UC Agriculture & Natural Resources

Proceedings of the Vertebrate Pest Conference

Title

Cattle Metabolic Fingerprinting to Predict Acute Stress Response Post-Wolf Encounters

Permalink

<https://escholarship.org/uc/item/8gz6f41p>

Journal

Proceedings of the Vertebrate Pest Conference, 28(28)

ISSN

0507-6773

Authors

Valerio, Azzurra
Wielgus, Robert
Valerio, Mariacristina
et al.

Publication Date

2018

DOI

10.5070/V42811015

Cattle Metabolic Fingerprinting to Predict Acute Stress Response Post-Wolf Encounters

Azzurra Valerio and Robert Wielgus

School of the Environment, Washington State University, Pullman, Washington

Mariacristina Valerio and Luca Casadei

Department of Chemistry, University of Rome La Sapienza, Italy

ABSTRACT: As wolves recolonize across their former range in western North America, encounters between livestock and wolves are expected to increase in frequency. Understanding the physiological state of the prey, as a response to stress imposed by the presence of a predator (trait-mediated effects), will help with predicting the total effect of predators on their prey beyond direct consumption (density-mediated effects). Fecal glucocorticoid (GCM) is widely used to measure stress response, but provides inconclusive results, particularly when applied to a finer spatio-temporal variation in predation risk (i.e., wolf-livestock encounters). Since the impact of external stressors (i.e., wolf encounters) on the body influences ultimately the physical and metabolic state of the animal, we purpose to investigate: 1) if the fecal metabolome extracted from cattle fecal samples reflects changes in the GCM levels, and 2) if the cattle metabolome can better predict stress response than GCM post-wolf encounter. We first performed a controlled stress experiment on five captive cows in a pasture. We also conducted a field study in Washington where we fit GPS collars equipped with proximity sensors on two wolves in two separate packs, and on 40 range cows in four different livestock herds. When a wolf and a cow equipped with proximity sensors are within 128 meters from one another, the GPS collars send a real-time message that allows us to assess the physiological state of the prey at a finer spatial-temporal scale. In both studies, we collected fecal samples from GPS radio-collared cattle and compared their metabolome to GCM levels. Our results clearly show that the metabolomic analysis of the cattle gut microbiome can better predict acute stress response than the GCM, in both the controlled and free-ranging environment.

KEY WORDS: acute stress-response, *Bos taurus*, *Canis lupus*, cattle, fecal glucocorticoids, fecal metabolome, GPS collars, metabolomic fingerprinting, predator-prey interactions, proximity sensors, wolves

Proc. 28th Vertebr. Pest Conf. (D. M. Woods, Ed.)
Published at Univ. of Calif., Davis. 2018. Pp. 77-84.

INTRODUCTION

As wolves (*Canis lupus*) recolonize across their former range in western North America, encounters between livestock and wolves are expected to increase in frequency. Understanding the physiological state of the prey, as a response to stress imposed by the presence of a predator (trait-mediated effects), will help with predicting the total effect of predators on their prey beyond direct consumption (density-mediated effects).

Fecal glucocorticoid (GCM) is widely used to measure acute stress response, but provides inconclusive results when applied to free-ranging animals (Cook 2012), particularly at a finer spatio-temporal variation in predation risk (i.e., wolf-livestock encounters). Since the impact of external stressors (i.e., wolf encounters) on the body ultimately influences the physical and metabolic state of the animal (Broom and Johnson 1993), we purpose to investigate changes in cattle metabolism by means of metabolomic analysis.

Metabolomics is a high-throughput technique that allows for the determination of all metabolites of a biological system (Oliver et al. 1998). The generation of high-density data (metabolites) from biological samples can be explored using multivariate mathematical modeling tools to reveal the subset of metabolites (fingerprint pattern) that defines the biochemical phenotype or metabolome of an organism. The metabolome thus reflects the combined effects of nutrient status, gender, age, and genetic background of an organism (Brindle et al. 2002,

Coen et al. 2005, Plumb et al. 2005, Stanley et al. 2005, Rosenblum et al. 2006). Since the metabolome responds to stimuli within seconds, it is possible to follow the rapid changes that occur in an organism in response to external environmental perturbations (i.e., stress), (Andrew et al. 2006, Lindon et al. 2007). Therefore, metabolomics provide a direct fingerprint of a biological system at a specific time, allowing the identification of any potential intrinsic and extrinsic factors that may influence the physiological state of cattle. Furthermore, because the metabolome can be extracted by means of Proton Nuclear Magnetic Resonance (¹H-NMR) Spectroscopy from cattle fecal samples (fecal metabolome), metabolomics can be used as an effective tool for non-invasive stress monitoring in wildlife research.

