blood smears examined.

The proportions of samples from pups with prepatent, patent, and postpatent hookworm infection that demonstrated a regenerative erythroid response were 66.7% (CI 22.3–95.7%), 65.0% (CI 58.7–71.0%), and 29.1% (CI 19.8–39.9%), respectively.

3.2. Factors explaining haematological parameter variability

For pups with patent hookworm infection, significant correlations were identified between most haematological parameters examined

Table 1
Nonparametric 95% haematological reference intervals and median values (with 95% confidence intervals) for neonatal Australian sea lion pups (*Neophoca cinerea*), partitioned by hookworm infection status. Values were calculated by bootstrap estimation which adjusted for repeated measures. Due to the small sample size for pups with prepatent hookworm infection, reference intervals for this group are presented as minimum–maximum with non-bootstrapped weighted median values.

Hookworm infection status	Prepatent			Patent			Postpatent		
Number of pups sampled	6			213			83		
	Min–max	Median	n[a]	95% RI	Median	n[a]	95% RI	Median	n[a]
PCV (L/L)	0.355–0.520	0.390	7	0.261–0.410 (0.245–0.278; 0.397–0.429)	0.340 (0.335–0.350)	244	0.284–0.440 (0.280–0.306; 0.430–0.455)	0.380 (0.370–0.389)	88
RBC ($\times 10^{12}$/L)	4.34–5.19	4.51	6	2.85–4.88 (2.54–3.03; 4.62–4.93)	3.75 (3.69–3.80)	236	3.43–5.34 (3.36–3.87; 5.13–5.46)	4.44 (4.35–4.53)	88
MCV (fL)	78.5–100.3	82.0	6	76.1–103.6 (74.5–77.2; 101.3–107.7)	90.1 (88.9–91.3)	233	65.1–96.2 (61.2–71.4; 94.7–100.5)	84.3 (82.5–86.3)	88
RET ($\times 10^9$/L)	0.0–117.2	78.5	6	13.0–203.8 (6.3–19.2; 180.1–209.1)	83.2 (75.1–87.2)	236	5.1–106.4 (4.0–10.0; 96.1–117.0)	45.1 (39.6–53.6)	85
nRBC ($\times 10^6$/L)	0.0–130.7	27.2	7	0.0–550.1 (0.0–0.0; 466.2–597.1)	92.6 (78.1–122.3)	224	0.0–127.1 (0.0–0.0; 97.7–135.1)	0.0 (0.0–0.0)	78
TPP (g/L)	58.0–75.0	70.5	7	51.8–86.2 (48.0–53.0; 81.3–87.7)	69.0 (66.0–70.0)	251	63.7–87.0 (62.0–66.1; 83.3–88.0)	72.0 (71.0–73.0)	89
cWBC ($\times 10^9$/L)	6.03–15.02	11.29	7	6.48–25.02 (5.40–6.88; 23.65–26.58)	12.12 (11.50–13.00)	237	3.74–15.18 (3.58–4.65; 13.57–18.53)	8.73 (7.92–9.25)	82
Neutrophils ($\times 10^9$/L)	3.26–9.54	7.91	7	2.47–20.13 (1.93–2.86; 17.30–21.01)	6.83 (6.37–7.49)	237	1.42–11.71 (1.08–2.14; 9.24–13.78)	4.91 (4.34–5.49)	82
Lymphocytes ($\times 10^9$/L)	1.30–3.57	2.85	7	1.44–5.99 (1.19–1.60; 5.65–6.27)	3.18 (2.99–3.40)	241	0.94–5.01 (0.67–1.19; 4.55–5.08)	2.55 (2.34–2.85)	85
Monocytes ($\times 10^9$/L)	0.11–0.47	0.45	6	0.00–1.07 (0.00–0.00; 0.90–1.18)	0.31 (0.28–0.34)	240	0.00–0.70 (0.00–0.00; 0.54–0.73)	0.17 (0.12–0.23)	83
Eosinophils ($\times 10^9$/L)	0.24–2.18	1.13	7	0.05–3.54 (0.00–0.08; 3.22–3.80)	1.13 (0.95–1.41)	246	0.06–1.24 (0.03–0.11; 1.00–1.28)	0.42 (0.34–0.50)	82

[a] Sample size after outlier removal. Abbreviations: cWBC — corrected leucocyte count; MCV — mean cell volume; nRBC — absolute nucleated erythrocyte count; PCV — packed cell volume; RBC — erythrocyte count; RET — absolute reticulocyte count; RI — reference interval; TPP — total plasma protein.

(26 of 36 pairs; Table 2), whereas for postpatent pups, fewer significant correlations were observed (15 of 36 pairs; Table 3). Strong correlations ($\rho \geq 0.75$) were not identified in either dataset. The moderate correlations ($0.35 \leq \rho < 0.75$) between haematological parameters are summarised below; all pairwise correlation coefficients (with 95% CI) are presented in Tables 2 and 3.

