

1
2
3 1 **Evaluating non-invasive markers of non-human primate immune activation and**
4
5 2 **inflammation**
6
7

8 3 James P. Higham^{1,2*}, Cornelia Kraus³, Christiane Stahl-Hennig⁴, Antje Engelhardt², Dietmar
9 4 Fuchs⁵, Michael Heistermann⁶
10

11 5 1 Dept. of Anthropology, New York University, 25 Waverly Place, New York, NY 10003
12

13 6 2 Jr Research Group “Sexual Selection”, German Primate Center, Kellnerweg 4, 37077
14 7 Göttingen
15

16 8 3 Department of Sociobiology/Anthropology, University of Göttingen, Kellnerweg 6, 37077
17 9 Göttingen, Germany
18

19 10 4 Unit of Infection Models, German Primate Center, Kellnerweg 4, 37077 Göttingen
20

21 11 5 Division of Biological Chemistry, Biocenter Innsbruck Medical University, Center for
22 12 Chemistry and Biomedicine, Innrain 80, 6020 Innsbruck, Austria
23

24 13 6 Endocrinology Laboratory, German Primate Center, Kellnerweg 4, 37077 Göttingen
25

26 14
27

28 15
29

30 16 Abbreviated running title: Non-invasive markers of immune activation
31

32 17 Keywords: Health, body condition, disease, urine, feces.
33

34 18 *Address for correspondence: Department of Anthropology, New York University, 25
35

36 19 Waverly Place, New York, NY 10003, USA
37

38 20 jhigham@nyu.edu
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 21 **Abstract**
4
5

6 22 Objectives: Health, disease and immune function are key areas of research in studies of
7
8 23 ecology and evolution, but work on free-ranging primates has been inhibited by a lack of
9
10 24 direct non-invasive measures of condition. Here, we evaluate the potential usefulness of
11
12 25 non-invasive measurement of three biomarkers, the acute-phase proteins C-reactive protein
13
14 26 (CRP) and haptoglobin, and neopterin, a byproduct of macrophage activity.
15
16
17

18 27 Materials and Methods: We took advantage of veterinary checks on captive rhesus (24) and
19
20 28 long-tailed (3) macaques at the German Primate Center (DPZ) to compare serum marker
21
22 29 measures, before measuring concentrations in feces and urine, and evaluating relationships
23
24 30 between matched serum, urine and fecal concentrations. In a second study, we monitored
25
26 31 excretion of these markers in response to simian immunodeficiency virus (SIV) infection and
27
28 32 surgical tissue trauma, undertaken for a separate study.
29
30
31
32

33 33 Results: We found that each biomarker could be measured in each matrix. Serum and
34
35 34 urinary concentrations of neopterin were strongly and significantly correlated, but neither
36
37 35 haptoglobin nor CRP concentrations in excreta proxied circulating serum concentrations.
38
39 36 Our infection study confirmed that urinary neopterin in particular is a reliable marker of
40
41 37 viral infection in macaques, but also indicated the potential of urinary and fecal CRP and
42
43 38 haptoglobin as indicators of inflammation.
44
45
46
47

48 39 Discussion: We highlight the potential of noninvasive markers of immune function,
49
50 40 especially of urinary neopterin, which correlates strongly with serum neopterin, and is
51
52 41 highly responsive to infection.
53
54
55

56 42
57
58
59
60

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;

1
2
3 66 Weyher et al., 2006). Although useful, these measures are crude and only indirectly (if at all)
4
5 67 reflect the immune status of an individual, and measures such as inter-individual differences
6
7 68 in macroparasite loads measured at individual timepoints can be particularly hard to
8
9
10 69 interpret and misleading with respect to aspects of immunity (e.g. Habig and Archie 2015).
11
12 70 As such, new non-invasive markers of immune activity and health would be highly valuable.
13
14

15
16 71 In the present study, we investigate several non-invasive (urinary and fecal) markers
17
18 72 of immune responses that might potentially be useful to assess individual health in field
19
20 73 studies of non-human primates. We focus specifically on macaques (where much work on
21
22 74 non-invasive physiological assessment has been undertaken, e.g. Engelhardt et al., 2004,
23
24 75 2005; Brauch et al., 2008; Heistermann et al., 2006; Girard-Buttoz et al., 2009, 2011; Ostner
25
26 76 et al., 2008; Higham et al., 2011a, 2013). Potential markers of the inflammatory immune
27
28 77 response include cytokines and chemokines (e.g. urinary IL-8, IL-6; serum values of such
29
30 78 cytokines have recently been published from free-ranging rhesus macaques, Hoffman et al.,
31
32 79 2011), acute phase proteins, and surrogate markers of immune responses. We chose three
33
34 80 markers for further investigation. The first two of these are the acute phase proteins C-
35
36 81 reactive protein (CRP) and haptoglobin, which are secreted by the liver in response to most
37
38 82 forms of tissue damage, infection, inflammation and neoplasia. They are therefore useful
39
40 83 nonspecific biochemical inflammatory markers (Pepys and Hirschfield, 2003; Gabay and
41
42 84 Kushner, 1999). An acute phase protein is defined as a protein that responds to
43
44 85 inflammation with a change in concentration of at least 25% (Gabay and Kushner, 1999), but
45
46 86 responses are usually much more substantial. In humans for example, CRP can increase in
47
48 87 response to inflammation by more than 1000% (Gabay and Kushner, 1999; Pepys and
49
50 88 Hirschfield, 2003) and in dogs CRP increases markedly (up to 45 fold) in response to surgery
51
52
53
54
55
56
57
58
59
60

1
2
3 89 (Yamamoto et al., 1993; Michelsen et al., 2012). Increased expression of such acute phase
4
5 90 proteins is often associated with long-term chronic health consequences (e.g. CRP and
6
7
8 91 cardiovascular disease, Ridker et al. 2000). The third biomarker we assessed was neopterin,
9
10 92 which is a byproduct of macrophage activity upon stimulation by γ -interferon secretion from
11
12 93 activated T-lymphocytes, and is regarded as an early marker of the Th1 response of cell-
13
14 94 mediated immunity (Widner et al., 2000). Apart from the general availability of assays to
15
16 95 measure these analytes in biological samples of primates, the fact that they are broadly
17
18 96 implicated in many immune responses and are not related to any specific infection makes
19
20 97 them highly suitable for primate field studies, where researchers will very rarely know the
21
22 98 precise infection or disease that the animals are suffering from.
23
24
25
26

27 99 In addition, these markers are commonly measured in blood and used in studies of
28
29 100 infection and disease in humans (neopterin, Plata-Nazar et al., 2010, Rho et al., 2011; CRP,
30
31 101 Rudzite et al. 2003), but also in macaques (e.g. neopterin, Heyes et al., 1991; CRP, Hart et
32
33 102 al., 1998; Jinbo et al., 1998, 1999; Klingstroem et al., 2002), and in other mammals including
34
35 103 mice (CRP; Huntoon et al., 2008), dogs (CRP; Yamamoto et al., 1993), pigs (CRP; Breineková
36
37 104 et al., 2007) and other livestock (haptoglobin and CRP, Peterson et al., 2004). They have also
38
39 105 been measured in excretory products (urine and feces) of humans and have been utilized as
40
41 106 non-invasive markers of infection and immune activation, including in studies of intestinal
42
43 107 infection, inflammation and macrophage activity (fecal neopterin, Ledjeff et al., 2001;
44
45 108 Campbell et al., 2004; urinary and fecal neopterin, Husain et al., 2013), intestinal health
46
47 109 (fecal haptoglobin; Matsumoto et al., 2001), general immune status (urinary neopterin,
48
49 110 Baydar et al., 2011) and gynecological cancer (urinary neopterin, Melichar et al., 2006). In
50
51 111 such cases they may not be measured because excreta concentrations indicate systemic
52
53
54
55
56
57
58
59
60

1
2
3 112 infectious status, but because they are indicative of more specific local infections in tissues
4
5 113 related to urinary or fecal excretion pathways, such as the kidneys and the gut. Finally, some
6
7 114 have been investigated and/or utilized as non-invasive markers of immune function in non-
8
9 115 human animals, including primates. For example, urinary neopterin has been used to
10
11 116 monitor simian immunodeficiency virus (SIV) infection in rhesus macaques (Fendrich et al.,
12
13 117 1989; Stahl-Hennig et al., 2002), while urinary neopterin (Amann et al., 2001) and salivary
14
15 118 haptoglobin and CRP (Gómez-Laguna et al., 2010) have been used to document immune
16
17 119 activation and monitor herd health in pigs.

21
22
23 120 To assess the validity of measurements of these immune markers in non-invasive
24
25 121 samples (urine, feces) of macaques, we took two approaches. In study 1, we took advantage
26
27 122 of the regular health monitoring that is undertaken on macaques at the German Primate
28
29 123 Center to obtain temporally-matched blood, fecal and urine samples from non-infected
30
31 124 individuals. Using these samples, we assessed relationships between serum and urinary, and
32
33 125 serum and fecal, concentrations of each marker to determine whether these correlate, and
34
35 126 hence whether the non-invasive measures might serve as proxy for the serum measures,
36
37 127 and also whether both non-invasive measures might be equally suitable proxies.

