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1 **Evaluating non-invasive markers of non-human primate immune activation and**
2 **inflammation**

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17 Keywords: Health, body condition, disease, urine, feces.

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Abstract

Objectives: Health, disease and immune function are key areas of research in studies of ecology and evolution, but work on free-ranging primates has been inhibited by a lack of direct non-invasive measures of condition. Here, we evaluate the potential usefulness of non-invasive measurement of three biomarkers, the acute-phase proteins C-reactive protein (CRP) and haptoglobin, and neopterin, a byproduct of macrophage activity.

Materials and Methods: We took advantage of veterinary checks on captive rhesus (24) and long-tailed (3) macaques at the German Primate Center (DPZ) to compare serum marker measures, before measuring concentrations in feces and urine, and evaluating relationships between matched serum, urine and fecal concentrations. In a second study, we monitored excretion of these markers in response to simian immunodeficiency virus (SIV) infection and surgical tissue trauma, undertaken for a separate study.

Results: We found that each biomarker could be measured in each matrix. Serum and urinary concentrations of neopterin were strongly and significantly correlated, but neither haptoglobin nor CRP concentrations in excreta proxied circulating serum concentrations. Our infection study confirmed that urinary neopterin in particular is a reliable marker of viral infection in macaques, but also indicated the potential of urinary and fecal CRP and haptoglobin as indicators of inflammation.

Discussion: We highlight the potential of noninvasive markers of immune function, especially of urinary neopterin, which correlates strongly with serum neopterin, and is highly responsive to infection.

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43 **Introduction**

44 Health in general, and immune function in particular, are key areas of both applied and
45 basic research in the study of ecology and evolution (Kappeler & Nunn 2015). Areas of
46 research that include immune function as central elements include primate disease ecology
47 (e.g. Nunn, 2006, 2012), , MHC function and its role in pathogen responsiveness
48 (Schwensow et al., 2007) and mate choice (e.g. Schwensow et al., 2008), and the
49 importance of environmental and social stress and its effects on health and disease (e.g.
50 Gordis et al., 2008; Jemmott et al., 1988; Cavigelli and Chaudry 2012). Though the
51 assessment of immune function and activation is of great relevance for many studies, it has
52 proven difficult to measure in studies of large-bodied free-ranging mammals, where it is
53 often not possible to trap individuals for the collection of blood.

54 In recent decades, the non-invasive measurement of physiological parameters has
55 revolutionized studies of captive and free-ranging mammals, allowing unprecedented
56 investigation of the proximate factors mediating behavioral and life history variation. Such
57 techniques are particularly commonly used in larger-bodied animals such as elephants and
58 non-human primates. Established examples include the measurement of steroid hormones
59 (see Wheaton et al. 2011, for a review) as well as proteins and peptides, such as
60 concentrations of urinary C-peptide of insulin (Sherry and Ellison, 2007). One element of
61 physiology that is usually missing from field studies is a direct measure of infection or
62 immune activation. Physical health has instead been commonly assessed by using visual
63 estimates of physical condition, for example the estimation of body fat (e.g. Berman and
64 Schwarz, 1988; Koenig et al., 1997), and wounds (e.g. Archie et al., 2012), or by the
65 quantification of fecal parasite load (e.g. Gillespie et al., 2005; Gillespie and Chapman, 2006;

Weyher et al., 2006). Although useful, these measures are crude and only indirectly (if at all) reflect the immune status of an individual, and measures such as inter-individual differences in macroparasite loads measured at individual timepoints can be particularly hard to interpret and misleading with respect to aspects of immunity (e.g. Habig and Archie 2015). As such, new non-invasive markers of immune activity and health would be highly valuable.

In the present study, we investigate several non-invasive (urinary and fecal) markers of immune responses that might potentially be useful to assess individual health in field studies of non-human primates. We focus specifically on macaques (where much work on non-invasive physiological assessment has been undertaken, e.g. Engelhardt et al., 2004, 2005; Brauch et al., 2008; Heistermann et al., 2006; Girard-Buttoz et al., 2009, 2011; Ostner et al., 2008; Higham et al., 2011a, 2013). Potential markers of the inflammatory immune response include cytokines and chemokines (e.g. urinary IL-8, IL-6; serum values of such cytokines have recently been published from free-ranging rhesus macaques, Hoffman et al., 2011), acute phase proteins, and surrogate markers of immune responses. We chose three markers for further investigation. The first two of these are the acute phase proteins C-reactive protein (CRP) and haptoglobin, which are secreted by the liver in response to most forms of tissue damage, infection, inflammation and neoplasia. They are therefore useful nonspecific biochemical inflammatory markers (Pepys and Hirschfield, 2003; Gabay and Kushner, 1999). An acute phase protein is defined as a protein that responds to inflammation with a change in concentration of at least 25% (Gabay and Kushner, 1999), but responses are usually much more substantial. In humans for example, CRP can increase in response to inflammation by more than 1000% (Gabay and Kushner, 1999; Pepys and Hirschfield, 2003) and in dogs CRP increases markedly (up to 45 fold) in response to surgery

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89 (Yamamoto et al., 1993; Michelsen et al., 2012). Increased expression of such acute phase
90 proteins is often associated with long-term chronic health consequences (e.g. CRP and
91 cardiovascular disease, Ridker et al. 2000). The third biomarker we assessed was neopterin,
92 which is a byproduct of macrophage activity upon stimulation by γ -interferon secretion from
93 activated T-lymphocytes, and is regarded as an early marker of the Th1 response of cell-
94 mediated immunity (Widner et al., 2000). Apart from the general availability of assays to
95 measure these analytes in biological samples of primates, the fact that they are broadly
96 implicated in many immune responses and are not related to any specific infection makes
97 them highly suitable for primate field studies, where researchers will very rarely know the
98 precise infection or disease that the animals are suffering from.

