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

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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.

Introduction

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

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

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

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

infectious status, but because they are indicative of more specific local infections in tissues related to urinary or fecal excretion pathways, such as the kidneys and the gut. Finally, some have been investigated and/or utilized as non-invasive markers of immune function in non-human animals, including primates. For example, urinary neopterin has been used to monitor simian immunodeficiency virus (SIV) infection in rhesus macaques (Fendrich et al., 1989; Stahl-Hennig et al., 2002), while urinary neopterin (Amann et al., 2001) and salivary haptoglobin and CRP (Gómez-Laguna et al., 2010) have been used to document immune activation and monitor herd health in pigs.

To assess the validity of measurements of these immune markers in non-invasive samples (urine, feces) of macaques, we took two approaches. In study 1, we took advantage of the regular health monitoring that is undertaken on macaques at the German Primate Center to obtain temporally-matched blood, fecal and urine samples from non-infected individuals. Using these samples, we assessed relationships between serum and urinary, and serum and fecal, concentrations of each marker to determine whether these correlate, and hence whether the non-invasive measures might serve as proxy for the serum measures, and also whether both non-invasive measures might be equally suitable proxies.

In study 2, we took advantage of a SIV infection experiment in combination with medical interventions and surgery in six rhesus macaques (carried out as part of a separate study by the German Primate Center´s Unit of Infection Models), to assess the response patterns of the three immune markers in urine and feces to infection and surgery. In contrast to the cross-sectional correlative data collected from healthy animals, this experimental approach should provide more direct information on the potential usefulness of each marker in each matrix for assessing macaque immune activation and inflammatory

responses. Collectively, our analyses represent an initial assessment of the feasibility of measuring these markers in primate excreta, provide baseline data for levels of these markers in healthy animals, and assess their usefulness in reflecting immune activation and inflammation in response to an experimentally induced acute infection and surgical tissue trauma.

Methods

Research Ethics

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

Study animals and sample collection

Study 1: Measurement of immune markers in healthy macaques

This study was conducted between Aug 2011 and Mar 2012 on 24 rhesus macaques (18 males, 6 non-pregnant females) and 3 male long-tailed macaques, which were housed at the German Primate Centre, Göttingen, Germany. Animals ranged in age between 3 and 11 years, with an average age (± 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

0.1 to 0.2 g were accurately weighted into 15 ml polypropylene tubes for future extraction. Urine samples were also aliquoted, and all aliquots of each sample type were then stored frozen at -20°C until analysis.

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

This study was undertaken between February and April 2014 on 6 rhesus macaques (3 males, 3 females) which were infected with SIV as part of a separate study undertaken by the German Primate Center's Unit of Infection Models. Animals ranged in age between 4 and 5 years, with an average age (± SEM) of 4.6 ±0.2 years. Average body weight was 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 of individuals fluctuated by less than 1% during the study period. The study was approved by the Lower Saxony State Office for Consumer Protection and Food Safety and performed with the project license 33.9-42502-04-12/0758-08. For infection, which required a deeper anesthesia, animals received a mixture of ketamine, xylazine and atropine. Each monkey was inoculated with 50% 1000 tissue culture infectious doses of the virus intravenously. The infection was confirmed by determining plasma viral RNA load.

During the experiment animals were subject to minor medical interventions, such as bone marrow aspiration and colon biopsies (all under anesthesia). They also underwent (together with bone marrow aspiration and colon biopsy) one surgical removal of peripheral lymph nodes two weeks post infection. In particular the latter likely involved surgical tissue trauma which is known to result in an acute phase protein response (e.g. Yamamoto et al. 1993; Michelsen et al. 2012). This situation thus provided a useful test case for assessing the

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

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

Sample measurement for immune marker analysis

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

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

Laboratory Analyses

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

All CRP and HPT measurements were carried out using ELISA kits for monkey CRP (Cat. No. 2210-4) and monkey haptoglobin (Cat. No. 2410-5) from Life Diagnostics, Inc., 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.

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.

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.

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).

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.