38
39
40
41
42 128 In study 2, we took advantage of a SIV infection experiment in combination with
43
44 129 medical interventions and surgery in six rhesus macaques (carried out as part of a separate
45
46 130 study by the German Primate Center's Unit of Infection Models), to assess the response
47
48 131 patterns of the three immune markers in urine and feces to infection and surgery. In
49
50 132 contrast to the cross-sectional correlative data collected from healthy animals, this
51
52 133 experimental approach should provide more direct information on the potential usefulness
53
54 134 of each marker in each matrix for assessing macaque immune activation and inflammatory
55
56
57
58
59
60

1
2
3 135 responses. Collectively, our analyses represent an initial assessment of the feasibility of
4
5 136 measuring these markers in primate excreta, provide baseline data for levels of these
6
7 137 markers in healthy animals, and assess their usefulness in reflecting immune activation and
8
9 138 inflammation in response to an experimentally induced acute infection and surgical tissue
10
11 139 trauma.

12
13
14
15
16 140

17 18 19 141 **Methods**

20 21 22 142 Research Ethics

23
24
25 143 All samples were collected during health checks of the macaque colony (e.g. annual health
26
27 144 check) or when animals were already immobilized for other purposes. Samples were
28
29 145 collected according to the ASAB/ABS guidelines on the ethical treatment of animals, and the
30
31 146 International Primatological Society guidelines on the ethical treatment of primates in
32
33 147 research. Urine and fecal samples collected from the SIV-infected animals were all collected
34
35 148 non-invasively without animal handling.

36
37
38
39
40 149

41 42 43 150 Study animals and sample collection

44 45 46 151 Study 1: Measurement of immune markers in healthy macaques

47
48
49 152 This study was conducted between Aug 2011 and Mar 2012 on 24 rhesus macaques (18
50
51 153 males, 6 non-pregnant females) and 3 male long-tailed macaques, which were housed at the
52
53 154 German Primate Centre, Göttingen, Germany. Animals ranged in age between 3 and 11
54
55 155 years, with an average age (\pm SEM) of 7.4 ± 0.5 years. Average body weight was 7.6 ± 0.4 kg
56
57
58
59
60

1
2
3 156 (males: 6.7 ± 0.5 kg; females: 10.3 ± 0.5 kg; overall range: 4.6-11.7 kg). Individuals were
4
5 157 housed either as same-sex pairs or in small same-sex groups in indoor cages and were fed
6
7 158 twice a day with commercial monkey chow supplemented with fruits and vegetables. Water
8
9
10 159 was available ad libitum.

11
12
13 160 From each study animal, matching urine, fecal and blood samples were collected
14
15 161 between 6.00 and 10.00 am for the measurement of neopterin (NEO), C-reactive protein
16
17 162 (CRP), and haptoglobin (HPT) concentrations as well as for the determination of
18
19
20 163 hematological parameters. At the time of sample collection, all animals were in good body
21
22 164 condition (mean BMI: 27.4 ± 1.1 ; range 20.8-45.0), visually healthy and showed no obvious
23
24 165 signs of any disease, except for one male who exhibited diarrhea. Veterinarians made the
25
26
27 166 decision to euthanize this animal 3 weeks after sample collection due to severe gut
28
29
30 167 problems and substantial weight loss. For urine and fecal sample collection, a study animal
31
32 168 was usually separated from its group members in the early morning (6.00 - 6.30 am) and
33
34 169 samples were collected upon urination and defecation on a plastic mat placed underneath
35
36
37 170 the cage. Only urine and fecal samples not obviously cross-contaminated with each other
38
39 171 were collected. Urine samples were immediately protected from light. For blood collection,
40
41 172 animals were subsequently (between 8.30 and 10.00 a.m. the same day) anesthetized with
42
43 173 an intra-muscular injection of ketamine hydrochloride (10mg/kg; Ketavet®). A blood sample
44
45 174 (4-8 ml) was drawn from the femoral vein of the animal and collected into a heparinized
46
47
48 175 tube. All samples were kept cold (4° - 7° C) upon collection and transferred to the
49
50
51 176 endocrinology laboratory within 4 hours of collection for further processing. Blood samples
52
53 177 were centrifuged at 1800 g for 10 min and plasma subsequently recovered and aliquoted.
54
55
56 178 Fresh fecal samples were well mixed using a spatula and from each sample two aliquots of
57
58
59
60

1
2
3 179 0.1 to 0.2 g were accurately weighted into 15 ml polypropylene tubes for future extraction.
4

5 180 Urine samples were also aliquoted, and all aliquots of each sample type were then stored
6

7 181 frozen at -20°C until analysis.
8
9

10
11 182
12

13
14 183 Study 2: Measurement of immune markers in response to SIV-infection
15

16
17 184 This study was undertaken between February and April 2014 on 6 rhesus macaques (3
18

19 185 males, 3 females) which were infected with SIV as part of a separate study undertaken by
20

21
22 186 the German Primate Center's Unit of Infection Models. Animals ranged in age between 4
23

24 187 and 5 years, with an average age (\pm SEM) of 4.6 ± 0.2 years. Average body weight was
25

26 188 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
27

28
29 189 of individuals fluctuated by less than 1% during the study period. The study was approved
30

31 190 by the Lower Saxony State Office for Consumer Protection and Food Safety and performed
32

33
34 191 with the project license 33.9-42502-04-12/0758-08. For infection, which required a deeper
35

36 192 anesthesia, animals received a mixture of ketamine, xylazine and atropine. Each monkey
37

38 193 was inoculated with 50% 1000 tissue culture infectious doses of the virus intravenously. The
39

40
41 194 infection was confirmed by determining plasma viral RNA load.
42

43
44 195 During the experiment animals were subject to minor medical interventions, such as bone
45

46 196 marrow aspiration and colon biopsies (all under anesthesia). They also underwent (together
47

48
49 197 with bone marrow aspiration and colon biopsy) one surgical removal of peripheral lymph
50

51 198 nodes two weeks post infection. In particular the latter likely involved surgical tissue trauma
52

53 199 which is known to result in an acute phase protein response (e.g. Yamamoto et al. 1993;
54

55
56 200 Michelsen et al. 2012). This situation thus provided a useful test case for assessing the
57
58
59
60

1
2
3 201 potential of the urinary and fecal CRP and haptoglobin measurements in indicating
4
5 202 inflammatory processes.
6
7

8
9 203 Urine and fecal samples for immune marker measurements were collected once
10
11 204 weekly for 4 weeks prior to virus inoculation and at least 3 times a week for 31 days
12
13 205 thereafter. Samples were collected, processed and stored as described for study 1.
14
15

16
17 206

18
19
20 207 Sample measurement for immune marker analysis
21

22
23 208 Plasma, urine and fecal samples were analyzed for concentrations of NEO, CRP and HPT
24
25 209 using commercial enzyme-immunoassay (ELISA) kits (see below). While plasma and urine
26
27 210 samples were taken unextracted to assay following appropriate dilution with assay buffer
28
29 211 (NEO) or sample diluent (CRP, HPT) provided with the respective kits, fecal samples had to
30
31 212 be extracted prior to analysis. For NEO, the extraction followed the procedure described by
32
33 213 Campbell et al. (2004) with small modifications. Specifically, fecal aliquots were thawed at
34
35 214 room temperature and one ml of 0.9% saline was added to all samples which were then
36
37 215 agitated for 10 min on a multi-tube vortexer. Samples were then centrifuged at 1800 g for
38
39 216 15 min and the supernatant recovered for analysis. Extraction of the two acute phase
40
41 217 proteins was carried out according to a protocol provided by Immundiagnostic AG,
42
43 218 Bensheim, Germany. Specifically, defrosted fecal samples were mixed with 1 ml of CRP
44
45 219 washing buffer and agitated for 10 min on a multi-tube vortexer. Samples were then
46
47 220 centrifuged at 1800 g for 15 min, the supernatant transferred into a 1.5ml polypropylene
48
49 221 tube, and centrifuged at 7500 g rpm for 5 min. 100 µl of the resulting supernatant was then
50
51 222 taken to CRP and HPT analysis. In order to compensate for the potential effect of differences
52
53
54
55
56
57
58
59
60

1
2
3 223 in water content of fecal samples on immune marker concentrations, following extraction
4
5 224 fecal dry weights for each sample were determined by drying samples in an oven at 50°C to
6
7
8 225 a constant weight. Fecal concentrations of each marker are expressed as ng per g of dried
9
10 226 feces (Campbell et al. 2004). Concentrations of urinary analytes were indexed by urinary
11
12 227 creatinine, measured as described (Bahr et al. 2000).