99 In addition, these markers are commonly measured in blood and used in studies of
100 infection and disease in humans (neopterin, Plata-Nazar et al., 2010, Rho et al., 2011; CRP,
101 Rudzite et al. 2003), but also in macaques (e.g. neopterin, Heyes et al., 1991; CRP, Hart et
102 al., 1998; Jinbo et al., 1998, 1999; Klingstroem et al., 2002), and in other mammals including
103 mice (CRP; Huntoon et al., 2008), dogs (CRP; Yamamoto et al., 1993), pigs (CRP; Breineková
104 et al., 2007) and other livestock (haptoglobin and CRP, Peterson et al., 2004). They have also
105 been measured in excretory products (urine and feces) of humans and have been utilized as
106 non-invasive markers of infection and immune activation, including in studies of intestinal
107 infection, inflammation and macrophage activity (fecal neopterin, Ledjeff et al., 2001;
108 Campbell et al., 2004; urinary and fecal neopterin, Husain et al., 2013), intestinal health
109 (fecal haptoglobin; Matsumoto et al., 2001), general immune status (urinary neopterin,
110 Baydar et al., 2011) and gynecological cancer (urinary neopterin, Melichar et al., 2006). In
111 such cases they may not be measured because excreta concentrations indicate systemic

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3 112 infectious status, but because they are indicative of more specific local infections in tissues
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5 113 related to urinary or fecal excretion pathways, such as the kidneys and the gut. Finally, some
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7 114 have been investigated and/or utilized as non-invasive markers of immune function in non-
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9 115 human animals, including primates. For example, urinary neopterin has been used to
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11 116 monitor simian immunodeficiency virus (SIV) infection in rhesus macaques (Fendrich et al.,
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13 117 1989; Stahl-Hennig et al., 2002), while urinary neopterin (Amann et al., 2001) and salivary
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15 118 haptoglobin and CRP (Gómez-Laguna et al., 2010) have been used to document immune
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17 119 activation and monitor herd health in pigs.
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23 120 To assess the validity of measurements of these immune markers in non-invasive
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25 121 samples (urine, feces) of macaques, we took two approaches. In study 1, we took advantage
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27 122 of the regular health monitoring that is undertaken on macaques at the German Primate
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29 123 Center to obtain temporally-matched blood, fecal and urine samples from non-infected
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31 124 individuals. Using these samples, we assessed relationships between serum and urinary, and
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33 125 serum and fecal, concentrations of each marker to determine whether these correlate, and
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35 126 hence whether the non-invasive measures might serve as proxy for the serum measures,
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37 127 and also whether both non-invasive measures might be equally suitable proxies.
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43 128 In study 2, we took advantage of a SIV infection experiment in combination with
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45 129 medical interventions and surgery in six rhesus macaques (carried out as part of a separate
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47 130 study by the German Primate Center's Unit of Infection Models), to assess the response
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49 131 patterns of the three immune markers in urine and feces to infection and surgery. In
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51 132 contrast to the cross-sectional correlative data collected from healthy animals, this
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53 133 experimental approach should provide more direct information on the potential usefulness
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55 134 of each marker in each matrix for assessing macaque immune activation and inflammatory
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135 responses. Collectively, our analyses represent an initial assessment of the feasibility of
136 measuring these markers in primate excreta, provide baseline data for levels of these
137 markers in healthy animals, and assess their usefulness in reflecting immune activation and
138 inflammation in response to an experimentally induced acute infection and surgical tissue
139 trauma.

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141 **Methods**

142 Research Ethics

143 All samples were collected during health checks of the macaque colony (e.g. annual health
144 check) or when animals were already immobilized for other purposes. Samples were
145 collected according to the ASAB/ABS guidelines on the ethical treatment of animals, and the
146 International Primatological Society guidelines on the ethical treatment of primates in
147 research. Urine and fecal samples collected from the SIV-infected animals were all collected
148 non-invasively without animal handling.

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150 Study animals and sample collection

151 Study 1: Measurement of immune markers in healthy macaques

152 This study was conducted between Aug 2011 and Mar 2012 on 24 rhesus macaques (18
153 males, 6 non-pregnant females) and 3 male long-tailed macaques, which were housed at the
154 German Primate Centre, Göttingen, Germany. Animals ranged in age between 3 and 11
155 years, with an average age (\pm SEM) of 7.4 ± 0.5 years. Average body weight was 7.6 ± 0.4 kg

(males: 6.7 ± 0.5 kg; females: 10.3 ± 0.5 kg; overall range: 4.6-11.7 kg). Individuals were housed either as same-sex pairs or in small same-sex groups in indoor cages and were fed twice a day with commercial monkey chow supplemented with fruits and vegetables. Water was available ad libitum.

From each study animal, matching urine, fecal and blood samples were collected between 6.00 and 10.00 am for the measurement of neopterin (NEO), C-reactive protein (CRP), and haptoglobin (HPT) concentrations as well as for the determination of hematological parameters. At the time of sample collection, all animals were in good body condition (mean BMI: 27.4 ± 1.1 ; range 20.8-45.0), visually healthy and showed no obvious signs of any disease, except for one male who exhibited diarrhea. Veterinarians made the decision to euthanize this animal 3 weeks after sample collection due to severe gut problems and substantial weight loss. For urine and fecal sample collection, a study animal was usually separated from its group members in the early morning (6.00 - 6.30 am) and samples were collected upon urination and defecation on a plastic mat placed underneath the cage. Only urine and fecal samples not obviously cross-contaminated with each other were collected. Urine samples were immediately protected from light. For blood collection, animals were subsequently (between 8.30 and 10.00 a.m. the same day) anesthetized with an intra-muscular injection of ketamine hydrochloride (10mg/kg; Ketavet®). A blood sample (4-8 ml) was drawn from the femoral vein of the animal and collected into a heparinized tube. All samples were kept cold (4° - 7° C) upon collection and transferred to the endocrinology laboratory within 4 hours of collection for further processing. Blood samples were centrifuged at 1800 g for 10 min and plasma subsequently recovered and aliquoted. Fresh fecal samples were well mixed using a spatula and from each sample two aliquots of

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179 0.1 to 0.2 g were accurately weighted into 15 ml polypropylene tubes for future extraction.

180 Urine samples were also aliquoted, and all aliquots of each sample type were then stored

181 frozen at -20°C until analysis.

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183 Study 2: Measurement of immune markers in response to SIV-infection

184 This study was undertaken between February and April 2014 on 6 rhesus macaques (3

185 males, 3 females) which were infected with SIV as part of a separate study undertaken by

186 the German Primate Center’s Unit of Infection Models. Animals ranged in age between 4

187 and 5 years, with an average age (\pm SEM) of 4.6 ± 0.2 years. Average body weight was

188 5.6 ± 0.3 kg (males: 5.7 ± 0.2 kg; females: 5.5 ± 0.6 kg; overall range: 4.4-6.4 kg). Body weight

189 of individuals fluctuated by less than 1% during the study period. The study was approved

190 by the Lower Saxony State Office for Consumer Protection and Food Safety and performed

191 with the project license 33.9-42502-04-12/0758-08. For infection, which required a deeper

192 anesthesia, animals received a mixture of ketamine, xylazine and atropine. Each monkey

193 was inoculated with 50% 1000 tissue culture infectious doses of the virus intravenously. The

194 infection was confirmed by determining plasma viral RNA load.