Results

Study 1

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

Serum–urinary and serum–fecal correlations

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

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

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

Study 2

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

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

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

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

Discussion

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

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

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

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

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

Urinary and fecal measures did not correlate with serum values for either CRP or haptoglobin. It is worth considering that we might expect correlations between concentrations of analytes in blood and urine rather than in feces. Both blood and urine concentrations represent relatively short-term measures, with excretion times usually much quicker for urine than feces (Hodges and Heistermann, 2011), making it more likely that the former would correlate with measures in blood. In contrast, concentrations in fecal samples represent the integration of circulating levels over longer periods, and so may not necessarily be expected to correlate with levels found in blood when analyzing cross-sectional data. Hence, the lack of a correlation between fecal (as well as urinary) and serum CRP and haptoglobin levels might reflect the rapid and extreme changes in this acute phase protein during a response which renders it highly unlikely that a snapshot measure such as serum values corresponds to more long-term measures (see also Touma and Palme, 2005). It is important also to remember that our sample size in Study 1 of 27 animals is relatively small, and as all animals were healthy, this might have reduced variation in the dataset hindering our ability to detect significant correlations. Standardization for creatinine may also add variation to urinary measures given differences in weight of our study animals (Crockett et al. 1993). That said, known relationships such as that between serum and urinary NEO were clearly demonstrated using our sample, indicating that our power was

sufficient to find such relationships where they exist and are strong. In general though, it is also worth remembering that in humans these markers are also measured in feces and urine rather than blood despite the easy availability of the latter specifically because fecal and urinary measurements are indicative of disease and infection in tissues associated with excretion pathways, such as the kidneys and the gut, rather than of general systemic infection. It may therefore be no surprise that correlations between serum, urine and fecal measures were not found.

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

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

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

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

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

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

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

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Figure Legends

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

Figure 2. Patterns of urinary and fecal concentrations of neopterin (NEO) in the 6 animals infected with SIV. Note the different scales.

Figure 3. Patterns of urinary and fecal excretion of C-reactive protein (CRP) in the 6 animals infected with SIV. Arrows 1-3 indicate the date of SIV infection (1), first bone marrow aspiration (2) and surgery for lymph node extirpation combined with second bone marrow aspiration and colon biopsy (3). Note the different scales.

Figure 4. Patterns of urinary and fecal excretion of haptoglobin (HPT) in the 6 animals infected with SIV. Arrows 1-3 indicate the timing of SIV infection (1), first bone marrow aspiration (2) and surgery for lymph node extirpation combined with second bone marrow aspiration and colon biopsy (3). Note the different scales.

Figure 5. Concentrations of (A) urinary and fecal C-reactive protein (CRP) and (B) urinary and fecal haptoglobin (HPT) in samples collected within 6 days before and 6 days after surgery for lymph node extirpation/intestinal biopsy sampling. Bars represent mean + SEM values. Differences were statistically significant in all cases (see text).

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 µg/ml (CRP and haptoglobin)

All urinary concentrations are given as ng/mg Cr

All fecal concentrations are given as ng/g dry weight

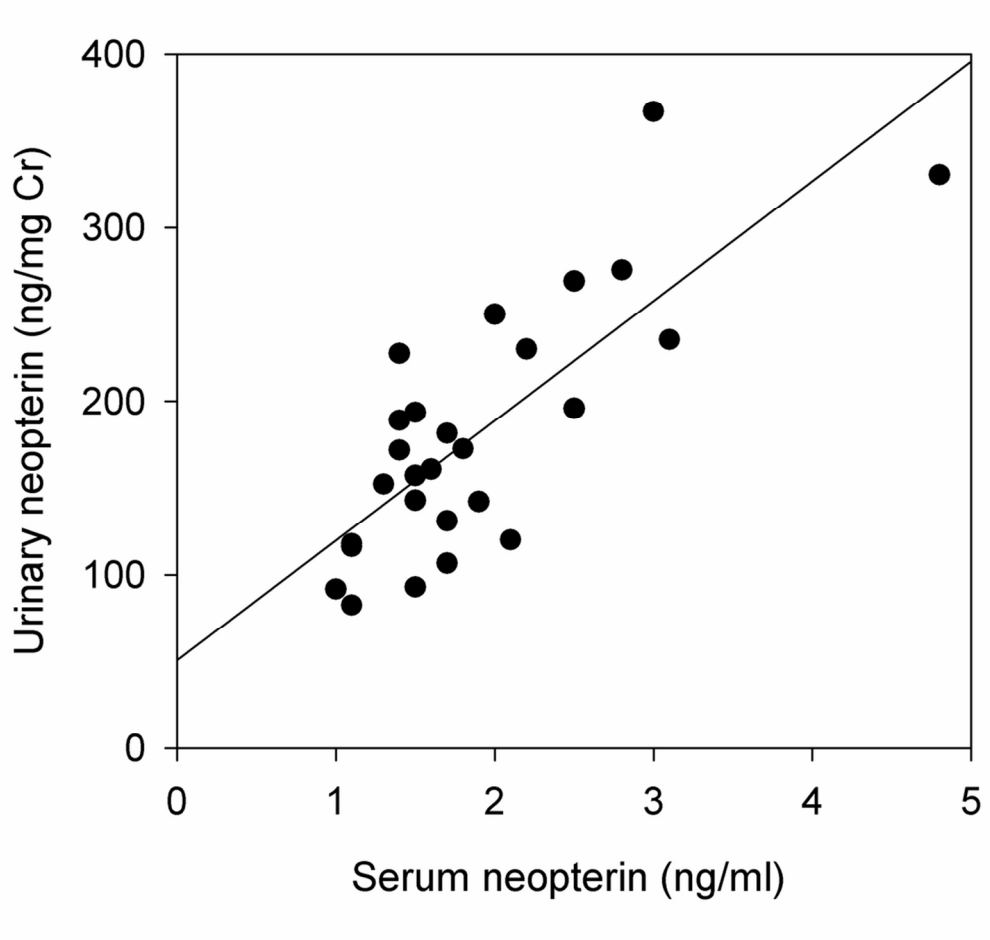
Table 2. Baseline concentrations of biomarkers and ranges of peak-to-baseline (P/B) ratios measured in Study 2 animals