13
14
15
16 228

17
18
19 229 Laboratory Analyses

20
21
22 230 NEO concentrations were determined using a human ELISA kit (Art. No. RE59321) from IBL
23
24 231 International GmbH, Hamburg, Germany. The assay was performed according to the
25
26
27 232 manufacturer's instructions. While plasma and fecal extracts were assayed undiluted, urine
28
29 233 samples were diluted 1:10 – 1:100 with assay buffer to bring sample concentrations into the
30
31 234 working range of the assay. For Study 2, prior to ELISA analysis (see above) urine samples
32
33 235 were initially measured via HPLC (Schroecksnadel et al., 2006) (data not shown). This
34
35
36 236 enabled us to reduce analytical costs by restricting our ELISA analysis to the most important
37
38 237 samples as indicated by the HPLC data. NEO measures generated by ELISA vs HPLC were
39
40 238 strongly and highly significantly correlated with an r-value of 0.96 (n=84, p<0.001).
41
42
43 239 Detection limit of the ELISA assay was 0.18 ng/ml. Inter-assay coefficients of variation,
44
45 240 determined by repeated measurement of high and low value quality controls in each assay
46
47
48 241 and across studies, were 12.0% and 6.6%, respectively.

49
50
51 242 All CRP and HPT measurements were carried out using ELISA kits for monkey CRP
52
53 243 (Cat. No. 2210-4) and monkey haptoglobin (Cat. No. 2410-5) from Life Diagnostics, Inc.,
54
55
56 244 West Chester, USA. Both assays were performed according to the manufacturer's
57
58
59
60

1
2
3 245 instructions. For both assays, fecal extracts were taken undiluted to assay, except for two
4
5 246 samples which were diluted 1:10 for HPT. While urine samples were usually diluted 1:2 for
6
7 247 both assays, plasma samples were normally diluted 1:1,000 for CRP measurements and
8
9 248 1:100,000 for HPT determinations. Detection limits of the assays were 1.17 ng/ml for CRP
10
11 249 and 1.56 ng/ml for HPT and inter-assay coefficients of variation of a high and low
12
13 250 concentrated quality control were 9.6% and 10.3% for CRP and 8.4% and 9.6% for HPT. All
14
15 251 measures of intra- and inter-assay variation were within accepted norms.
16
17
18
19
20
21 252
22
23
24 253 Statistical Analyses
25
26
27 254 To assess potential sex or age effects on immune marker concentrations we examined
28
29 255 serum levels of the three immune markers in the Study 1 animals. One animal that was
30
31 256 known to be sick (n=1) was excluded from this analysis in order to remove any effects of this
32
33 257 individual on age or sex differences. Residual values of parametric analyses did not meet
34
35 258 model assumptions even if dependent variables were log-transformed, as determined by
36
37 259 inspection of residual QQ plots. Visual inspection reveals the distributions of several
38
39 260 variables to be non-normally distributed, but as expected for markers that show huge
40
41 261 responsiveness to infection/inflammation, exhibiting numerous similar lower values but
42
43 262 with occasional much higher values. We undertook univariate general linear model (GLM)
44
45 263 analyses on each serum marker separately (fixed factor, sex; covariate, age) so that both
46
47 264 variables could be assessed in the same model. However, we also tested each variable
48
49 265 separately using non-parametric statistics (Mann Whitney U test, Spearman's rank
50
51 266 correlation), and present these results in addition where they differ from those of the
52
53 267 parametric statistics.
54
55
56
57
58
59
60

1
2
3 268 We used bivariate correlations to assess serum to urinary and serum to fecal
4
5 269 relationships for each marker. Variables were not normally distributed, and this was still the
6
7 270 case even after log-transformation. We therefore undertook non-parametric Spearman's
8
9 271 rank correlations throughout. Sample sizes sometimes change slightly between analyses as
10
11 272 in one or two cases there was insufficient urine volume to measure all variables. Tests were
12
13 273 one-tailed as we clearly predicted a positive correlation between these variables.
14
15
16
17

18 274 In order to present descriptive statistics for the magnitude of biomarker responses
19
20 275 to SIV infection (NEO) and surgical trauma (CRP, HPT) in Study 2 animals, we determined for
21
22 276 both urine and feces the peak-to-baseline ratios of each marker (for males and females
23
24 277 separately, and combined). For calculating baseline values we took the period prior to SIV
25
26 278 infection up to 3 days thereafter when biomarker levels were still unaffected by the
27
28 279 treatment (see Results). We examined whether the acute phase proteins (CRP and HPT) in
29
30 280 the Study 2 animals increased in response to surgical trauma by comparing urinary and fecal
31
32 281 CRP and HPT concentrations in the period within 6 days before versus 6 days after the
33
34 282 surgery for lymph node extirpation using the Wilcoxon signed rank test (due to the small
35
36 283 sample size).
37
38
39
40
41

42 284 Probability values < 0.05 were considered statistically significant. As our aim was to
43
44 285 discover whether markers were measurable and potentially useful and informative in
45
46 286 different matrices, we considered our analyses exploratory rather than definitive and did
47
48 287 not correct for multiple testing.
49
50
51

52 288
53
54

55 289
56
57
58
59
60

1
2
3 290 **Results**

4
5
6 291 *Study 1*

7
8
9 292 Concentrations of all markers in each matrix are presented in Table 1. Values for the 3 long-
10
11 293 tailed macaques fell within the range of those of the rhesus macaques. There were no sex
12
13 294 differences in serum levels for any of the three immune markers when analyzed using GLMs
14
15 295 also containing age as a variable (NEO: $F_{1,25} = 0.094$, $p = 0.763$; CRP: $F_{1,24} = 0.027$, $p = 0.871$;
16
17 296 HPT: $F_{1,25} = 0.179$, $p = 0.676$). While we found no effects of age on serum concentrations when
18
19 297 corrected for sex using GLMs (NEO, $F_{1,25} = 3.297$, $p = 0.082$; CRP, $F_{1,24} = 1.167$, $p = 0.292$; HPT,
20
21 298 $F_{1,25} = 0.473$, $p = 0.498$), Spearman's rank correlations showed significant correlations for NEO
22
23 299 ($r_s = 0.513$, $n = 26$, $p = 0.007$) and CRP ($r_s = 0.498$, $n = 26$, $p = 0.010$), with older individuals having
24
25
26
27 300 higher concentrations of both markers.

28
29
30
31 301

32
33
34 302 Serum–urinary and serum–fecal correlations

35
36
37 303 *Neopterin*: Serum NEO concentrations were strongly and significantly correlated with
38
39 304 urinary ($r_s = 0.664$, $n = 27$, $p < 0.001$; Fig. 1) but not fecal ($r_s = 0.171$, $n = 27$, $p = 0.196$)
40
41 305 concentrations.

42
43
44
45 306 *CRP*: Serum CRP concentrations were not correlated with either urinary ($r_s = -0.037$, $n = 26$,
46
47 307 $p = 0.429$) or fecal CRP measures ($r_s = -0.003$, $n = 27$, $p = 0.493$).

48
49
50
51 308 *Haptoglobin*: Serum and urinary HPT concentrations were not correlated ($r = 0.264$, $n = 26$,
52
53 309 $p = 0.096$). HPT levels in fecal samples were either low or below the detection limit of the
54
55 310 assay.

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: $z = 1.887$, $p = 0.030$).

1
2
3 333 *Haptoglobin*: Urinary and fecal HPT excretion usually remained consistently low throughout
4
5 334 most of the experimental period. In the majority of animals (5/6) however, an increase was
6
7 335 recorded in levels of urinary HPT in the periods following first bone marrow aspiration, and
8
9 336 in particular in response to the surgery for lymph node extirpation/intestinal biopsy
10
11 337 sampling (Figures 4 and 5). As for CRP, the elevation in levels following surgery was short-
12
13 338 lived but statistically significant ($z = 1.739$, $p = 0.037$). The rise in HPT levels following lymph
14
15 339 node extirpation/intestinal biopsy sampling was also recorded in fecal samples where it was
16
17 340 much more marked though (Figure 5; $z = 2.201$, $p = 0.014$).
18
19
20
21
22
23
24
25

341

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)

1
2
3 356 haptoglobin may nevertheless be useful non-invasive markers of inflammation given their
4
5 357 significant, although short-lived, elevation in response to surgical tissue trauma.
6
7

8
9 358 Urinary neopterin concentrations correlate positively and significantly with those
10
11 359 found in serum (Figure 1), and respond consistently to SIV infection (Figure 2), a finding in
12
13 360 line with earlier studies (Fendrich et al., 1989; Stahl-Hennig, 2002). Measurement of this
14
15 361 marker in urine from free-ranging macaques is likely to reveal the presence of infections
16
17 362 associated with macrophage activation and the Th 1 response, which promote cellular
18
19 363 immunity in response to intracellular pathogens (e.g bacteria, viruses, fungi or parasites;
20
21 364 Elenkov and Chrousos, 1999). Regular measurement might allow the development of such
22
23 365 an infection to be tracked. Though this requires regular sampling, such sampling regimes
24
25 366 are a common requirement for other markers too. For example, the tracking of ovulation
26
27 367 through the measurement of estrogen and progesterone metabolites excreted in feces
28
29 368 and/or urine also requires frequent sampling (Hodges and Heistermann 2011). Studies of
30
31 369 free-ranging primates often assess the onset of the luteal phase of the cycle through the
32
33 370 detection of increased progesterone concentrations greater than 2 SDs above the previous 3-
34
35 371 5 baseline (follicular phase) values, and maintained for at least 3 consecutive samples
36
37 372 (following Jeffcoate 1983). Similar assessment criteria might be used for urinary neopterin
38
39 373 to determine whether an infection has occurred.
40
41
42
43
44
45