195 During the experiment animals were subject to minor medical interventions, such as bone

196 marrow aspiration and colon biopsies (all under anesthesia). They also underwent (together

197 with bone marrow aspiration and colon biopsy) one surgical removal of peripheral lymph

198 nodes two weeks post infection. In particular the latter likely involved surgical tissue trauma

199 which is known to result in an acute phase protein response (e.g. Yamamoto et al. 1993;

200 Michelsen et al. 2012). This situation thus provided a useful test case for assessing the

201 potential of the urinary and fecal CRP and haptoglobin measurements in indicating
202 inflammatory processes.

203 Urine and fecal samples for immune marker measurements were collected once
204 weekly for 4 weeks prior to virus inoculation and at least 3 times a week for 31 days
205 thereafter. Samples were collected, processed and stored as described for study 1.

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207 Sample measurement for immune marker analysis

208 Plasma, urine and fecal samples were analyzed for concentrations of NEO, CRP and HPT
209 using commercial enzyme-immunoassay (ELISA) kits (see below). While plasma and urine
210 samples were taken unextracted to assay following appropriate dilution with assay buffer
211 (NEO) or sample diluent (CRP, HPT) provided with the respective kits, fecal samples had to
212 be extracted prior to analysis. For NEO, the extraction followed the procedure described by
213 Campbell et al. (2004) with small modifications. Specifically, fecal aliquots were thawed at
214 room temperature and one ml of 0.9% saline was added to all samples which were then
215 agitated for 10 min on a multi-tube vortexer. Samples were then centrifuged at 1800 g for
216 15 min and the supernatant recovered for analysis. Extraction of the two acute phase
217 proteins was carried out according to a protocol provided by Immundiagnostic AG,
218 Bensheim, Germany. Specifically, defrosted fecal samples were mixed with 1 ml of CRP
219 washing buffer and agitated for 10 min on a multi-tube vortexer. Samples were then
220 centrifuged at 1800 g for 15 min, the supernatant transferred into a 1.5ml polypropylene
221 tube, and centrifuged at 7500 g rpm for 5 min. 100 µl of the resulting supernatant was then
222 taken to CRP and HPT analysis. In order to compensate for the potential effect of differences

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223 in water content of fecal samples on immune marker concentrations, following extraction
224 fecal dry weights for each sample were determined by drying samples in an oven at 50°C to
225 a constant weight. Fecal concentrations of each marker are expressed as ng per g of dried
226 feces (Campbell et al. 2004). Concentrations of urinary analytes were indexed by urinary
227 creatinine, measured as described (Bahr et al. 2000).

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229 Laboratory Analyses

230 NEO concentrations were determined using a human ELISA kit (Art. No. RE59321) from IBL
231 International GmbH, Hamburg, Germany. The assay was performed according to the
232 manufacturer’s instructions. While plasma and fecal extracts were assayed undiluted, urine
233 samples were diluted 1:10 – 1:100 with assay buffer to bring sample concentrations into the
234 working range of the assay. For Study 2, prior to ELISA analysis (see above) urine samples
235 were initially measured via HPLC (Schroecksnadel et al., 2006) (data not shown). This
236 enabled us to reduce analytical costs by restricting our ELISA analysis to the most important
237 samples as indicated by the HPLC data. NEO measures generated by ELISA vs HPLC were
238 strongly and highly significantly correlated with an r-value of 0.96 (n=84, p<0.001).
239 Detection limit of the ELISA assay was 0.18 ng/ml. Inter-assay coefficients of variation,
240 determined by repeated measurement of high and low value quality controls in each assay
241 and across studies, were 12.0% and 6.6%, respectively.

242 All CRP and HPT measurements were carried out using ELISA kits for monkey CRP
243 (Cat. No. 2210-4) and monkey haptoglobin (Cat. No. 2410-5) from Life Diagnostics, Inc.,
244 West Chester, USA. Both assays were performed according to the manufacturer’s

instructions. For both assays, fecal extracts were taken undiluted to assay, except for two samples which were diluted 1:10 for HPT. While urine samples were usually diluted 1:2 for both assays, plasma samples were normally diluted 1:1,000 for CRP measurements and 1:100,000 for HPT determinations. Detection limits of the assays were 1.17 ng/ml for CRP and 1.56 ng/ml for HPT and inter-assay coefficients of variation of a high and low concentrated quality control were 9.6% and 10.3% for CRP and 8.4% and 9.6% for HPT. All measures of intra- and inter-assay variation were within accepted norms.

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253 Statistical Analyses

To assess potential sex or age effects on immune marker concentrations we examined serum levels of the three immune markers in the Study 1 animals. One animal that was known to be sick (n=1) was excluded from this analysis in order to remove any effects of this individual on age or sex differences. Residual values of parametric analyses did not meet model assumptions even if dependent variables were log-transformed, as determined by inspection of residual QQ plots. Visual inspection reveals the distributions of several variables to be non-normally distributed, but as expected for markers that show huge responsiveness to infection/inflammation, exhibiting numerous similar lower values but with occasional much higher values. We undertook univariate general linear model (GLM) analyses on each serum marker separately (fixed factor, sex; covariate, age) so that both variables could be assessed in the same model. However, we also tested each variable separately using non-parametric statistics (Mann Whitney U test, Spearman's rank correlation), and present these results in addition where they differ from those of the parametric statistics.

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268 We used bivariate correlations to assess serum to urinary and serum to fecal
relationships for each marker. Variables were not normally distributed, and this was still the
case even after log-transformation. We therefore undertook non-parametric Spearman’s
rank correlations throughout. Sample sizes sometimes change slightly between analyses as
in one or two cases there was insufficient urine volume to measure all variables. Tests were
one-tailed as we clearly predicted a positive correlation between these variables.

274 In order to present descriptive statistics for the magnitude of biomarker responses
to SIV infection (NEO) and surgical trauma (CRP, HPT) in Study 2 animals, we determined for
both urine and feces the peak-to-baseline ratios of each marker (for males and females
separately, and combined). For calculating baseline values we took the period prior to SIV
infection up to 3 days thereafter when biomarker levels were still unaffected by the
treatment (see Results). We examined whether the acute phase proteins (CRP and HPT) in
the Study 2 animals increased in response to surgical trauma by comparing urinary and fecal
CRP and HPT concentrations in the period within 6 days before versus 6 days after the
surgery for lymph node extirpation using the Wilcoxon signed rank test (due to the small
sample size).

284 Probability values < 0.05 were considered statistically significant. As our aim was to
discover whether markers were measurable and potentially useful and informative in
different matrices, we considered our analyses exploratory rather than definitive and did
not correct for multiple testing.