The aim of this study was to assess metabolic profiles of cattle feces to predict acute stress response in relation to wolf presence. To achieve this goal, the first step was to perform a biological validation of metabolomic analysis to measure acute stress response, as to our knowledge there are no such studies. We conducted a stress-induced experiment on beef cows (*Bos taurus*) in controlled settings, with the objective of monitoring and comparing changes in fecal metabolome and fecal GCM in relation to the adrenal activity. Our hypothesis was that fecal metabolome reflects changes in fecal GCM levels.

After the validation of metabolomic analysis on cattle fecal samples to assess acute stress response, the second step was to conduct a field study. Our hypothesis was that,

due to the limitations of fecal GCM analysis in free ranging conditions, cattle fecal metabolome can better predict acute stress response post-wolf encounter. We fit GPS collars equipped with proximity sensors on wolves and cows in Washington state. When a wolf and a cow equipped with proximity sensors are within a pre-set distance from one another, the GPS collars send a real-time message of an interaction, and allows researchers to assess the physiological state of the prey at a finer spatial-temporal scale. We monitored and compared changes in fecal metabolome and fecal GCM collecting fecal samples from cows equipped with proximity sensors.

METHODS

Experimental Study

We performed a stress-induced experiment on five lactating cows at the Washington State University (WSU) Ensminger Beef Center. We used two Angus and three Angus/Simmental cows, between seven to nine years of age and mean body weight of 748 kg (range: 680-816 kg). The cows and their calves were housed in a pasture (3.88 ha) with access to shelter, forage grasses, and water *ab libitum*. All the protocols for this research were approved by the WSU Institutional Animal Care and Use Committee, (IACUC# 04684-002).

We collected cattle fecal samples before and after an induced-stressful event to monitor changes in fecal metabolome and fecal GCM in relation to adrenal activity. During the experiment, the cows remained in their home pasture to minimize the study-induced disturbances. We disrupted the daily routine of the cows only during the day of the stress-induced test by separating the cows from their calves, and restraining them into a small pen located outside the home pasture. After 2.5 h, we moved individual cows into a squeeze chute to collect three stress-induced blood samples per animal from the coccygeal vein in the tail. The blood samples were stored briefly on ice, centrifuged at 1,500 rpm for 15 min and then frozen at -20°C until extraction and assay.

Fecal collection was performed for a total of 14 days divided in three experimental phases: pre-stress, stress, and post-stress. During the pre-stress and post-stress phases we collected one feces per cow per day for a total of 12 days (six days per phase). During the stress phase, we collected all the cows' spontaneous defecations for 48 hours immediately following the stress-induced test. From each fecal sample, we collected two sub-samples to perform both the metabolomic and fecal GCM analysis. All sub-samples were homogenized and immediately frozen after collection: GCM sub-samples were stored in sterile bags and frozen at -20 °C; metabolomic sub-samples were collected in cryovials and frozen in liquid nitrogen at -80 °C.

Field Study

Study Area

We conducted a pilot study during 2016 grazing season (May-October) in two wolf pack territories in North-Central and North-Eastern Washington (Figure 1). The mean number of wolves in both packs during the study was 10 (adults and pups), and the mean home range size was 942 km². Within each wolf pack territory, we

Figure 1. Map of the two pilot study areas in North Central and North-East Washington, with minimum convex polygons of wolf packs (n = 2) and with cattle grazing allotments (n = 4). In both study areas we monitored wolf-livestock interactions by using GPS collars with proximity sensors fit on wolves (n = 2) and cattle (n = 40) during the grazing season 2016.

monitored two cattle herds with varying degrees of spatial overlap of the wolf pack. All four cattle operations in the study were cow-calf operations, and the mean number of individual livestock per herd was 130. The cows, primarily Angus and Charolais breeds, were free ranging from May through October within grazing allotments on the Colville National Forest and the Confederated Tribes of the Colville Reservation. The average size of the four grazing allotments was 65 km² (ranging from 47 to 207 km²). Some cattle grazed beyond the assigned allotment boundaries due to lack of fencing.

Large native prey for wolves in the study areas included moose (*Alces alces*), Rocky Mountain elk (*Cervus elaphus nelsoni*), mule deer (*Odocoileus hemionus*), and white-tailed deer (*O. virginianus*). Other carnivores that shared the landscape with wolves included black bears (*Ursus americanus*), cougars (*Puma concolor*), coyotes (*Canis latrans*), and bobcats (*Lynx rufus*).