For both groups of pups there was significant moderate positive correlation between PCV/RBC and RET/nRBC; however, only pups with patent hookworm infection demonstrated evidence for regenerative responses with significant moderate negative correlation of PCV/nRBC and RBC/nRBC and significant weak negative correlation of PCV/RET and RBC/RET. Postpatent pups had non-significant correlations between PCV/nRBC, RBC/nRBC, PCV/RET, and RBC/RET. For pups with patent hookworm infection, there was significant moderate positive correlation between RBC/TPP, significant moderate negative correlation between RBC/neutrophil-count and TPP/eosinophil-count, and significant weak negative correlation between RBC/eosinophil-count. In contrast, postpatent pups had significant weak negative correlation between RBC/TPP and RBC/neutrophil-count, non-significant correlation between TPP/eosinophil-count, and significant moderate negative correlation between RBC/eosinophil-count. Postpatent pups also had significant moderate positive correlation between TPP/lymphocyte-count and nRBC/monocyte-count, whilst pups with patent hookworm infection had significant weak negative correlation and non-significant correlation for these pairs, respectively.

The final linear mixed models assessing the effects of pathogen, host, and environment factors on the variability of pup haematological parameters are presented in Table 4 (effects; erythrocytes and TPP),

Table 2
Spearman's rank correlation of haematological parameters in neonatal Australian sea lion pups (*Neophoca cinerea*) with patent hookworm (*Uncinaria sanguinis*) infection, calculated by bootstrap estimation to adjust for repeated measures. Median bootstrap replicate values (with 95% confidence intervals) of the correlation coefficient (top right triangle) and sample sizes (bottom left triangle) are presented. Values indicated in **bold** were statistically significant ($P < 0.05$).

	PCV	RBC	RET	nRBC	TPP	Neutrophils	Lymphocytes	Monocytes	Eosinophils
PCV		**0.71 (0.62, 0.78)**	**−0.26 (−0.38, −0.14)**	**−0.41 (−0.51, −0.30)**	**0.20 (0.07, 0.32)**	**−0.30 (−0.41, −0.17)**	**−0.24 (−0.36, −0.11)**	**0.17 (0.06, 0.30)**	−0.06 (−0.18, 0.07)
RBC	242		**−0.21 (−0.32, −0.07)**	**−0.37 (−0.48, −0.26)**	**0.37 (0.24, 0.49)**	**−0.37 (−0.49, −0.25)**	**−0.26 (−0.38, −0.13)**	0.04 (−0.10, 0.18)	**−0.13 (−0.25, −0.005)**
RET	240	243		**0.43 (0.32, 0.53)**	−0.07 (−0.20, 0.06)	0.08 (−0.07, 0.20)	0.10 (−0.02, 0.23)	−0.03 (−0.16, 0.10)	**0.19 (0.06, 0.31)**
nRBC	247	242	242		**−0.19 (−0.32, −0.07)**	0.04 (−0.08, 0.16)	**0.22 (0.09, 0.34)**	−0.04 (−0.16, 0.09)	**0.25 (0.13, 0.37)**
TPP	251	241	239	246		0.09 (−0.04, 0.20)	**−0.17 (−0.29, −0.04)**	**0.17 (0.05, 0.30)**	**−0.35 (−0.46, −0.23)**
Neutrophils	247	242	242	250	246		**0.18 (0.06, 0.30)**	**0.16 (0.03, 0.29)**	**−0.22 (−0.34, −0.10)**
Lymphocytes	247	242	242	250	246	250		−0.05 (−0.17, 0.07)	**0.29 (0.17, 0.40)**
Monocytes	247	242	242	250	246	250	250		**−0.17 (−0.29, −0.04)**
Eosinophils	247	242	242	250	246	250	250	250	

Table 3
Spearman's rank correlation of haematological parameters in neonatal Australian sea lion pups (*Neophoca cinerea*) with postpatent hookworm (*Uncinaria sanguinis*) infection, calculated by bootstrap estimation to adjust for repeated measures. Median bootstrap replicate values (with 95% confidence intervals) of the correlation coefficient (top right triangle) and sample sizes (bottom left triangle) are presented. Values indicated in **bold** were statistically significant ($P < 0.05$).