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290 Results

291 Study 1

292 Concentrations of all markers in each matrix are presented in Table 1. Values for the 3 long-
 293 tailed macaques fell within the range of those of the rhesus macaques. There were no sex
 294 differences in serum levels for any of the three immune markers when analyzed using GLMs
 295 also containing age as a variable (NEO: $F_{1,25} = 0.094$, $p = 0.763$; CRP: $F_{1,24} = 0.027$, $p = 0.871$;
 296 HPT: $F_{1,25} = 0.179$, $p = 0.676$). While we found no effects of age on serum concentrations when
 297 corrected for sex using GLMs (NEO, $F_{1,25} = 3.297$, $p = 0.082$; CRP, $F_{1,24} = 1.167$, $p = 0.292$; HPT,
 298 $F_{1,25} = 0.473$, $p = 0.498$), Spearman's rank correlations showed significant correlations for NEO
 299 ($r_s = 0.513$, $n = 26$, $p = 0.007$) and CRP ($r_s = 0.498$, $n = 26$, $p = 0.010$), with older individuals having
 300 higher concentrations of both markers.

301

302 Serum–urinary and serum–fecal correlations

303 *Neopterin*: Serum NEO concentrations were strongly and significantly correlated with
 304 urinary ($r_s = 0.664$, $n = 27$, $p < 0.001$; Fig. 1) but not fecal ($r_s = 0.171$, $n = 27$, $p = 0.196$)
 305 concentrations.

306 *CRP*: Serum CRP concentrations were not correlated with either urinary ($r_s = -0.037$, $n = 26$,
 307 $p = 0.429$) or fecal CRP measures ($r_s = -0.003$, $n = 27$, $p = 0.493$).

308 *Haptoglobin*: Serum and urinary HPT concentrations were not correlated ($r = 0.264$, $n = 26$,
 309 $p = 0.096$). HPT levels in fecal samples were either low or below the detection limit of the
 310 assay.

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311 *Study 2*

312 Generally, for the 3 males and 3 females used in this study baseline values of all three
313 immune markers (calculated for the period pre-treatment up to 3 days after infection) in
314 both urine and feces (Table 2) were in the same range exhibited by the healthy animals of
315 study 1.

316 *Neopterin*: Consistent with prior studies, urinary NEO showed a strong response to SIV
317 infection (Figure 2). Values began to elevate from around one week post-infection, and
318 typically rose to around 10-25 times baseline levels around day 15 which coincided with
319 peak viremia. NEO concentrations typically remained elevated for several weeks, though
320 concentrations greater than 10 times baseline were only seen for around a week. Small
321 spikes in fecal values around this time were inconsistent in their duration and timing. Given
322 the vast differences in concentration of NEO detected in feces vs urine (peak levels per ml
323 urine are about 100 fold higher than baseline fecal levels per g feces; data not shown) , this
324 is likely due to occasional small (drop-sized) contamination of fecal samples with urine.

325 *CRP*: Urinary and fecal CRP excretion patterns showed rises and falls in concentrations that
326 were not obviously related to the timing of the SIV infection event (Figure 2). In the majority
327 of animals (4/6) there was on average however an approximately 2.5 fold elevation in CRP
328 levels in both urine and feces in the days immediately following lymph node
329 extirpation/intestinal biopsy sampling compared to the days prior to surgery (Figures 3 and
330 5). Although this elevation was short-lived, lasting for a couple of days at most (see Figure
331 3), it nonetheless represented a statistically significant increase in both matrices (Figure 5;
332 urine: $z = 1.992$, $p = 0.023$; feces: $z = 1.887$, $p = 0.030$).

333 *Haptoglobin*: Urinary and fecal HPT excretion usually remained consistently low throughout
 334 most of the experimental period. In the majority of animals (5/6) however, an increase was
 335 recorded in levels of urinary HPT in the periods following first bone marrow aspiration, and
 336 in particular in response to the surgery for lymph node extirpation/intestinal biopsy
 337 sampling (Figures 4 and 5). As for CRP, the elevation in levels following surgery was short-
 338 lived but statistically significant ($z = 1.739$, $p = 0.037$). The rise in HPT levels following lymph
 339 node extirpation/intestinal biopsy sampling was also recorded in fecal samples where it was
 340 much more marked though (Figure 5; $z = 2.201$, $p = 0.014$).

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342 Discussion

343 Our study sought to assess whether several markers of health and immune activity could be
 344 measured non-invasively in non-human primates, and to see how these responded to
 345 medical intervention and infection. Our results demonstrate that it is possible to do this
 346 reliably, and provide baseline data on values of these markers in blood, urine and feces for
 347 visually healthy captive macaques. Our data also show a significant positive correlation
 348 between blood and urinary concentrations of neopterin, further highlighting its potential as
 349 non-invasive markers of changes in circulating blood concentrations. Moreover, consistent
 350 with studies in the pathology literature, tracking of individuals through medical
 351 interventions and following SIV infection shows urinary neopterin to be a highly reliable
 352 marker of infection, with a 10-25 fold increase in excretion in response to SIV infection.
 353 Urinary and fecal levels of the two acute phase proteins did not correlate significantly with
 354 serum values, suggesting that they may be of limited applicability for assessing lower level
 355 inflammation. However, our data do suggest that urinary and fecal CRP and (especially)

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356 haptoglobin may nevertheless be useful non-invasive markers of inflammation given their
357 significant, although short-lived, elevation in response to surgical tissue trauma.

358 Urinary neopterin concentrations correlate positively and significantly with those
359 found in serum (Figure 1), and respond consistently to SIV infection (Figure 2), a finding in
360 line with earlier studies (Fendrich et al., 1989; Stahl-Hennig, 2002). Measurement of this
361 marker in urine from free-ranging macaques is likely to reveal the presence of infections
362 associated with macrophage activation and the Th 1 response, which promote cellular
363 immunity in response to intracellular pathogens (e.g bacteria, viruses, fungi or parasites;
364 Elenkov and Chrousos, 1999). Regular measurement might allow the development of such
365 an infection to be tracked. Though this requires regular sampling, such sampling regimes
366 are a common requirement for other markers too. For example, the tracking of ovulation
367 through the measurement of estrogen and progesterone metabolites excreted in feces
368 and/or urine also requires frequent sampling (Hodges and Heistermann 2011). Studies of
369 free-ranging primates often assess the onset of the luteal phase of the cycle through the
370 detection of increased progesterone concentrations greater than 2 SDs above the previous 3-
371 5 baseline (follicular phase) values, and maintained for at least 3 consecutive samples
372 (following Jeffcoate 1983). Similar assessment criteria might be used for urinary neopterin
373 to determine whether an infection has occurred.