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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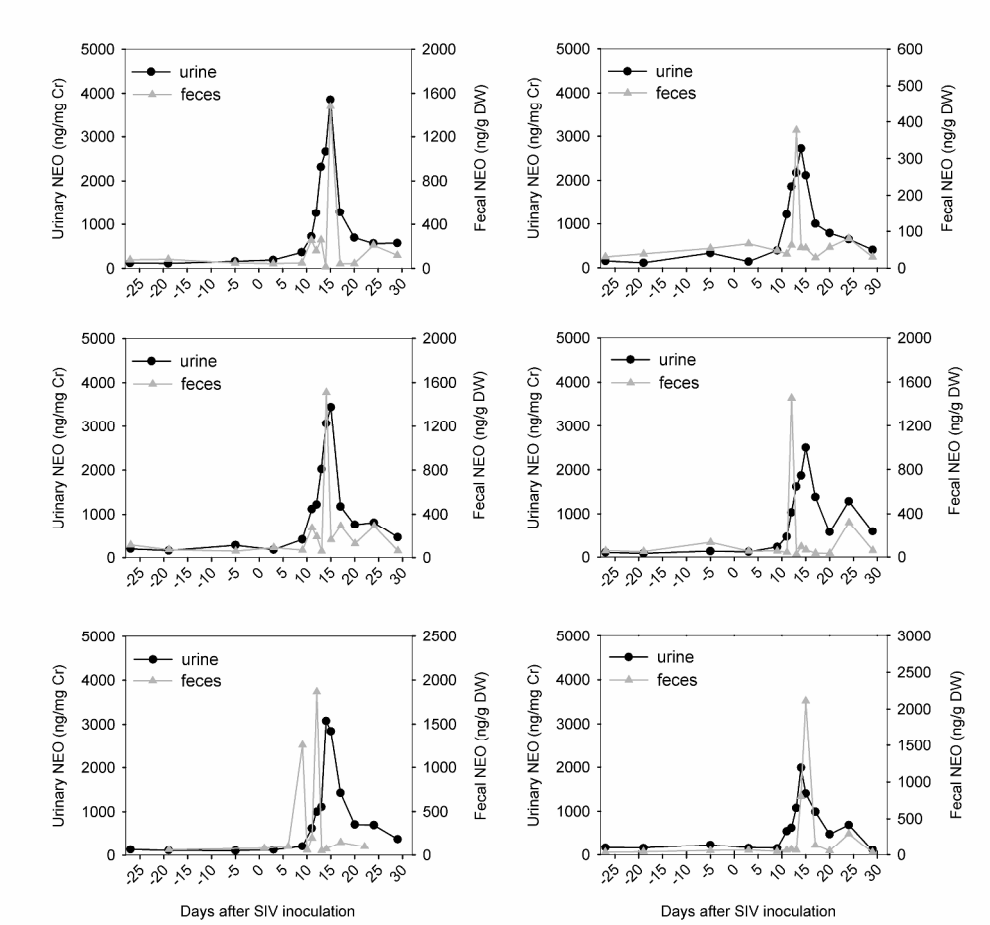