46
47 374 Although fecal neopterin concentrations did not correlate with serum or urinary
48
49 375 concentrations in our study and, in contrast to urinary neopterin, did not show a consistent
50
51 376 response to SIV infection, they are sometimes used not as a general method for measuring
52
53 377 infection in the body, but specifically as a measure of inflammatory gut disease and
54
55 378 infections in humans (Ledjeff et al., 2001; Campbell et al., 2004; Husain et al., 2013). It still
56
57
58
59
60

1
2
3 379 therefore retains potential as a method of testing for intestinal macrophage activity in non-
4
5 380 human primates. Data are required in which fecal neopterin concentrations can be
6
7
8 381 compared for healthy individuals and individuals known to have inflammatory gut infections
9
10 382 (e.g. see Husain et al. 2013 for humans), or on the same individuals from periods of both gut
11
12 383 infection and health.

13
14
15 384 Urinary and fecal measures did not correlate with serum values for either CRP or
16
17 385 haptoglobin. It is worth considering that we might expect correlations between
18
19
20 386 concentrations of analytes in blood and urine rather than in feces. Both blood and urine
21
22 387 concentrations represent relatively short-term measures, with excretion times usually much
23
24 388 quicker for urine than feces (Hodges and Heistermann, 2011), making it more likely that the
25
26 389 former would correlate with measures in blood. In contrast, concentrations in fecal samples
27
28 390 represent the integration of circulating levels over longer periods, and so may not
29
30 391 necessarily be expected to correlate with levels found in blood when analyzing cross-
31
32 392 sectional data. Hence, the lack of a correlation between fecal (as well as urinary) and serum
33
34 393 CRP and haptoglobin levels might reflect the rapid and extreme changes in this acute phase
35
36 394 protein during a response which renders it highly unlikely that a snapshot measure such as
37
38 395 serum values corresponds to more long-term measures (see also Touma and Palme, 2005).
39
40 396 It is important also to remember that our sample size in Study 1 of 27 animals is relatively
41
42 397 small, and as all animals were healthy, this might have reduced variation in the dataset
43
44 398 hindering our ability to detect significant correlations. Standardization for creatinine may
45
46 399 also add variation to urinary measures given differences in weight of our study animals
47
48 400 (Crockett et al. 1993). That said, known relationships such as that between serum and
49
50 401 urinary NEO were clearly demonstrated using our sample, indicating that our power was
51
52
53
54
55
56
57
58
59
60

1
2
3 402 sufficient to find such relationships where they exist and are strong. In general though, it is
4
5 403 also worth remembering that in humans these markers are also measured in feces and urine
6
7 404 rather than blood despite the easy availability of the latter specifically because fecal and
8
9 405 urinary measurements are indicative of disease and infection in tissues associated with
10
11 406 excretion pathways, such as the kidneys and the gut, rather than of general systemic
12
13 407 infection. It may therefore be no surprise that correlations between serum, urine and fecal
14
15 408 measures were not found.
16
17
18
19

20 409 Although urinary haptoglobin concentrations did not correlate with those in serum,
21
22 410 the potential usefulness of urinary haptoglobin measurements for monitoring inflammatory
23
24 411 processes is nonetheless suggested by our finding of markedly elevated levels in response to
25
26 412 bone marrow aspiration and lymph node extirpation. In particular, the surgery for lymph
27
28 413 node extirpation is likely to have resulted in tissue trauma, which is known to stimulate an
29
30 414 increase in acute phase protein secretion (Yamamoto et al. 1993; Michelsen et al. 2012).
31
32 415 Haptoglobin concentrations in blood increase in response to infections associated with
33
34 416 inflammation, but typically show a broader and less acute response curve when compared
35
36 417 to other acute phase proteins such as CRP (Gabay and Kushner, 1999). Regular
37
38 418 measurements of urinary haptoglobin might therefore potentially allow inflammatory
39
40 419 infections to be detected and monitored in wild mammals, particularly as haptoglobin
41
42 420 shows a relatively long release function in response to infection (Gabay and Kushner, 1999).
43
44
45
46
47
48

49 421 Fecal haptoglobin levels showed a similar response to surgery, with elevations even
50
51 422 more pronounced than those found in urine. Some limited evidence also emerged from
52
53 423 animals of study 1 to suggest that high levels of fecal haptoglobin excretion may be
54
55 424 indicative of health issues. Within the cohort of healthy individuals in three animals ≥ 15
56
57
58
59
60

1
2
3 425 times higher concentrations were found compared with the rest of the animals, with one
4
5 426 animal showing an extreme value of >10,000 ng/g (~200 fold elevation above average).
6
7 427 Interviewing the animal keepers and the vet and looking at animal history reports revealed
8
9 428 that in the past these three animals have exhibited symptoms of gut problems, such as
10
11 429 diarrhea or Giardia infection, relatively often. The individual with the highest fecal
12
13 430 haptoglobin level also showed markedly elevated concentrations in fecal and serum CRP
14
15 431 (both 5-fold above the study sample mean) and serum and urinary neopterin (3-fold and 2-
16
17 432 fold above the mean, respectively) as well as serum haptoglobin (2 fold above the mean).
18
19 433 This animal was the individual confirmed to be suffering from severe diarrhea and weight
20
21 434 loss during the time of sample collection (see Methods). Information on the gut status of
22
23 435 the two other animals with elevated haptoglobin levels in feces was not available, but
24
25 436 visually they appeared to be healthy (e.g. no diarrhea) when samples were collected. Taken
26
27 437 together, our results are tentative but promising, and suggest that measurement of
28
29 438 haptoglobin in urine and, in particular, feces may have potential for tracking both more
30
31 439 systemic as well as local inflammatory processes in macaques non-invasively. Since the
32
33 440 responses found were short-lived (lasting a few days at most), frequent sampling would be
34
35 441 necessary to detect acute occurrences of inflammation reliably.
36
37
38
39
40
41
42
43

44 442 Similarly to haptoglobin, we found elevated urinary and fecal CRP concentrations in
45
46 443 response to the surgical tissue trauma associated with lymph node extirpation. This also
47
48 444 suggests that non-invasive measure of CRP may be of potential value for tracking
49
50 445 inflammation in macaques. In contrast to haptoglobin however, CRP excretion patterns
51
52 446 were overall more variable, and the rise in fecal CRP in response to surgery was markedly
53
54 447 weaker than that for haptoglobin.
55
56
57
58
59
60

1
2
3 448 Before further studies seek to utilize these or any other markers, it will also be
4
5 449 important to investigate their stability under conditions of contamination with dirt or (in the
6
7 450 case of urine) feces, as well as issues related to how they must be stored and transported.
8
9
10 451 When careful analyses of such issues are undertaken, detailed recommendations can then
11
12 452 be made to fieldworkers on how to collect, store and transport samples for analysis in a way
13
14 453 that minimizes analyte contamination and degradation (see Higham et al., 2011b for
15
16
17 454 macaque C-peptides). In addition to urinary and fecal markers, some studies may also wish
18
19 455 to consider measuring relevant analytes from saliva. Methods for saliva collection from
20
21 456 primates have been used in free-ranging settings (Higham et al., 2010), and similar or
22
23 457 adapted methods are probably feasible for numerous (though clearly not all) primate
24
25
26 458 species in free-ranging populations. In saliva, many native analytes can be measured,
27
28 459 including sympathetic axis correlates such as alpha-amylase (e.g. rhesus macaques, Higham
29
30 460 et al., 2010; bonobos, Beringer et al., 2012), haptoglobin (e.g. pigs, Gómez-Laguna et al.,
31
32 461 2010) and CRP (e.g. humans, Rao et al., 2010).
33
34
35

36
37 462 There have been several recent and exciting developments in evolutionary studies of
38
39 463 primate immune function, including publications showing that high-ranking baboon males
40
41 464 heal faster than low-ranking males (Archie et al., 2012), and that rhesus macaque females
42
43 465 experimentally assigned low ranks show increased immune marker and receptor gene
44
45 466 expression (Tung et al., 2012). Hopefully, our study will encourage further investigations of
46
47 467 the non-invasive measurement of immune function. As methods that enable multiple
48
49 468 measurement of many analytes from the same sample become more reliable and
50
51 469 widespread (e.g. Hauser et al., 2011; Weltring et al., 2012), the direct measurement of
52
53
54 470 multiple markers may hopefully become more common-place. Multi-assays are now
55
56
57
58
59
60