374 Although fecal neopterin concentrations did not correlate with serum or urinary
375 concentrations in our study and, in contrast to urinary neopterin, did not show a consistent
376 response to SIV infection, they are sometimes used not as a general method for measuring
377 infection in the body, but specifically as a measure of inflammatory gut disease and
378 infections in humans (Ledjeff et al., 2001; Campbell et al., 2004; Husain et al., 2013). It still

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3 379 therefore retains potential as a method of testing for intestinal macrophage activity in non-
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5 380 human primates. Data are required in which fecal neopterin concentrations can be
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8 381 compared for healthy individuals and individuals known to have inflammatory gut infections
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10 382 (e.g. see Husain et al. 2013 for humans), or on the same individuals from periods of both gut
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12 383 infection and health.

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15 384 Urinary and fecal measures did not correlate with serum values for either CRP or
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18 385 haptoglobin. It is worth considering that we might expect correlations between
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20 386 concentrations of analytes in blood and urine rather than in feces. Both blood and urine
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22 387 concentrations represent relatively short-term measures, with excretion times usually much
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24 388 quicker for urine than feces (Hodges and Heistermann, 2011), making it more likely that the
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26 389 former would correlate with measures in blood. In contrast, concentrations in fecal samples
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28 390 represent the integration of circulating levels over longer periods, and so may not
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30 391 necessarily be expected to correlate with levels found in blood when analyzing cross-
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32 392 sectional data. Hence, the lack of a correlation between fecal (as well as urinary) and serum
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34 393 CRP and haptoglobin levels might reflect the rapid and extreme changes in this acute phase
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36 394 protein during a response which renders it highly unlikely that a snapshot measure such as
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38 395 serum values corresponds to more long-term measures (see also Touma and Palme, 2005).
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40 396 It is important also to remember that our sample size in Study 1 of 27 animals is relatively
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42 397 small, and as all animals were healthy, this might have reduced variation in the dataset
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44 398 hindering our ability to detect significant correlations. Standardization for creatinine may
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46 399 also add variation to urinary measures given differences in weight of our study animals
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48 400 (Crockett et al. 1993). That said, known relationships such as that between serum and
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50 401 urinary NEO were clearly demonstrated using our sample, indicating that our power was
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402 sufficient to find such relationships where they exist and are strong. In general though, it is
403 also worth remembering that in humans these markers are also measured in feces and urine
404 rather than blood despite the easy availability of the latter specifically because fecal and
405 urinary measurements are indicative of disease and infection in tissues associated with
406 excretion pathways, such as the kidneys and the gut, rather than of general systemic
407 infection. It may therefore be no surprise that correlations between serum, urine and fecal
408 measures were not found.

409 Although urinary haptoglobin concentrations did not correlate with those in serum,
410 the potential usefulness of urinary haptoglobin measurements for monitoring inflammatory
411 processes is nonetheless suggested by our finding of markedly elevated levels in response to
412 bone marrow aspiration and lymph node extirpation. In particular, the surgery for lymph
413 node extirpation is likely to have resulted in tissue trauma, which is known to stimulate an
414 increase in acute phase protein secretion (Yamamoto et al. 1993; Michelsen et al. 2012).
415 Haptoglobin concentrations in blood increase in response to infections associated with
416 inflammation, but typically show a broader and less acute response curve when compared
417 to other acute phase proteins such as CRP (Gabay and Kushner, 1999). Regular
418 measurements of urinary haptoglobin might therefore potentially allow inflammatory
419 infections to be detected and monitored in wild mammals, particularly as haptoglobin
420 shows a relatively long release function in response to infection (Gabay and Kushner, 1999).

421 Fecal haptoglobin levels showed a similar response to surgery, with elevations even
422 more pronounced than those found in urine. Some limited evidence also emerged from
423 animals of study 1 to suggest that high levels of fecal haptoglobin excretion may be
424 indicative of health issues. Within the cohort of healthy individuals in three animals ≥ 15

425 times higher concentrations were found compared with the rest of the animals, with one
 426 animal showing an extreme value of >10,000 ng/g (~200 fold elevation above average).
 427 Interviewing the animal keepers and the vet and looking at animal history reports revealed
 428 that in the past these three animals have exhibited symptoms of gut problems, such as
 429 diarrhea or Giardia infection, relatively often. The individual with the highest fecal
 430 haptoglobin level also showed markedly elevated concentrations in fecal and serum CRP
 431 (both 5-fold above the study sample mean) and serum and urinary neopterin (3-fold and 2-
 432 fold above the mean, respectively) as well as serum haptoglobin (2 fold above the mean).
 433 This animal was the individual confirmed to be suffering from severe diarrhea and weight
 434 loss during the time of sample collection (see Methods). Information on the gut status of
 435 the two other animals with elevated haptoglobin levels in feces was not available, but
 436 visually they appeared to be healthy (e.g. no diarrhea) when samples were collected. Taken
 437 together, our results are tentative but promising, and suggest that measurement of
 438 haptoglobin in urine and, in particular, feces may have potential for tracking both more
 439 systemic as well as local inflammatory processes in macaques non-invasively. Since the
 440 responses found were short-lived (lasting a few days at most), frequent sampling would be
 441 necessary to detect acute occurrences of inflammation reliably.

442 Similarly to haptoglobin, we found elevated urinary and fecal CRP concentrations in
 443 response to the surgical tissue trauma associated with lymph node extirpation. This also
 444 suggests that non-invasive measure of CRP may be of potential value for tracking
 445 inflammation in macaques. In contrast to haptoglobin however, CRP excretion patterns
 446 were overall more variable, and the rise in fecal CRP in response to surgery was markedly
 447 weaker than that for haptoglobin.

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448 Before further studies seek to utilize these or any other markers, it will also be
449 important to investigate their stability under conditions of contamination with dirt or (in the
450 case of urine) feces, as well as issues related to how they must be stored and transported.
451 When careful analyses of such issues are undertaken, detailed recommendations can then
452 be made to fieldworkers on how to collect, store and transport samples for analysis in a way
453 that minimizes analyte contamination and degradation (see Higham et al., 2011b for
454 macaque C-peptides). In addition to urinary and fecal markers, some studies may also wish
455 to consider measuring relevant analytes from saliva. Methods for saliva collection from
456 primates have been used in free-ranging settings (Higham et al., 2010), and similar or
457 adapted methods are probably feasible for numerous (though clearly not all) primate
458 species in free-ranging populations. In saliva, many native analytes can be measured,
459 including sympathetic axis correlates such as alpha-amylase (e.g. rhesus macaques, Higham
460 et al., 2010; bonobos, Beringer et al., 2012), haptoglobin (e.g. pigs, Gómez-Laguna et al.,
461 2010) and CRP (e.g. humans, Rao et al., 2010).