	PCV	RBC	RET	nRBC	TPP	Neutrophils	Lymphocytes	Monocytes	Eosinophils
PCV		**0.57 (0.39, 0.70)**	−0.13 (−0.36, 0.09)	−0.15 (−0.36, 0.05)	−0.08 (−0.29, 0.14)	**−0.28 (−0.49, −0.06)**	0.02 (−0.18, 0.23)	0.21 (−0.01, 0.43)	**−0.31 (−0.50, −0.10)**
RBC	90		−0.17 (−0.36, 0.05)	−0.21 (−0.40, 0.001)	**−0.33 (−0.53, −0.13)**	**−0.34 (−0.55, −0.14)**	−0.05 (−0.26, 0.15)	−0.03 (−0.25, 0.19)	**−0.39 (−0.55, −0.20)**
RET	85	86		**0.39 (0.20, 0.57)**	0.12 (−0.10, 0.34)	**0.27 (0.06, 0.48)**	0.06 (−0.17, 0.28)	0.06 (−0.14, 0.27)	**0.28 (0.07, 0.48)**
nRBC	85	86	85		0.06 (−0.15, 0.28)	**0.25 (0.07, 0.44)**	−0.09 (−0.29, 0.13)	**0.37 (0.17, 0.53)**	**0.25 (0.03, 0.43)**
TPP	90	91	86	86		**0.30 (0.08, 0.49)**	**0.36 (0.16, 0.55)**	−0.13 (−0.33, 0.08)	0.05 (−0.15, 0.27)
Neutrophils	85	86	85	86	86		0.12 (−0.10, 0.33)	0.06 (−0.14, 0.29)	0.15 (−0.07, 0.36)
Lymphocytes	85	86	85	86	86	86		−0.17 (−0.35, 0.04)	−0.01 (−0.21, 0.22)
Monocytes	85	86	85	86	86	86	86		**0.23 (0.002, 0.43)**
Eosinophils	85	86	85	86	86	86	86	86	

Table 5 (effects; leucocytes), and Supplementary Table S3 (P-values), and are summarised below. The final models accounted for 26–65% of the total variance observed in haematological parameters with differences between individual pups explaining up to 28% of the variability.

3.2.1. Effects of pathogen factors

Patent hookworm infection was associated with significantly lower PCV, RBC, and TPP values and significantly higher nRBC and eosinophil counts, relative to pups with prepatent infection. Postpatent hookworm infection status was associated with significantly higher RBC and TPP values and significantly lower nRBC, lymphocyte, and eosinophil counts, relative to pups with patent hookworm infection. However, PCV and RBC values remained significantly lower compared to those pups with prepatent hookworm infection. Hookworm infection status was not significantly associated with RET, neutrophil, or monocyte counts.

The presence of lice (identified in 73.3% of sampling events; see Supplementary Table S4) was also associated with a significant decrease in PCV, although the magnitude of effect was markedly less than that for patent hookworm infection (Table 4). In contrast to patent hookworm infection, lice infestation was associated with significantly increased TPP concentrations. No other significant effects were identified for lice infestation for the remainder of the haematological parameters.

3.2.2. Effects of host factors

Increases in standard length were associated with significant increases in PCV, RBC, and TPP values and significant decreases in RET, nRBC, and all leucocyte parameters. The presence of moult was also associated with significant decreases in RET, but did not have significant effects on any of the other haematological parameters.

Body condition was a significant host factor in the final models. Pups in excellent body condition had significantly higher nRBC compared to all other pups. Similarly, TPP values significantly increased with improving body condition although the effect of poor body condition was not significantly different from the other categorical levels. Pups in fair body condition had significantly lower eosinophil counts compared to pups in good or excellent body condition; however, as for TPP, the effect of poor body condition on eosinophil counts was not significantly different from the other categorical levels. Compared to female pups, male pups had significantly lower PCV and RBC values. The presence of lesions was associated with significantly higher neutrophil counts. No other significant effects were identified for body condition, pup sex, or the presence of lesions for the remainder of the haematological parameters. Body weight was not associated with significant effects on any of the haematological parameters after accounting for the effects of other factors.

3.2.3. Effects of environment factors

Pups captured whilst awake or mobile had significantly higher lymphocyte counts relative to pups that were captured whilst asleep. There was no significant difference in lymphocyte counts between awake and mobile pup captures and no other significant effects were identified for capture-type for any of the other haematological parameters. Year of sampling was associated with significant effects for all haematological parameters except for PCV, RBC, and RET. At Seal Bay, pups sampled during the summer breeding season (2012) had significantly higher neutrophil and eosinophil counts and significantly lower monocyte counts, relative to pups sampled during the winter breeding season (2010). At Dangerous Reef, pups sampled during the winter breeding season (2011) had significantly higher nRBC, lymphocyte, and eosinophil counts, and significantly lower TPP, neutrophil, and monocyte counts, relative to pups sampled during the summer breeding season (2013). Overall, pups sampled at Seal Bay had significantly higher nRBC and eosinophil counts and significantly lower TPP values and neutrophil counts compared to pups sampled at Dangerous Reef.

4. Discussion

The current study established haematological reference intervals for free-ranging neonatal Australian sea lion pups within the context of endemic hookworm infection and estimated the impact of *U. sanguinis* and *A. microchir*, and the concurrent effects of host and environment factors, on the variability of haematological parameters of pups. By investigating markers for erythroid regeneration, the current study demonstrated that anaemia in neonatal Australian sea lion pups is not solely a benign physiological response to host–environment changes, but largely reflects a significant pathological process that adversely impacts pup health with potential implications for the population demography and viability of this species.