1
2
3 471 available that simultaneously measure up to 20 different cytokines and chemokines in non-
4
5 472 human primate blood samples (Giavedoni, 2005). Such methods offer great promise,
6
7 473 particularly if they can be applied to non-invasive samples such as urine. We therefore
8
9 474 encourage further evaluations and validations of non-invasive markers in the area of
10
11 475 immune activation and primate health. The development and validation of more non-
12
13 476 invasive immune markers is likely to expand our ability to investigate primate behavior,
14
15 477 ecology and evolution considerably. Such measures will prove crucial to establishing the
16
17 478 physiological links connecting variation in behavioral strategies to long-term life-history
18
19 479 outcomes such as mortality, so linking the “short-term behavioral study” and “long-term
20
21 480 demographic study” elements of primatology.
22
23
24
25
26
27
28
29
30
31
32

33 481

34 482

35 483 **Acknowledgements**

36
37 484 We are very grateful to A. Schrod, K. Raue, N. Stolte-Leeb, A. Klippert for helping to organize
38
39 485 sample collection and the animal keepers of the macaque colony, in particular P. Mueller
40
41 486 and G. Marschhausen for collecting the samples for this study. We would also like to thank
42
43 487 K. Fuhrmann and P. Kiesel for help in sample preparation and, in particular, A. Heistermann
44
45 488 for carrying out all enzyme-immunoassays. We are grateful to the Associate Editor and
46
47 489 Reviewers for constructive comments on an earlier version of the manuscript. We also
48
49 490 thank the members of the research group “Sociality and Health in Primates” (DFG FOR 2136)
50
51 491 for stimulating discussions on the topic. A. Engelhardt was funded by a grant from the
52
53 492 German Research Council (DFG EN 719/2).
54
55
56
57
58
59
60

1
2
3 493 **References**4
5 494

6 495 Amann A, Widner B, Rieder J, Antretter H, Hoffmann G, Mayr V, Strohmenger H-U, Fuchs D.

7
8 496 2001. Monitoring of immune activation using biochemical changes in a porcine9
10 497 model of cardiac arrest. *Mediators of Inflammation* 10:343–34611
12 498 Archie EA, Altmann J, Alberts SC. 2012. Social status predicts wound healing in wild13
14 499 baboons. *Proceedings of the National Academy of Sciences USA* 109:9017-902215
16
17 500 Bahr NI, Palme R, Möhle U, Hodges JK, Heistermann M. 2000. Comparative aspects of the18
19 501 metabolism and excretion of cortisol in three individual nonhuman primates.20
21 502 *General and Comparative Endocrinology* 117:427-438.22
23 503 Baydar M, Capan Z, Girgin G, Baydar T, Sahin G. 2011. Evaluation of changes in immune24
25 504 system of operating room personel by measurement of urinary neopterin26
27 505 concentrations. *Pteridines* 22:13-17.28
29 506 Beringer V, Deschner T, Möstl E, Selzer D, Hohmann G. 2012. Stress affects salivary alpha-30
31 507 Amylase activity in bonobos. *Physiology and Behavior* 105:476-48232
33 508 Berman CM, Schwartz S. 1988. A non-intrusive method for determining relative body-fat in34
35 509 free-ranging monkeys. *American Journal of Primatology* 14:53-64.36
37 510 Brauch K, Hodges JK, Engelhardt A, Fuhrmann K, Shaw E, Heistermann M. 2008. Sex-specific38
39 511 reproductive behaviours and paternity in free-ranging Barbary macaques (*Macaca*40
41 512 *sylvanus*). *Behavioral Ecology and Sociobiology* 62:1453-66.42
43 513 Breineková K, Svoboda M, Smutná M, Vorlová L. 2007. Markers of acute stress in pigs.44
45 514 *Physiological Research* 56: 323-32946
47 515 Campbell DI, McPhail G, Lunn PG, Elia M, Jeffries DJ. 2004. Intestinal inflammation48
49 516 measured by fecal neopterin in Gambian children with enteropathy: association with50
51 517 growth failure, *Giardia lamblia*, and intestinal permeability. *Journal of Pediatric*52
53 518 *Gastroenterology and Nutrition* 39:153–157.54
55
56
57
58
59
60

- 1
2
3 519 Cavigelli SA, Chaudry HS. 2012. Social status, glucocorticoids, immune function, and health:
4
5 520 Can animal studies help to us understand human socioeconomic-status-related
6
7 521 health disparities? *Hormones and Behavior* 62:295-313.
8
9 522 Crockett CM, Bowers CL, Sackett GP, Bowden DM. 1993. Urinary cortisol responses of
10
11 523 longtailed macaques to five cage sizes, tethering, sedation, and room change.
12
13 524 *American Journal of Primatology* 30:55-74.
14
15 525 Deschner T, Kratzsch J, Hohmann G. 2008. Urinary C-peptide as a method for monitoring
16
17 526 body mass changes in captive bonobos (*Pan paniscus*). *Hormones and Behavior* 54:
18
19 527 620-626.
20
21 528 Elenkov IJ, Chrousos GP. 1999. Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory
22
23 529 cytokines and susceptibility to disease. *Trends in Endocrinology and Metabolism* 10:
24
25 530 359-368.
26
27 531 Emery Thompson M, Knott CD 2008. Urinary C-peptide of insulin as a non-invasive marker of
28
29 532 energy balance in wild orangutans. *Hormones and Behavior* 53:526-535.
30
31
32 533 Emery Thompson M, Muller MN, Wrangham RW, Lwanga JS, Potts K. 2009. Urinary C-
33
34 534 peptide tracks seasonal and individual variation in energy balance in wild
35
36 535 chimpanzees. *Hormones and Behavior* 55:299-305.
37
38 536 Engelhardt A, Hodges JK, Niemitz C, Heistermann M. 2005. Female sexual behavior, but not
39
40 537 sex skin swelling reliably indicates the timing of the fertile phase in wild long-tailed
41
42 538 macaques (*Macaca fascicularis*). *Hormones and Behavior* 47:195-204.
43
44 539 Engelhardt A, Pfeifer J-B, Heistermann M, Niemitz C, van Hooff JARAM, Hodges JK. 2004.
45
46 540 Assessment of female reproductive status by male long-tailed macaques (*Macaca*
47
48 541 *fascicularis*) under natural conditions. *Animal Behaviour* 67:915–924.
49
50 542 Fendrich C, Luke W, Stahl-Hennig C, Herchenroder O, Fuchs D, Wachter H, Hunsmann G.
51
52 543 1989. Urinary neopterin concentrations in rhesus monkeys after infection with
53
54 544 simian immunodeficiency virus (SIV_{mac} 251). *AIDS* 3:305-307
55
56
57
58
59
60

- 1
2
3 545 Georgiev AV, Emery Thompson M, Lokasola AL, Wrangham RW. 2011. Seed predation by
4 546 bonobos (*Pan paniscus*) at Kokolopori, Democratic Republic of Congo. *Primates*
5 547 52:309-314
6
7
8
9 548 Girard-Buttoz C, Heistermann M, Krummel S, Engelhardt A. 2009. Seasonal and social
10 549 influences on fecal androgen and glucocorticoid excretion in wild male long-tailed
11 550 macaques (*Macaca fascicularis*). *Physiology and Behavior* 98:168-75.
12
13
14
15 551 Girard-Buttoz C, Higham JP, Heistermann M, Wedergärtner S, Maestripieri D & Engelhardt A.
16 552 2011. Urinary C-peptide measurement as a marker of nutritional status in macaques.
17 553 *PLoS One* 6:e18042.
18
19
20
21 554 Gabay C, Kushner I. 1999. Acute-phase proteins and other systemic responses to
22 555 inflammation. *The New England Journal of Medicine* 340:448-454
23
24
25
26 556 Giavedoni LD. 2005. Simultaneous detection of multiple cytokines and chemokines from
27 557 nonhuman primates using luminex technology. *Immunological Methods* 301:89-101.
28
29
30 558 Gillespie TR, Chapman CA. 2006. Prediction of parasite infection dynamics in primate
31 559 metapopulations based on attributes of forest fragmentation. *Conservation Biology*
32 560 20:441-448
33
34
35
36 561 Gillespie TR, Chapman CA, Greiner EC. 2005. Affects of logging on gastrointestinal parasite
37 562 infections and infection risk in African primates. *Journal of Applied Ecology* 42:699-
38 563 707
39
40
41
42 564 Gómez-Laguna J, Gutiérrez A, Pallarés FJ, Salguero FJ, Cerón JJ, Carrasco L. 2010.
43 565 Haptoglobin and C-reactive protein as biomarkers in the serum, saliva and meat juice
44 566 of pigs experimentally infected with porcine reproductive and respiratory syndrome
45 567 virus. *The Veterinary Journal* 185:83-87.
46
47
48
49
50 568 Gordis EB, Granger DA, Susman EJ, Trickett PK. 2008. Salivary alpha amylase–cortisol
51 569 asymmetry in maltreated youth. *Hormones and Behavior* 53:96-103.
52
53
54
55 570 Habig B and Archie EA. 2015. Social status, immune response and parasitism in males: a
56 571 meta-analysis. *Philosophical Transactions Royal Society B* 370:20140109.
57
58
59
60