462 There have been several recent and exciting developments in evolutionary studies of
463 primate immune function, including publications showing that high-ranking baboon males
464 heal faster than low-ranking males (Archie et al., 2012), and that rhesus macaque females
465 experimentally assigned low ranks show increased immune marker and receptor gene
466 expression (Tung et al., 2012). Hopefully, our study will encourage further investigations of
467 the non-invasive measurement of immune function. As methods that enable multiple
468 measurement of many analytes from the same sample become more reliable and
469 widespread (e.g. Hauser et al., 2011; Weltring et al., 2012), the direct measurement of
470 multiple markers may hopefully become more common-place. Multi-assays are now

471 available that simultaneously measure up to 20 different cytokines and chemokines in non-
 472 human primate blood samples (Giavedoni, 2005). Such methods offer great promise,
 473 particularly if they can be applied to non-invasive samples such as urine. We therefore
 474 encourage further evaluations and validations of non-invasive markers in the area of
 475 immune activation and primate health. The development and validation of more non-
 476 invasive immune markers is likely to expand our ability to investigate primate behavior,
 477 ecology and evolution considerably. Such measures will prove crucial to establishing the
 478 physiological links connecting variation in behavioral strategies to long-term life-history
 479 outcomes such as mortality, so linking the “short-term behavioral study” and “long-term
 480 demographic study” elements of primatology.

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704 **Figure Legends**

705 Figure 1. The relationship between values of serum and urinary neopterin.

706 Figure 2. Patterns of urinary and fecal concentrations of neopterin (NEO) in the 6 animals

707 infected with SIV. Note the different scales.

708 Figure 3. Patterns of urinary and fecal excretion of C-reactive protein (CRP) in the 6 animals

709 infected with SIV. Arrows 1-3 indicate the date of SIV infection (1), first bone marrow

710 aspiration (2) and surgery for lymph node extirpation combined with second bone marrow

711 aspiration and colon biopsy (3). Note the different scales.

712 Figure 4. Patterns of urinary and fecal excretion of haptoglobin (HPT) in the 6 animals

713 infected with SIV. Arrows 1-3 indicate the timing of SIV infection (1), first bone marrow

714 aspiration (2) and surgery for lymph node extirpation combined with second bone marrow

715 aspiration and colon biopsy (3). Note the different scales.

716 Figure 5. Concentrations of (A) urinary and fecal C-reactive protein (CRP) and (B) urinary and

717 fecal haptoglobin (HPT) in samples collected within 6 days before and 6 days after surgery

718 for lymph node extirpation/intestinal biopsy sampling. Bars represent mean + SEM values.

719 Differences were statistically significant in all cases (see text).

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Table 1. Concentrations of markers measured in Study 1, from 23 rhesus macaques and 3 long-tailed macaques (age 7.4 ± 0.5 (SEM) years, range = 3-11 ys). Body weights were 7.6 ± 0.4 kg (range = 4.6–11.7 kg), and BMIs were 27.4 ± 1.1 (range = 20.8–45.0).

Marker	Matrix		Mean	SEM	Range
NEO	Serum	Males	1.4	0.2	0.6-3.1
		Females	2.0	0.2	1.5-2.8
		All	1.6	0.1	0.6-3.1
	Urine	Males	171.9	16.0	84.0-366.7
		Females	185.4	26.0	92.9-265.6
		All	175.0	13.6	84.0-366.7
	Feces	Males	46.9	7.6	19.3-145.3
		Females	58.4	19.7	21.0-145.8
		All	49.6	7.3	19.3-145.8
CRP	Serum	Males	4.7	1.2	1.2-26.3
		Females	6.5	2.1	1.5-13.0
		All	5.1	1.0	1.2-26.3
	Urine	Males	22.4	6.8	1.0-94.3
		Females	63.8	34.5	5.2-220.0
		All	32.3	10.0	1.0-220.0
	Feces	Males	123.1	31.1	31.0-604.2
		Females	74.6	12.9	36.9-152.8
		All	111.9	24.4	31.0-604.2
HPT	Serum	Males	816.0	74.0	170-1260
		Females	641.7	150.4	190-1100
		All	775.8	66.9	170-1260
	Urine	Males	37.9	7.0	8.9-102.4
		Females	258.0	143.8	22.3-935.7
		All	90.7	37.8	8.9-935.7
	Feces	Not measureable in most samples from healthy individuals.			

Serum concentrations are given in ng/ml (NEO) or $\mu\text{g/ml}$ (CRP and haptoglobin)

All urinary concentrations are given as ng/mg Cr

All fecal concentrations are given as ng/g dry weight

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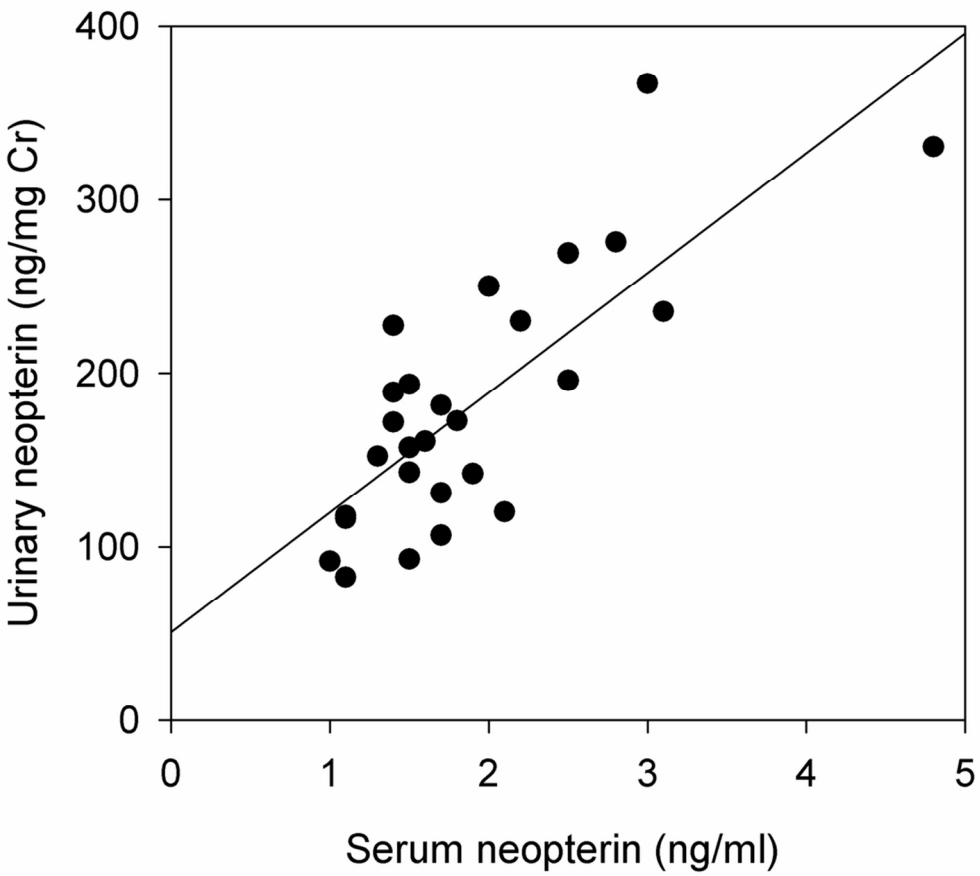
Table 2. Baseline concentrations of biomarkers and ranges of peak-to-baseline (P/B) ratios measured in Study 2 animals