4.1. Development of haematological reference intervals

The partitioning of neonatal Australian sea lion haematological reference intervals by hookworm infection status provides important age- and disease-specific context, enhancing their utility for future investigations. Reference intervals are generally developed by obtaining representative samples from a 'healthy' reference population, excluding subjects with clinical signs of disease that may affect the parameters of

Table 4
Final linear mixed models of the effect of host–pathogen–environment factors on the variability of neonatal Australian sea lion pup (*Neophoca cinerea*) erythrocyte parameters and total plasma protein. Presented values are the amount of variance explained by each model (R^2_m – fixed factors only; R^2_c – fixed and random factors) and the predicted regression coefficient ± standard error for the comparative level(s) for each factor, relative to the reference level. For each model, superscript symbols indicate which comparative level coefficients were significantly different ($P < 0.05$), within each factor; values indicated in **bold** were significantly different from the reference level. Dashes indicate factors which were excluded from the final model ($P > 0.05$). P-values are presented in Table S3.

			Body condition			Hookworm infection status		Lice	Moulting		Sex		Standard length	Year of sampling		
Reference level			Poor			Prepatent		Negative	Non-moulting		Female		–	2010		
Comparative level			Fair	Good	Excellent	Patent	Postpatent	Positive	Moulting		Male		5 cm increase	2011	2012	2013
Parameter	R^2_m	R^2_c														
PCV	37%	65%	–	–	–	**−0.098 ± 0.015**^	**−0.092 ± 0.016**^	**−0.019 ± 0.005**	–		**−0.016 ± 0.005**		**0.018 ± 0.002**	–	–	–
RBC	47%	64%	–	–	–	**−0.92 ± 0.20**^	**−0.56 ± 0.21***	–	–		**−0.18 ± 0.06**		**0.23 ± 0.02**	–	–	–
RET[a]	16%	26%	–	–	–	–	–	–	**−2.1 ± 0.4**		–		**−0.3 ± 0.1**	–	–	–
nRBC[a]	22%	32%	2.9 ± 3.7^	4.1 ± 3.8^	**8.3 ± 4.1***	**10.4 ± 3.7**^	4.1 ± 4.0*	–	–		–		**−2.6 ± 0.5**	**−4.5 ± 2.1**^	−1.5 ± 1.8^	**−8.0 ± 2.0***
TPP	33%	30%	0.5 ± 2.9^	2.5 ± 2.9*	5.6 ± 3.2#	**−6.8 ± 2.9**^	−0.9 ± 3.0*	**4.1 ± 0.9**	–		–		**1.1 ± 0.4**	**3.0 ± 1.5**^	−1.2 ± 1.3*	**6.6 ± 1.5#**

Abbreviations and units: see Table 1.
[a] Square-root transformed.

interest (Ceriotti et al., 2009). However, few individuals in free-ranging populations are likely to be considered completely disease-free, so reference intervals developed from biased sampling of 'healthy' or captive individuals have little utility for free-ranging populations (Schwacke et al., 2009). For this reason, the occurrence of endemic disease in free-ranging populations should be considered when establishing baseline data to ensure reference intervals reflect the 'normal' characteristics of the sampled population (Pacioni et al., 2013; Hufschmid et al., 2014).

Parturition marks an extreme life-history change, necessitating adaptation to dynamic nutritional and environmental challenges and the development of immunological responses. As such, consideration of the age of sampled pups is also important to appropriately partition the reference population and interpret haematological values. However, as the extended breeding season of the Australian sea lion (Higgins, 1993; McIntosh et al., 2012) precludes the routine collection of known-age pup data or the estimation of pup age from peak parturition dates, as utilised for other otariid species (Richmond et al., 2005; Trillmich et al., 2008), the development of reference intervals for pups partitioned by known-age categories has limited clinical and conservation utility in this species. Conversely, hookworm infection status may be readily determined, is biologically meaningful, and provides a proximate measure of age as the timing of patent hookworm infection (from 11–14 days of age to approximately 2–3 months of age) effectively delimits pups into three age groups (Marcus et al., 2014a). Thus, within the context of endemic hookworm infection, the haematological reference intervals developed in the current study provide a baseline reference for the interpretation of haematological data from individual neonatal Australian sea lion pups and facilitate the monitoring of population-level health trends *via* changes in the temporal proportions of outliers (Lander et al., 2014; see Supplementary Table S1).