- 1
2
3 572 Hart BA, Bank RA, de Roos JA, Brok H, Jonker M, Theuns HM, Hakimi J, Te Koppele JM. 1998.
4 573 Collagen-induced arthritis in rhesus monkeys: evaluation of markers for
5 574 inflammation and joint degredation. *British Journal of Rheumatology* 37:314-323.
6
7
8
9 575 Hauser B, Mugisha L, Preis A, Deschner T. 2011. LC-MS analysis of androgen metabolites in
10 576 serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*).
11 577 *General and Comparative Endocrinology* 170:92-98
12
13
14
15 578 Heistermann M, Palme R, Ganswindt A. 2006. Comparison of different
16 579 enzymeimmunoassays for assessment of adrenocortical activity in primates based on
17 580 fecal analysis. *American Journal of Primatology* 68:257-273.
18
19
20
21 581 Heyes MP, Lackner A, Kaufman S, Milstien S. 1991. Cerebrospinal fluid and serum neopterin
22 582 and biopterin in D-retrovirus-infected rhesus macaques (*Macaca mulatta*):
23 583 relationship to clinical and viral status. *AIDS* 5:477-616.
24
25
26
27 584 Higham JP, Vitale AB, Mas-Rivera A, Ayala JE, Maestriperi D. 2010. Measuring salivary
28 585 analytes from free-ranging monkeys. *Physiology and Behavior* 101:601-607.
29
30
31
32 586 Higham JP, Heistermann M, Maestriperi D. 2011a. The energetics of male-male endurance
33 587 rivalry in rhesus macaques (*Macaca mulatta*). *Animal Behaviour* 81:1001-1007.
34
35
36 588 Higham JP, Girard-Buttoz C, Engelhardt A, Heistermann M. 2011b. Urinary C-peptide of
37 589 insulin as a non-invasive marker of nutritional status: some practicalities. *PLoS*
38 590 *One* 6:e22398.
39
40
41
42 591 Higham JP, Heistermann M, Maestriperi D. 2013. The endocrinology of male rhesus
43 592 macaque social and reproductive status: a test of the challenge and social stress
44 593 hypotheses. *Behavioral Ecology and Sociobiology* 67:19-30.
45
46
47
48 594 Hodges JK, Heistermann M. 2011. Field endocrinology: monitoring hormonal changes in
49 595 free-ranging primates. In: *Field and Laboratory Methods in Primatology: A Practical*
50 596 *Guide*. (Eds. Setchell JM, Curtis DJ). Cambridge University Press: Cambridge, pp 353-
51 597 370.
52
53
54
55
56
57
58
59
60

- 1
2
3 598 Hoffman CL, Higham JP, Heistermann M, Prendergast B, Coe C, Maestripieri D. 2011.
4
5 599 Immune function and HPA axis activity in free-ranging rhesus macaques.
6
7 600 Physiology and Behavior 104:507-514
8
9 601 Huntoon KM, Wang Y, Eppolito CA, Barbour KW, Berger FG, Shrikant PA, Baumann H. 2008.
10
11 602 The acute phase protein haptoglobin regulates host immunity. Journal of Leukocyte
12
13 603 Biology 84:170-181
14
15 604 Husain N, Tokoro K, Popov JM, Naides SJ, Kwasny MJ, Buchman AL. 2013. Neopterin
16
17 605 concentration as an index of disease activity in Crohn's disease and ulcerative
18
19 606 colitis. Journal of Clinical Gastroenterology 47:246-251
20
21 607 Jeffcoate, SL. 1983. Ovulation: methods for its prediction and detection. John Wiley & Sons:
22
23 608 Chichester.
24
25 609 Jemmott JB, Magloire K. 1988. Academic stress, social support, and secretory
26
27 610 immunoglobulin A. Journal of Personality and Social Psychology 55:803-810.
28
29
30 611 Jinbo T, Hayashi S, Iguchi K, Shimizu M, Matsumoto T, Naiki M, Yamamoto S. 1998.
31
32 612 Development of monkey C-reactive protein CRP assay methods. Veterinary
33
34 613 Immunology and Immunopathology 61:195-202
35
36 614 Jinbo T, Ami Y, Suzaki Y, Kobune F, Ro S, Naiki M, Iguchi K, Yamamoto S. 1999.
37
38 615 Concentrations of C-reactive protein in normal monkeys (*Macaca irus*) and in
39
40 616 monkeys inoculated with *Bordetella bronchiseptica* R-5 and measles virus.
41
42 617 Veterinary Research Communications 23:265-274
43
44 618 Kappeler PM, Nunn CL. 2015. (Eds) The sociality-health-fitness nexus in animal societies.
45
46 619 Philosophical Transactions of the Royal Society 370 (1669)
47
48 620 Klingström J, Plyusnin A, Vaheeri A, Lundkvist A. 2002. Wild-type Puumala Hantavirus
49
50 621 infection induces cytokines, C-Reactive protein, creatinine, and nitric oxide in
51
52 622 *Cynomolgus* macaques. Journal of Virology 76:444-449
53
54
55
56
57
58
59
60

- 1
2
3 623 Koenig A, Borries C, Chalise MK, Winkler P. 1997. Ecology, nutrition and timing of
4 reproductive events in an Asian primate, the Hanuman langur (*Presbytis entellus*).
5 624
6 625 *Journal of Zoology* 243:215-235.
7
8
9 626 Ledjeff E, Artner-Dworzak E, Witasek A, Fuchs D, Hausen A. 2001. Neopterin concentrations
10 627 in colon dialysate. *Pteridines* 12:155-160
11
12
13 628 Matsumoto M, Ohishi H, Benno Y. 2001. Impact of LKM512 yogurt on improvement of
14 629 intestinal environment of the elderly. *FEMS Immunology and Medical Microbiology*,
15 630 31:181-186
16
17
18
19 631 Melichar B, Solichova D, Freedman RS. 2006. Neopterin as an indicator of immune activation
20 632 and prognosis in patients with gynaecological malignancies. *International Journal of*
21 633 *Gynaecological Cancer* 16: 240-252.
22
23
24
25 634 Michelsen J, Heller J, Wills F, Noble GK. 2012. Effect of surgeon experience on postoperative
26 635 plasma cortisol and C-reactive protein concentrations after ovariectomy in
27 636 the dog: a randomised trial. *Australian Veterinary Journal* 90: 474-478.
28
29
30
31
32 637 Nunn CL, Altizer SM. 2006. *Infectious Diseases in Primates: Behavior, Ecology and Evolution*.
33 638 Oxford University Press: Oxford.
34
35
36 639 Nunn CL. 2012. Primate disease ecology in comparative and theoretical perspective.
37 640 *American Journal of Primatology* 74:497-509
38
39
40 641 Nunn CL, Lindenfors P, Pursall ER, Rolff J. 2009. On sexual dimorphism in immune function.
41 642 *Philosophical transactions of the Royal Society of London Series B* 364:61-69.
42
43
44
45 643 Ostner J, Kappeler P, Heistermann M. 2008. Androgen and glucocorticoid levels reflect
46 644 seasonally occurring social challenges in male redfronted lemurs (*Eulemur fulvus*
47 645 *rufus*). *Behavioral Ecology Sociobiology* 62:627-38.
48
49
50
51 646 Pepys MB, Hirschfield GM. 2003. C-reactive protein: a critical update. *The Journal for Clinical*
52 647 *Investigation* 111: 1805-1812.
53
54
55 648 Petersen HH, Nielsen JP, Heegaard PMH. 2004. Application of acute phase protein
56 649 measurements in veterinary clinical chemistry. *Veterinary Research* 35:163–187
57
58
59
60