Marker	Matrix		Mean ± SEM	Range P/B-ratio
NEO	Urine	Males	145.8 ± 30.1	16.7-25.8
		Females	171.6 ± 13.7	10.8-26.7
		All	158.7 ± 15.9	10.8-26.7
	Feces	Males	74.4 ± 4.9	n.a.
		Females	53.2 ± 4.8	n.a.
		All	63.8 ± 5.6	n.a.
CRP	Urine	Males	30.8 ± 15.5	1.8-23.3
		Females	47.7 ± 21.4	3.1-6.5
		All	39.3 ± 12.4	1.8-23.3
	Feces	Males	123.6 ± 4.0	5.7-14.2
		Females	102.2 ± 21.8	1.3-6.4
		All	112.9 ± 11.0	1.3-14.2
HPT	Urine	Males	35.7 ± 2.1	3.2-16.0
		Females	74.6 ± 20.3	2.3-43.1
		All	55.1 ± 12.6	2.3-43.1
	Feces	Males	221.4 ± 105.4	11.7-74.6
		Females	103.0 ± 19.7	16.2-105.7
		All	162.2 ± 54.8	11.7-105.7

n.a. = not applicable (see Results)

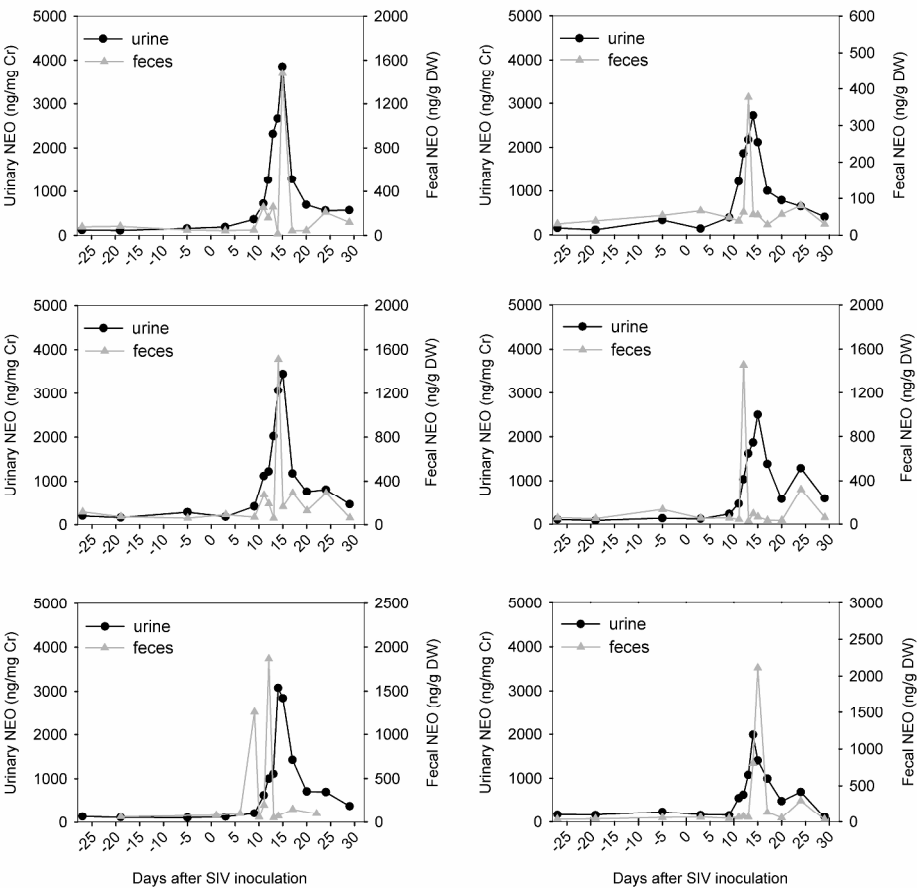
All urinary concentrations are given as ng/mg Cr