Weighted bootstrap estimation techniques were adopted in the current study to provide improved parameter estimation, facilitate the use of repeated measures data, and reduce the number of individuals required for sample collection. The traditional calculation of nonparametric reference intervals assumes sample independence; ignoring the fact that different numbers of measurements were obtained from different individuals can result in the development of incorrect reference intervals (Taylor et al., 1996). For this reason, to account for the correlation between repeated observations, weighted bootstrap estimation was used when developing nonparametric reference intervals to ensure equal contribution from individual pups whilst making efficient use of all available data (Taylor et al., 1996; Alatzas et al., 2014). Pairwise Spearman's rank correlations were estimated similarly. Alternative approaches, namely the exclusion of all but one sampling event per individual or the averaging of repeated measurements, are frequently employed in wildlife research but are relatively inefficient and can lead to the calculation of incorrect estimates (Taylor et al., 1996).

4.2. Investigation of haematological parameter variability

Determining the fundamental causes of variability in the haematological parameters of free-ranging populations is commonly confounded by the dynamic inter-related effects of pathogen, host, and environment factors (Beldomenico et al., 2008; Hufschmid et al., 2014). In the current study, haematological values and patterns of correlation differed for pups with patent or postpatent hookworm infection (Tables 1–3), yet the models attributed only part of this variation to the direct effects of hookworm infection (Tables 4 and 5). It is possible that the intensity of parasitic infection accounts for some of this variation. For example, in harbour seals (*Phoca vitulina*), the intensity of lice (*Echinophthirius horridus*) infestation was negatively correlated with PCV and RBC, however, no significant haematological differences were identified between infected and uninfected seals (Thompson et al., 1998). Similarly, hookworm infection intensity is inversely associated with growth rates of northern fur seal (*Callorhinus ursinus*), New Zealand sea lion (*Phocarctos hookeri*), and Australian sea lion

Table 5
Final linear mixed models of the effect of host-pathogen-environment factors on the variability of neonatal Australian sea lion pup (*Neophoca cinerea*) leucocyte parameters. Interpretation as per Table 4.

			Body condition			Capture-type		Hookworm infection status		Lesion	Standard length	Year of sampling		
Reference level			Poor			Sleeping		Prepatent		Absent	–	2010		
Comparative level			Fair	Good	Excellent	Awake	Mobile	Patent	Postpatent	Present	5 cm increase	2011	2012	2013
Parameter	R^2_m	R^2_c												
Neutrophils[b]	40%	51%	–	–	–	–	–	–	–	**0.24 ± 0.08**	**−0.07 ± 0.02**	**0.63 ± 0.13**^	**0.41 ± 0.11***	**0.86 ± 0.13#**
Lymphocytes[a]	24%	33%	–	–	–	**0.10 ± 0.05**^	**0.17 ± 0.08**^	0.25 ± 0.14^	0.09 ± 0.15*	–	**−0.05 ± 0.02**	**0.28 ± 0.09**^	−0.03 ± 0.08*	−0.05 ± 0.09*
Monocytes[a]	30%	39%	–	–	–	–	–	–	–	–	**−0.04 ± 0.01**	**−0.42 ± 0.05**^	**−0.15 ± 0.04***	0.05 ± 0.05#
Eosinophils[a]	42%	57%	−0.07 ± 0.14^	0.03 ± 0.14*	0.15 ± 0.15*	–	–	**0.34 ± 0.14**^	−0.13 ± 0.15*	–	**−0.09 ± 0.02**	**−0.19 ± 0.08**^	**0.15 ± 0.07***	**−0.53 ± 0.08#**

Abbreviations and units: see Table 1.
[a] Square-root transformed.
[b] Log_e transformed.

pups (Chilvers et al., 2009; DeLong et al., 2009; Marcus et al., 2014a). Hence, variance in haematological parameters related to the intensity of these parasitic infections could have been attributed to interrelated factors such as body condition, moult status, standard length, and the year of sampling, or may have contributed towards the total amount of unexplained variance in the models. In the current study, it was not possible to obtain this data as methods for determining the *in situ* intensity of hookworm infection have not been validated in pinnipeds. Conversely, the intensity of lice infestation may be determined by direct counting, although this was not undertaken in order to reduce individual pup handling time. Thus, because *U. sanguinis* and *A. microchir* infections were measured as categorical factors, their effects on haematological parameters could have been underestimated; further investigations utilising anthelmintics to prevent, reduce, or eliminate parasitic infections may help to refine estimates of their haematological effects (López-Olvera et al., 2006; Castinel, 2007). Regardless, the findings of the current study contribute towards a greater understanding of the pathogen, host, and environment factors that influence the values of haematological parameters in pinniped pups.