- 1
2
3 650 Plata-Nazar K, Luczak G, Gora-Gebka M, Liberek A, Kaminska B. 2010. Serum neopterin
4
5 651 concentrations in children with viral gastroenteritis. *Pteridines* 21:11-16
6
7 652 Preston BT, Capellini I, McNamara P, Barton RA, Nunn CL. 2009. Parasite resistance and the
8
9 653 adaptive significance of sleep. *BMC Evolutionary Biology* 9:7
10
11 654 Rao NL, Shetty S, Upadhyaya K, Prasad RM, Lobo EC, Kedilaya HP, Prasad G. 2010. Salivary C-
12
13 655 reactive protein in Hashimoto's Thyroiditis and subacute Thyroiditis. *International*
14
15 656 *Journal of Inflammation* 2010:514619
16
17 657 Rho YH, Solus J, Raggi P, Oeser A, Gebretsadik T, Shintani A, Stein CM. 2011. Macrophage
18
19 658 activation and coronary atherosclerosis in systemic Lupus erythematosus and
20
21 659 rheumatoid arthritis. *Arthritis Care and Research* 63:535-541
22
23 660 Rudzite V, Jurika E, Fuchs D, Kalnins U, Erglis A, Trusinskis K. 2003. Serum concentration of C-
24
25 661 reactive protein, neopterin and phospholipids in patients with different grade of
26
27 662 coronary heart disease. *Pteridines* 14:133-137
28
29 663 Schroecksadel K, Winkler C, Fuchs D. 2006. Method for urinary neopterin measurements
30
31 664 by HPLC. *Journal of Biochemical and Biophysical Methods* 66: 99-100.
32
33 665 Schwensow N, Fietz J, Dausmann KH, Sommer S. 2007. Neutral versus adaptive genetic
34
35 666 variation in parasite resistance: importance of major histocompatibility complex
36
37 667 supertypes in a free-ranging primate. *Heredity* 99:265-275.
38
39 668 Schwensow N, Fietz J, Dausmann KH, Sommer S. 2008. MHC-associated mating strategies
40
41 669 and the importance of overall genetic diversity in an obligate pair-living primate.
42
43 670 *Evolutionary Ecology* 22:617-636.
44
45 671 Sherry DS, Ellison PT. 2007. Potential applications or urinary C-peptide of insulin for
46
47 672 comparative energetic research. *American Journal of Physical Anthropology* 133:771-
48
49 673 778.
50
51 674 Stahl-Hennig C, Fendrich C, Lüke W, Widner B, Hunsmann G, Fuchs D. 2002. Urinary
52
53 675 neopterin indicates early infection and disease progression: Model studies with
54
55 676 simian and human immunodeficiency viruses in macaques. *Pteridines* 13:1-8
56
57
58
59
60

1
2
3 677 Touma C, Palme R. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds:
4 the importance of validation. *Annals of the New York Academy of Sciences* 1046:54–
5 678 74.
6
7 679

8
9 680 Tung J, Barreiro LB, Johnson ZP, Hansen KD, Michopoulos V, Toufexis D, Michelini KM,
10 Wilson ME, Gilad Y. 2012. Social environment is associated with gene regulatory
11 681 variation in the rhesus macaque immune system. *Proceedings of the National*
12 682 *Academy of Sciences USA* 109:6490-6495
13
14 683

15
16
17 684 Weltring A, Schaebs FS, Perry SE, Deschner T. 2012. Simultaneous measurement of
18 685 endogenous steroid hormones and their metabolites with LC-MS/MS in faeces of a
19 686 New World primate species, *Cebus capucinus*. *Physiology and Behavior* 105:510-521
20
21

22
23 687 Weyher A, Ross C, Semple S. 2006. A comparison of gastrointestinal parasites in a crop
24 688 raiding and a wild foraging troop of olive baboons in Nigeria. *International Journal of*
25 689 *Primateology* 27:1519-1534
26
27
28

29 690 Wheaton CJ, Savage A, Lasley BL. 2011. Advances in the understanding of primate
30 691 reproductive endocrinology. In: *Primates in Perspective (2nd Ed)* (Eds Campbell CJ,
31 692 Fuentes A, MacKinnon KC, Bearder SK & Stumpf RM). Oxford University Press:
32 693 Oxford, pp 377-389.
33
34
35
36

37 694 Widner B, Wirleitner B, Baier-Bitterlich G, Weiss G, Fuchs D. 2000. Cellular immune
38 695 activation, neopterin production, tryptophan degradation and the development of
39 696 immunodeficiency. *Archivum Immunologiae et Therapiae Experimentalis* 48:251-
40 697 258.
41
42
43
44

45 698 Yamamoto S, Shida T, Miyaji S, Santsuka H, Fujise H, Mukawa K, Furukawa E, Nagae T, Naiki
46 699 M. 1993. Changes in serum C-reactive protein levels in dogs with various disorders
47 700 and surgical traumas. *Veterinary Research Communications* 17: 85-93.
48
49
50

51 701

52
53
54 702
55
56
57
58 703
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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).

720

721

722

723

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

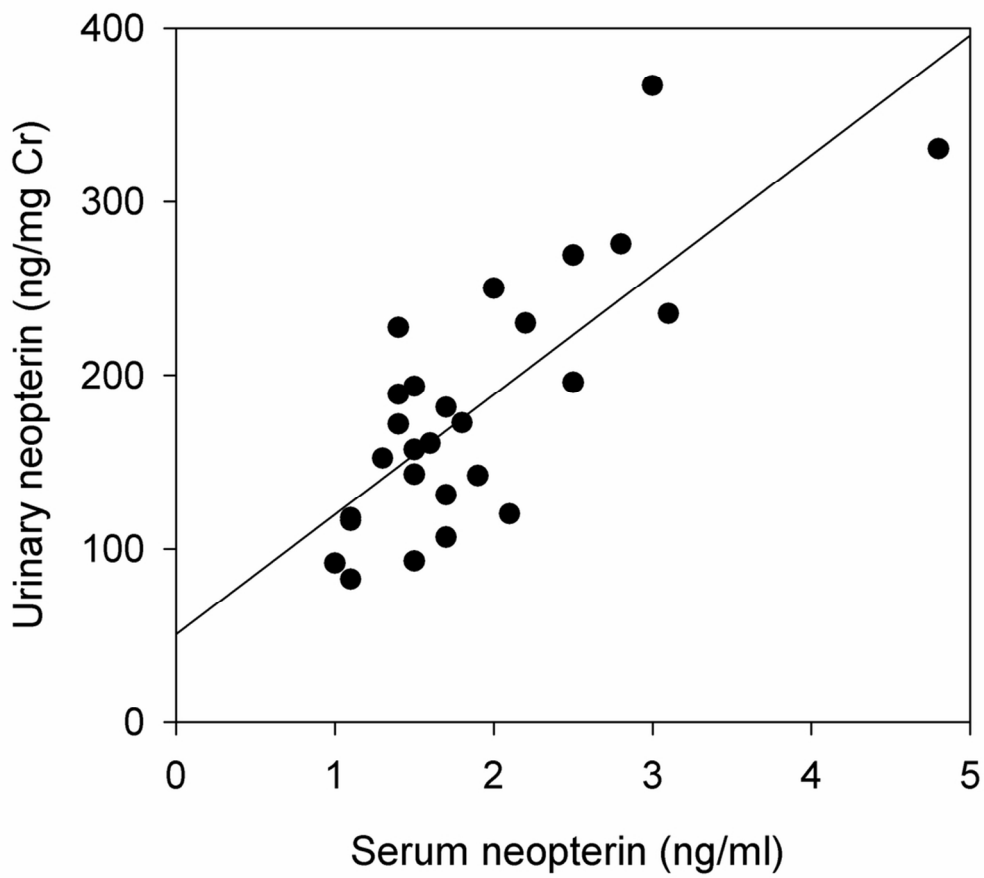
Table 2. Baseline concentrations of biomarkers and ranges of peak-to-baseline (P/B) ratios measured in Study 2 animals

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

n.a. = not applicable (see Results)

All urinary concentrations are given as ng/mg Cr

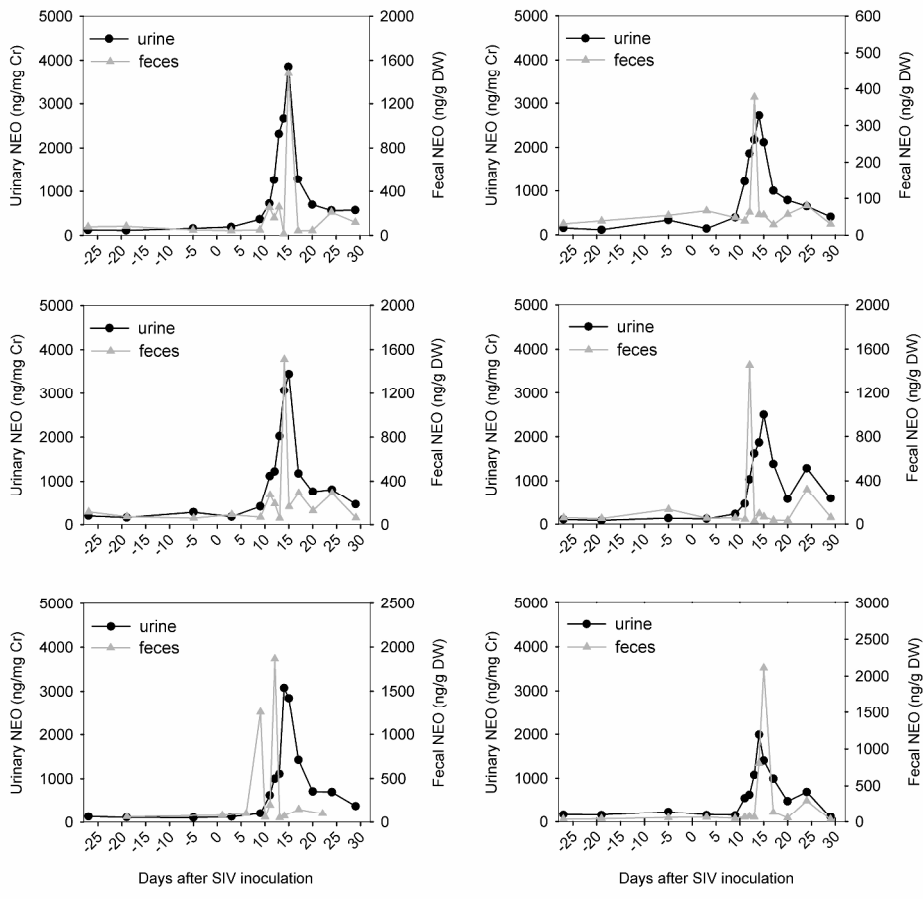
All fecal concentrations are given as ng/g dry weight