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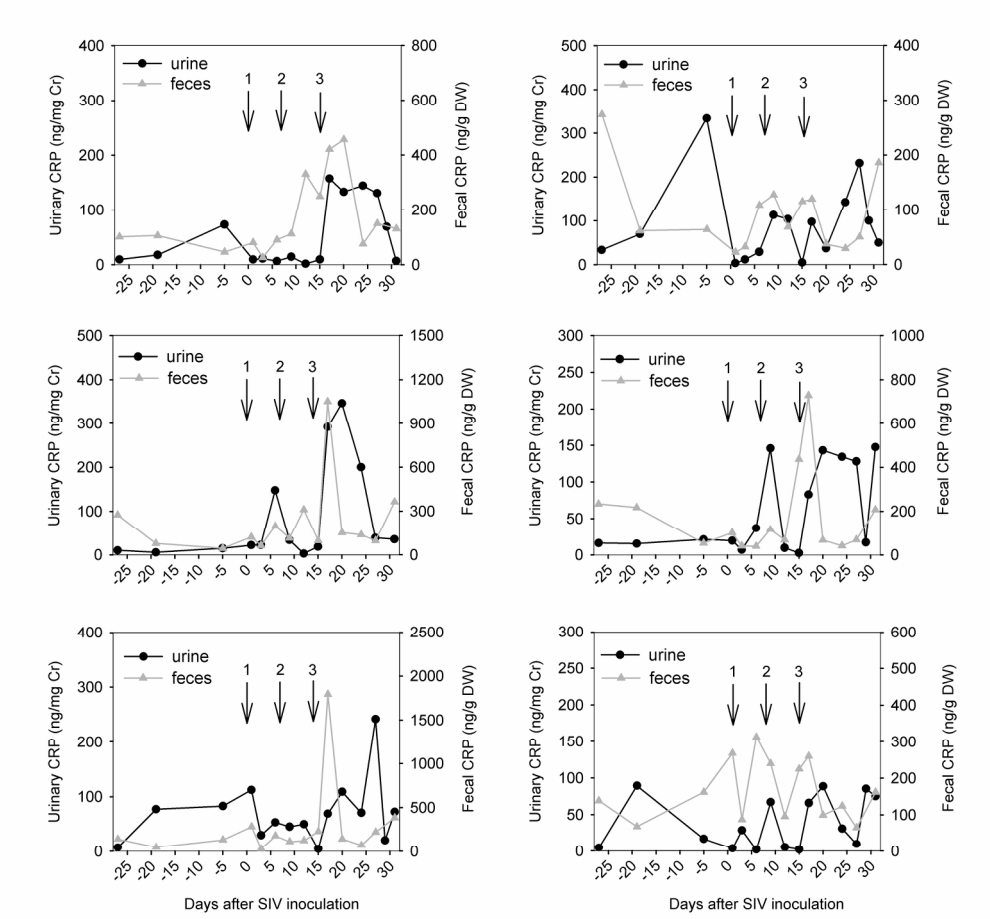

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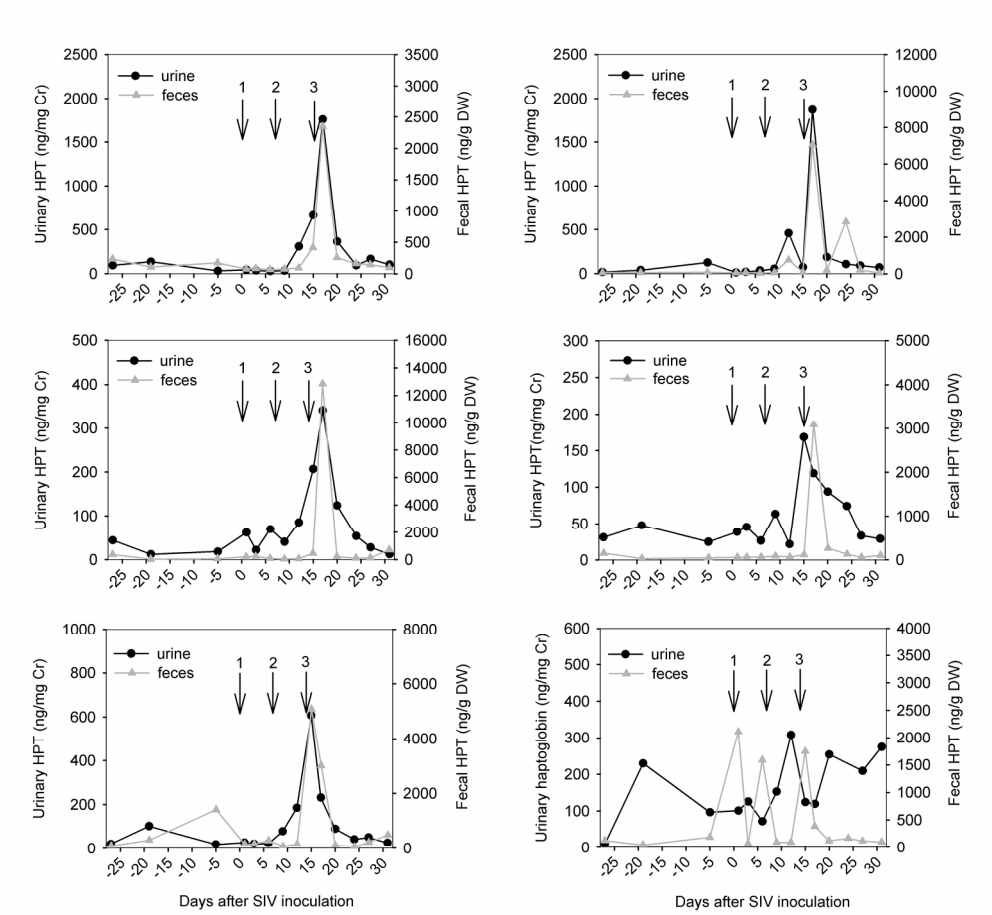