4.2.1. Effects of *U. sanguinis* on erythrocyte and TPP values

The presence of *U. sanguinis* infection was significantly associated with anaemia, implicating this parasite as an agent of disease and challenging assumptions about the non-pathological, physiological, nature of neonatal anaemia in pinnipeds. The pattern of anaemia observed in the current study was similar to that considered 'normal' for many pinniped species (Trillmich et al., 2008); Australian sea lion pups approximately two weeks to 2–3 months of age (that is, pups with patent hookworm infection) had lower erythrocyte values (RBC and PCV) than both younger pups with prepatent hookworm infection and older postpatent pups (Table 1). Total plasma protein values followed the same pattern, suggestive of haemorrhage. In contrast to a study of New Zealand sea lion pups in which neonatal anaemia was attributed to physiological causes rather than hookworm (*Uncinaria* sp.) infection (Castinel, 2007), the linear mixed models in the current study indicated that the erythrocyte and TPP changes in Australian sea lion pups were primarily attributable to patent hookworm infection (Table 4), suggestive of the occurrence of significant haemorrhagic anaemia. This is in agreement with earlier investigations in which infections of *Uncinaria lucasi* in northern fur seal pups and *Uncinaria lyonsi* in California sea lion (*Zalophus californianus*) pups were associated with anaemia (Olsen, 1958; Lyons et al., 2001; Kuzmina and Kuzmin, 2015).

The erythroid response to anaemia in Australian sea lion pups was characterised as regenerative for the majority of pups with hookworm infection, indicative of the presence of a pathological process leading to anaemia. In the absence of absolute reticulocyte count data, the erythrocyte changes identified in the current study could be attributed to the strong correlation of age with hookworm infection status (Fig. 1) and would not refute the hypothesis that neonatal anaemia is non-pathological, caused predominantly by physiological responses to host–environment changes. The classification of the erythroid response to anaemia aids in differentiating between pathological and physiological mechanisms; increased numbers of circulating reticulocytes are diagnostic of a regenerative erythroid response to pathological anaemia (Stockham and Scott, 2008). However, there is limited data for reticulocyte counts in pinnipeds and none from pups; in older cohorts of Australian sea lions, fewer than 1% of erythrocytes were identified as reticulocytes, indicating that a normal reticulocyte count in the Australian sea lion is expected to be less than 47.7–60.8 $\times 10^9$/L (Needham et al., 1980). As such, what constitutes an adequate reticulocytosis in pinnipeds is unknown. For this reason, guidelines recommended for dogs were applied in the current study to define a minimum regenerative threshold (reticulocyte count > 65.0 $\times 10^9$/L indicates regeneration; Hodges and Christopher, 2011). Increased nRBC values may also be supportive of a regenerative erythroid response (Jain, 1993); in the current study, pups with patent hookworm infection had significantly higher

nRBC compared to pre-patent and postpatent pups (Tables 1 and 4). Similarly, in anaemic northern fur seal pups infected with *U. lucasi*, increased numbers of nRBC were also observed (Olsen, 1958). Reference values for nRBC have also been reported for harbour seal pups (95% interval 0–8 nRBC/100 leucocytes), harp seal pups (*Phoca groenlandica*; range 0–50 nRBC/100 leucocytes), and hooded seal pups (*Cystophora cristata*; range 1–45 nRBC/100 leucocytes) (Trumble and Castellini, 2002; Boily et al., 2006); unfortunately, significant limitations to the comparative and clinical utility of these values arise due to the paucity of absolute nRBC count data (Allison and Meinkoth, 2007) and data on health status. In Australian sea lion pups with patent hookworm infection, both RET and nRBC were significantly negatively correlated with PCV and RBC; these changes were not observed in postpatent pups. Additionally, MCV values were increased and TPP values were decreased, relative to prepatent and postpatent pups. Overall, these findings are suggestive of a macrocytic regenerative response to hookworm-associated haemorrhage, providing a causative link between hookworm infection and anaemia and further implicating *U. sanguinis* as an important agent of disease in Australian sea lion pups. Hence, the effects of hookworm infection offer an alternative – or concurrent – explanation for the occurrence of neonatal anaemia in Australian sea lion pups to the hypothesis that neonatal anaemia results from physiological responses to non-pathological host–environment changes.

4.2.2. Effects of *A. microchir* on erythrocyte and TPP values

Relative to *U. sanguinis* infection, the presence of *A. microchir* infestation was associated with a smaller decrease in PCV values, no change in RBC, RET, or nRBC values, and an increase in TPP values, indicating that *A. microchir* infestation plays a lesser role in neonatal anaemia, although may contribute towards immunological stimulation or dehydration. Incidentally, the occurrence of lice infestation in pups in the current study (crude cumulative prevalence 79.3%, CI 74.2–83.8%; see Supplementary Table S4) was significantly higher (Fisher's exact test: $P < 0.001$) than that previously reported for Australian sea lion pups (48.9%, CI 34.1–63.9%; McIntosh and Murray, 2007). Differences in methodological approach (among others the calculation of cumulative prevalence *versus* cross-sectional prevalence) account for the higher prevalence observed in the current study. Additionally, the crude cumulative prevalence of lice infestation of pups with patent hookworm infection (81.2%, CI 75.3–86.2%) was significantly higher (Fisher's exact test: $P = 0.029$) than for postpatent pups (68.7%, CI 57.6–78.4%). The evidence that lice can directly cause disease in free-ranging pinnipeds is limited (Thompson et al., 1998), although they are capable of acting as vectors for other pathogens (Jellison and Milner, 1958; Geraci et al., 1981; Linn et al., 2001). Heavy lice infestations can cause pruritus, alopecia, and anaemia, however, they may be acquired secondary to other disease processes causing debilitation (Dailey, 2001), such as hookworm infection. Hence, the current study indicates that *A. microchir* is associated with mild disease and is unlikely to be having a significant impact on the health status of Australian sea lion pups. Further investigation of the epidemiology of *A. microchir* in Australian sea lion pups is necessary to determine the factors that influence the prevalence and intensity of this parasite.