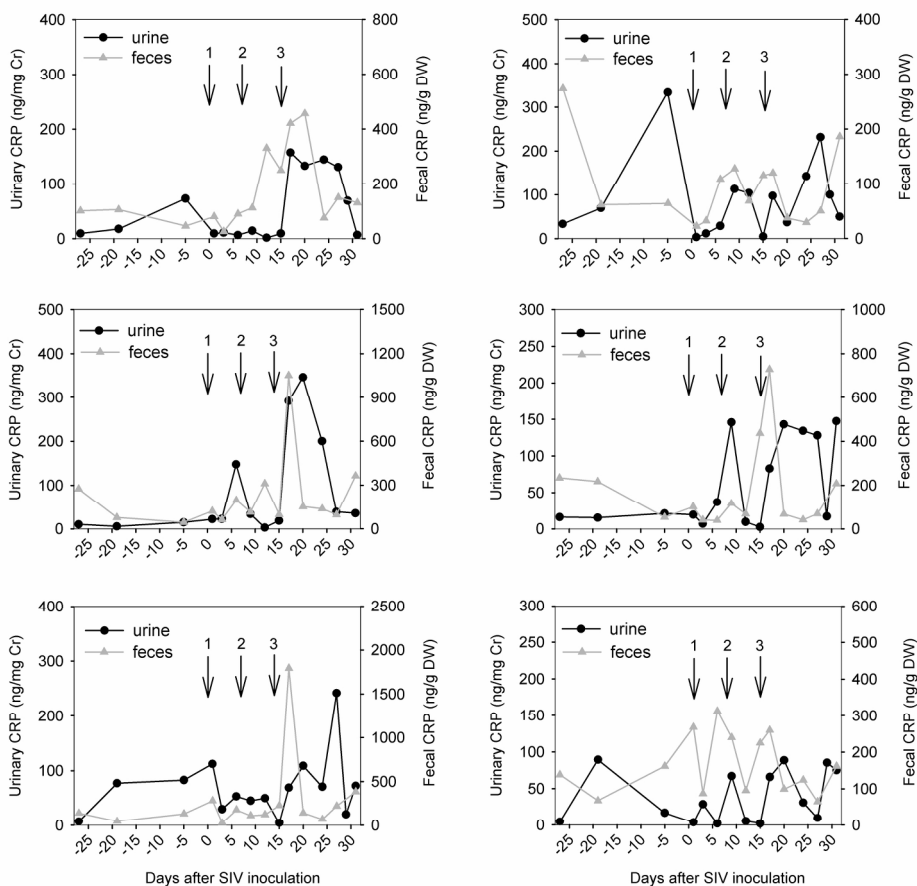
94x89mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



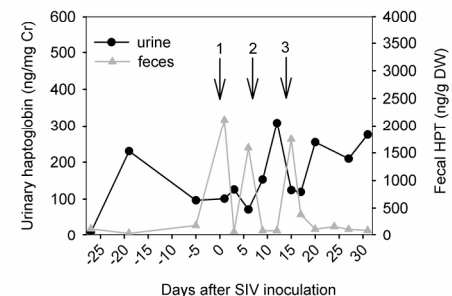
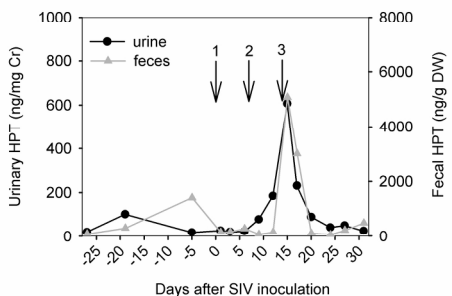
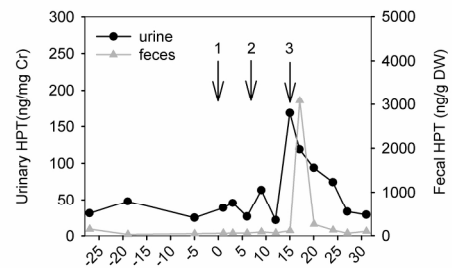
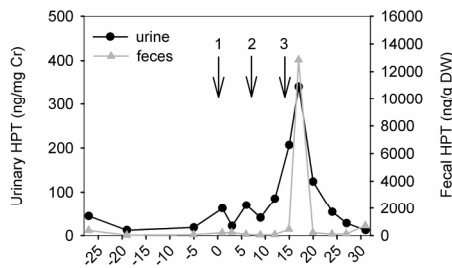
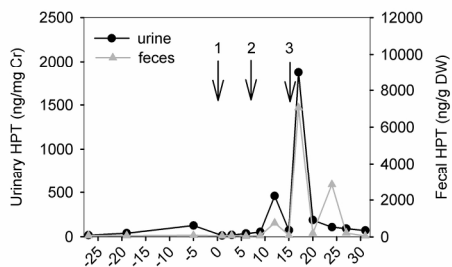
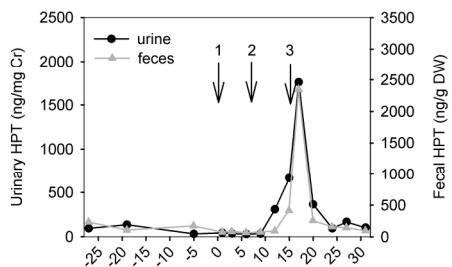
419x399mm (300 x 300 DPI)



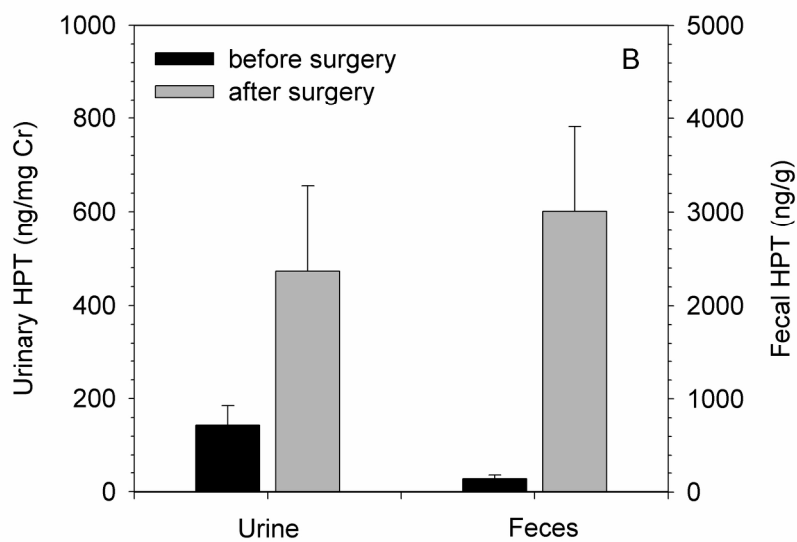
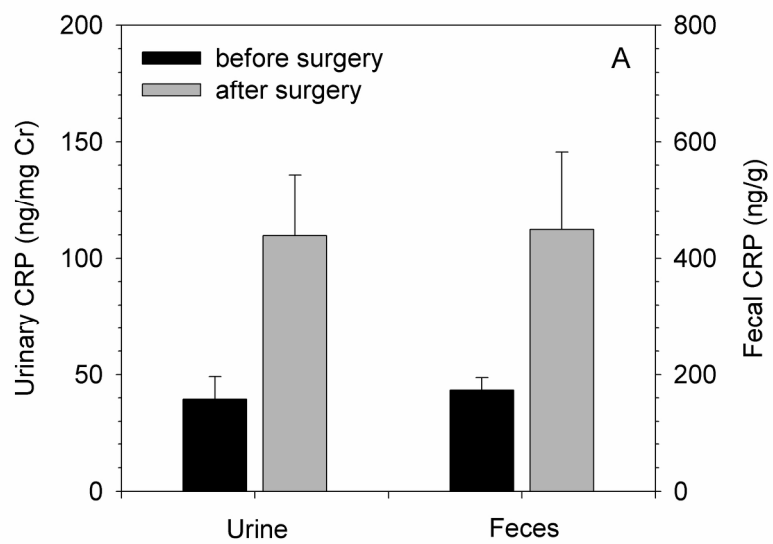
199x190mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



199x190mm (300 x 300 DPI)



180x249mm (300 x 300 DPI)