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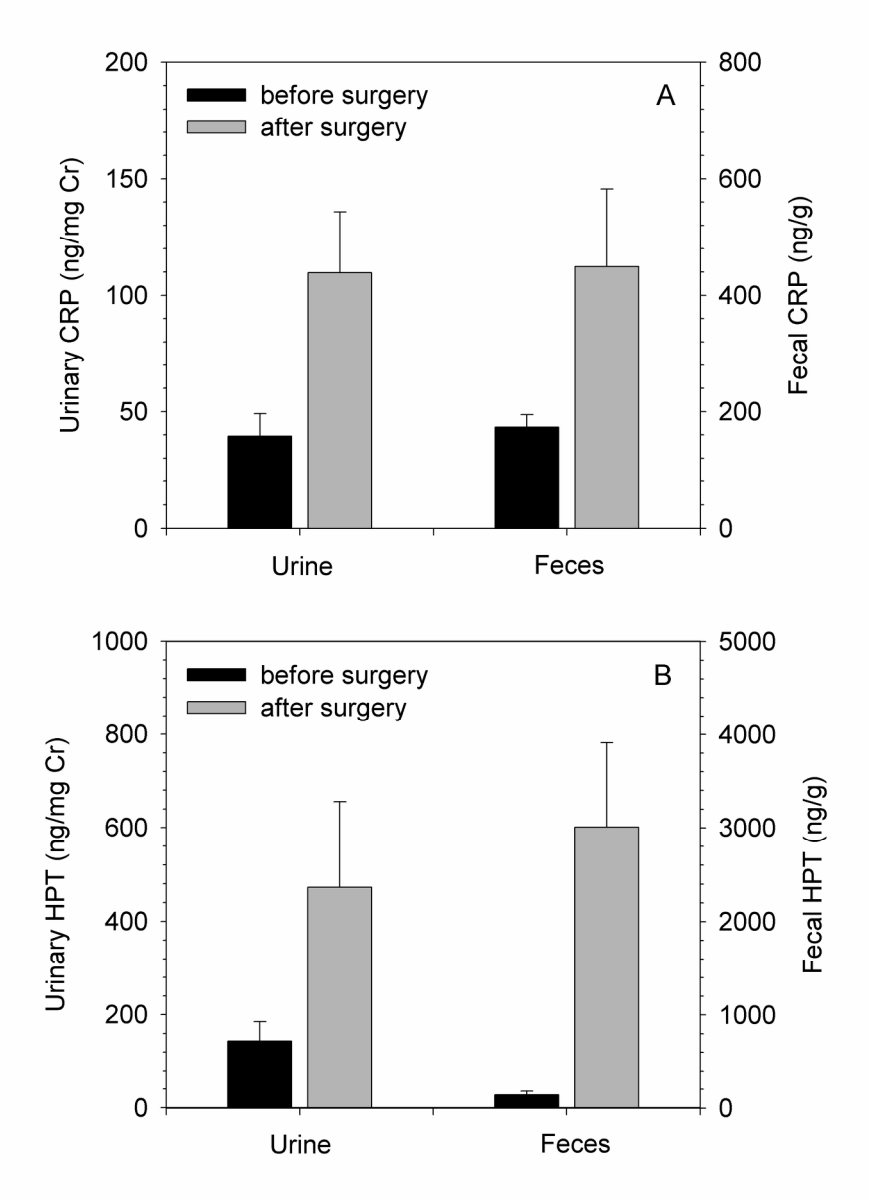

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