4.2.3. Effects of host factors on erythrocyte and TPP values

As expected, significant increases in PCV, RBC, and TPP values were associated with increases in standard length (Table 4), indicative of recovery from hookworm-associated haemorrhagic anaemia *via* the regenerative erythroid response and progressive 'normalisation' of haematological parameters in older pups. Additionally, increases in TPP values could be explained by increased exposure to antigenic stimuli (and therefore higher globulin values) in older pups, associated with the ontogeny of diving behaviour (Fowler et al., 2007; Brock et al., 2013). Further investigation to characterise changes in plasma protein fractions and their response to disease are required to further elucidate the physiological and pathological mechanisms contributing towards hypo- and hyperproteinaemia (Gray et al., 2005; Schmertmann, 2010).

Pup sex had a small but significant effect on PCV and RBC values (Table 4), a difference not identified in a limited study of Australian sea lions aged 6–23 months (Fowler et al., 2007). Sex-related neonatal erythrocyte differences have been reported in one longitudinal study of Steller sea lions (*Eumetopias jubatus*) in which male pups had significantly lower RBC and significantly higher PCV than female pups, although differences were considered clinically irrelevant (Lander et al., 2014). These differences were not identified in other studies of Steller sea lion pups (Rea et al., 1998; Richmond et al., 2005) nor in investigations of other pinniped pups (Lane et al., 1972; Horning and Trillmich, 1997; Hall, 1998; Beckmen et al., 2003; Castinel, 2007; Trillmich et al., 2008). The underlying mechanisms contributing to these sex-related differences are unclear as hookworm infection intensity does not appear to significantly differ between sexes (Marcus et al., 2014a) and major sex-related differences in physiology and behaviour are not expected in pinniped pups (Greig et al., 2010).

4.2.4. Effects of pathogen and host factors on leucocyte values

Temporal changes in leucocyte parameters in Australian sea lion pups followed the same general pattern described for Steller sea lion and Galapagos sea lion (*Zalophus wollebaeki*) pups, that is, median leucocyte counts were high shortly after birth and decreased with increasing age (Keogh et al., 2010; Brock et al., 2013). This pattern likely reflects a similar developmental history in which immunologically-naïve neonates are exposed to a range of novel environmental antigens and develop endogenous immune responses, resulting in a complex series of correlations between leucocytes (Tables 2 and 3). Neutrophils were the predominant leucocyte cell-type, followed by lymphocytes, eosinophils, and monocytes, the relative proportions of which were similar across all hookworm infection status groups and approximated those reported for older cohorts of Australian sea lions (Needham et al., 1980). Consistent with previous investigations, no basophils were identified in the pup blood smears examined (Needham et al., 1980; Clark et al., 2002; Schmertmann, 2010).

The leucocyte response to hookworm infection was characterised predominantly by a systemic lymphocytosis and eosinophilia (Table 5), reflective of the small-intestinal tissue response identified histologically (Larum, 2010), and was similar to the predominantly eosinophilic response observed in humans and dogs to hookworm infection (Fujiwara et al., 2006). In contrast, lice infestation was not associated with significant effects on leucocyte parameters in the current study. The observed lymphocyte values of Australian sea lion pups with patent hookworm infection (median 3.18×10^9/L, 95% RI $1.44–5.99 \times 10^9$/L) were similar-to-less than those observed in both New Zealand sea lion pups (1–58 days of age) with *Uncinaria* sp. infection (mean 2.32×10^9/L, range $0.21–16.04 \times 10^9$/L) and Steller sea lions pups (<2 months of age) of undetermined health status (median 3.17×10^9/L, 95% RI $1.13–8.99 \times 10^9$/L) (Castinel, 2007; Lander et al., 2014). In contrast, the eosinophil values of Australian sea lion pups (median 1.13×10^9/L, 95% RI 0.05–3.54) were markedly higher than those observed in both New Zealand sea lion pups (mean 0.05×10^9/L, range $0.00–1.46 \times 10^9$/L) and Steller sea lions pups (median 0.36×10^9/L, 95% RI $0.00–1.93 \times 10^9$/L) (Castinel, 2007; Lander et al., 2014). It is unclear whether this difference in eosinophil values is due to inherent immunological host-response differences or reflects the intensity and pathogenicity of hookworm infection in Australian sea lion pups compared to other pinniped hosts. The latter is more likely given the high intensity of infection (Marcus et al., 2014a) and marked intestinal pathology identified on histopathology sections (Larum, 2010). Interestingly, pups in better body condition tended to have higher eosinophil values (as well as higher nRBC and TPP values; see Tables 4 and 5), suggesting that eosinophilic inflammatory responses may have a protective effect as pup body condition is inversely associated with hookworm infection intensity (Marcus et al., 2014a). Further investigation to