All fecal concentrations are given as ng/g dry weight

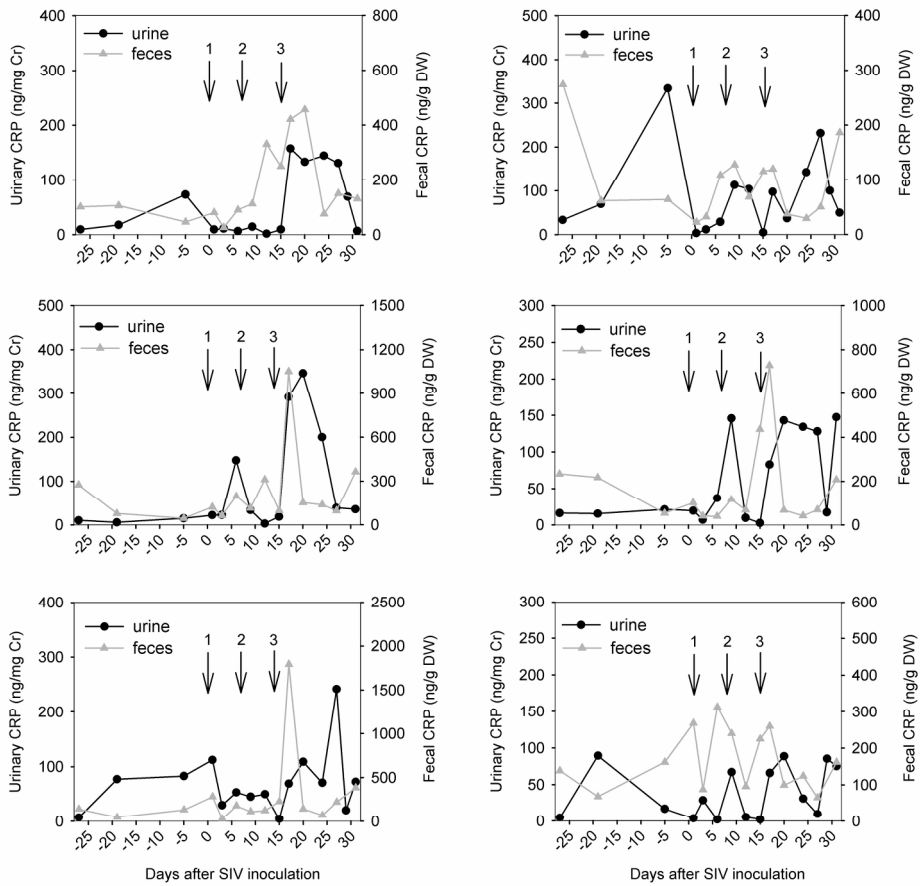


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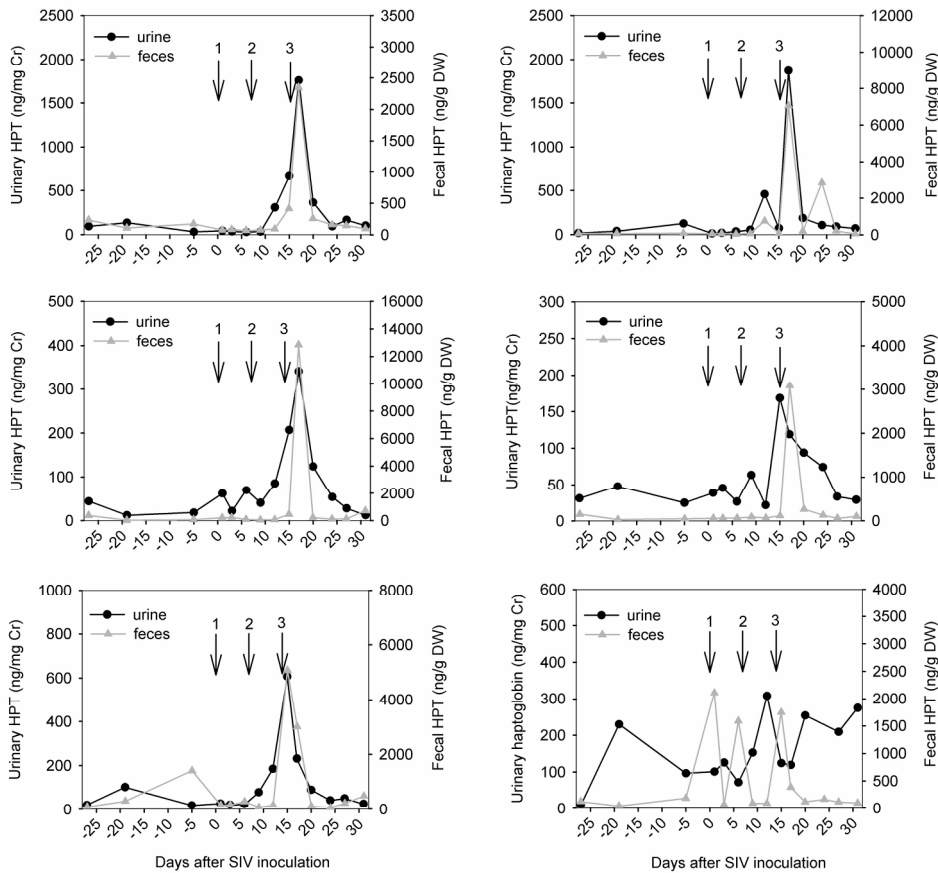


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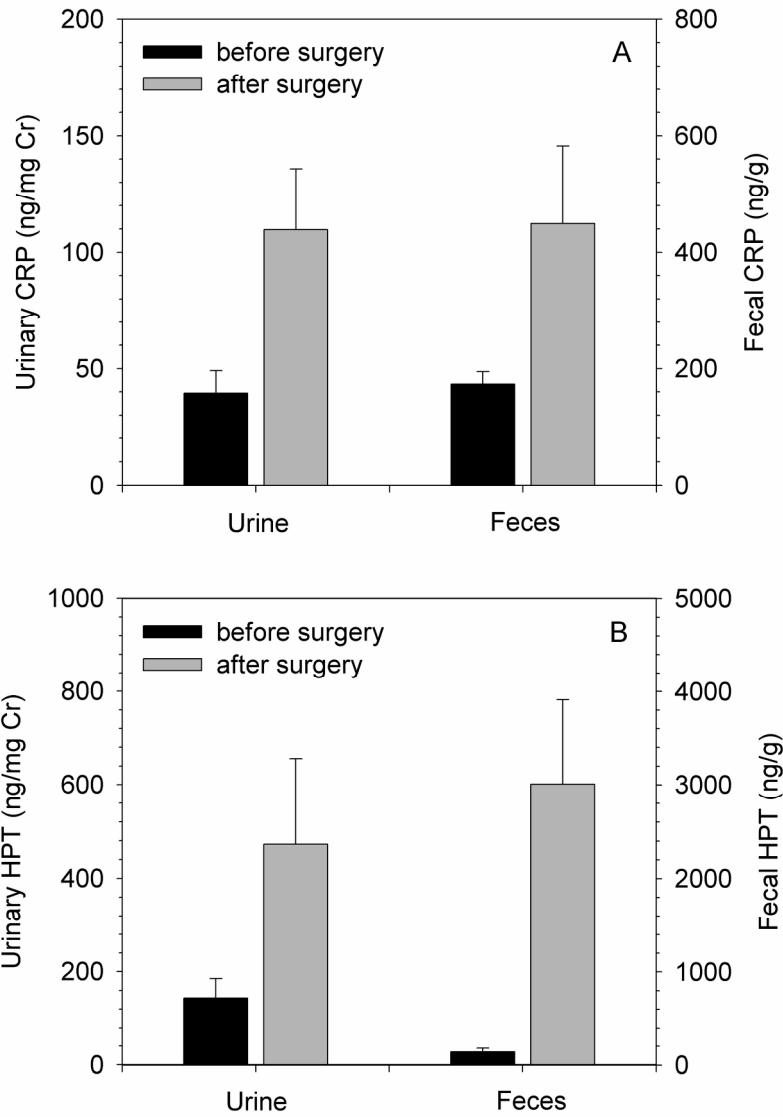


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