determine whether changes in leukocyte values are associated with the intensity and severity of parasitic infections are required to assess whether these immunological-responses are advantageous or deleterious to the host, and their implications for pup survival.

4.2.5. Effects of environment factors on haematological values

The effects of capture and manual restraint on observed haematological values warrant consideration, as the physiological fight-or-flight response can result in leucocytosis, due primarily to shifts in neutrophils, lymphocytes, and monocytes from the marginal pool to the circulating pool (Stockham and Scott, 2008). Additionally, splenic contraction can increase circulating erythrocytes, falsely elevating PCV, RBC, and nRBC values (Castellini et al., 1996; Stockham and Scott, 2008). In the current study, capture-type was identified as a significant factor influencing lymphocyte values, with pups captured whilst awake or mobile demonstrating a relative lymphocytosis compared to pups captured whilst asleep, supportive of a physiological lymphocytosis. Although differential effects of capture-type were not observed for other parameters, it is likely that capture-type was a relatively insensitive proxy of physiological stress levels and that the haematological parameters of all sampled pups were influenced to some degree by the acute effects of capture and manual restraint (Castellini et al., 1996). As such, the reported reference intervals reflect the haematological values of free-ranging manually-restrained neonatal pups; comparisons with captive-animal studies or those that utilise chemical restraint must be undertaken cautiously.

Finally, the relative magnitude and direction of effects attributed to the year of sampling were aligned with seasonal fluctuations in hookworm infection intensity for some haematological parameters. For example, pups demonstrated higher eosinophil counts and lower TPP values during the high-hookworm-infection-intensity season compared to the low-hookworm-infection-intensity season at both colonies (Tables 4 and 5). However, interpretation of these results is confounded as the variance associated with seasonal changes in categorically-scored factors was likely also attributed to the year of sampling (see Section 4.2). For example, the severity of cutaneous ulcerative lesions observed in pups during the Dangerous Reef summer breeding season (2013) was markedly increased compared to the other breeding seasons (unpubl. data), yet the associated variance in haematological parameters would not have been encompassed by the binomial factor 'presence of lesions' and likely was attributed to the year of sampling (see neutrophil count, Table 5). Conversely, and contrary to expectations, year of sampling had no significant effect on PCV, RBC, and RET values (Table 4), although it is possible that these effects were attributed to standard length as fluctuations in hookworm infection intensity are also expected to impact growth rates. The collection of data from additional breeding seasons is required to clarify the role of environmental seasonality and pathogen infection intensity on haematological parameters.

5. Conclusion

This is the first study to report haematological reference intervals for free-ranging neonatal Australian sea lion pups and to describe the effects of pathogen, host, and environment factors on the variability of haematological parameters in this species. *Uncinaria sanguinis* was identified as an important agent of disease for this species, with infection in pups characterised by regenerative anaemia, hypoproteinaemia, and a predominantly lymphocytic–eosinophilic systemic inflammatory response, with effects still evident in some postpatent pups. Conversely, the effects of *A. microchir* were less apparent with infestation unlikely to impact pup health. Importantly, this study demonstrated that anaemia in neonatal Australian sea lion pups is not solely a benign physiological response to host–environment changes, but largely reflects a significant pathological process that adversely impacts pup health. Predominantly benthic foragers, Australian sea lions operate at or near their physiological limits with limited capacity to cope with shifts in resource availability (Fowler et al., 2007; Peters et al., 2014). As such, the effects of *U. sanguinis* on the haematological values of pups might have implications for the development of foraging and diving behaviour, which would subsequently influence the independent survival of juveniles following weaning, significantly impacting the population demography and threatening the viability of this species. The haematological reference intervals developed in this study can facilitate the implementation of long-term health surveillance, which is critical for the early recognition of changes in disease impact and to inform conservation management strategies. The outcomes of this study contribute towards a greater understanding of the dynamic role of pathogen–host–environment relationships in influencing the values of haematological parameters in pinniped pups, whilst highlighting the difficulties associated with inferring cause and effect in free-ranging populations with endemic disease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2015.02.017.

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