Wolf and Cattle Telemetry Data

Wolves and cattle were captured, handled, and monitored using protocols reviewed and approved by

Table 1. Mean cortisol levels (ug/dl) extracted from cow blood samples collected during the stress experimental phase.

	Animal ID				
	Cow 1	Cow 2	Cow 3	Cow 4	Cow 5
Cortisol ug/dl	1.63	3.06	1.66	<1.0	<1.0

WSU's Institutional Animal Care and Use Committee (IACUC # 04661-002 and # 04687-001). We used GPS collars with proximity sensors to monitor interactions between wolves and cattle. When a wolf and a cow equipped with proximity sensors are within 128 meters from one another, the GPS collars send a real-time message of the encounter. Moreover, the GPS schedules switch to a higher frequency fix rate taking point locations every 65 seconds, instead of two hours (established fix rate), allowing to record a finer resolution of spatio-temporal variation in risk (i.e., physiological state of the prey at a finer spatial-temporal scale) (Drewe et al 2012). We define each proximity event as any time a wolf and a cow fit proximity sensor detect one another. So, this means that in some cases multiple proximity events can be recorded in the same interaction event.

We fit on two wolves in two separate packs GPS PLUS Iridium collars (VECTRONIC Aerospace GmbH Inc. Berlin, Germany) equipped with proximity sensors. In the study there were other pack members who were previously fit with GPS collars without proximity sensors by the Washington Department of Fish and Wildlife (WDFW). In addition, we fit GPS collars with proximity sensors (VECTRONIC Aerospace GmbH Inc. Berlin, Germany) on 10 lead cows, or matriarchs, in each of the four livestock herds (n = 40 total of collared cows).

Fecal Collection

We collected only fecal samples voided by cows fit with GPS collars. From May to October, we collected one fecal sample from each cow every two weeks. In addition, after any known interaction with wolves equipped with proximity sensors, we collected one fecal sample per cow/s per day for four days (96 h). We based the 96-hour period on the results of the experimental study. In fact, we observed the acute stress response lasting up to 96 h after the stress event in some cows.

From each fecal sample, we collected two sub-samples to perform both the metabolomic and fecal GCM analysis. Sample collection and storage are consistent with the methods used for the experimental study (see above). Fecal samples collected were labeled in two groups: interaction and non-interaction groups. We labeled as interaction all the fecal samples collected after a known interaction recorded by means of proximity sensors. We labeled as non-interaction all the feces collected from GPS-collared cows that were at a 10-km minimum distance from the GPS-collared wolves for at least 96 h, based on the results of the experimental study.

¹H Nuclear Magnetic Resonance (NMR) Spectroscopy Analysis

NMR Sample Preparation

From each sample, 2 g of feces were used and 6 ml of water were added. Then each sample was vortexed for 2 min and centrifuged for 10 min at 14,000g and 20 °C to obtain fecal water. We collected 4 ml of supernatant that was subsequently lyophilized for 48 h. Then, the powder was dissolved in 1 ml of 10 mM D₂O phosphate buffered saline solution at pH = 7.4 containing TSP 1 mM and NaN₃ 10 mM. After centrifugation, 800 µl of each resulting supernatant was transferred into a 5-mm NMR tube.

¹H-NMR Spectroscopy

For the analysis of fecal water samples, *J*-resolved pulse sequence was used to better observe resonances as these samples are partially or completely buried in a typical 1D spectrum. The two-dimensional (2D) ¹H *J*-resolved (JRES) NMR spectra were acquired on a Varian 400 MHz Spectrometer with a double spin echo sequence, using 16 scans per increment for a total of 32 increments and collecting 16 k data points. The spectral widths were 6 kHz in F2 and 40 Hz in F1. The relaxation delay was three seconds. The water resonance was suppressed using presaturation. Each Free Induction Decay (FID) was multiplied with a sine-bell window functions in both dimensions and Fourier transformed. JRES spectra were then tilted by 45°, symmetrised about F1 and referenced to acetate at $\delta_H = 1.90$ ppm. The proton-decoupled skyline projections (p-JRES) were exported using VnmrJ 3.2 software.

¹H-NMR Spectra Pre-processing Treatment

The 1D skyline projections exported were aligned and then reduced into spectral bins with widths ranging from 0.01 to 0.03 ppm by using the ACD intelligent bucketing method (1D NMR Manager software (ACD/Labs, Toronto, Canada). Binning was performed excluding the residual water region (δ 4.66–4.80 ppm). The resulting bins were integrated and normalized with respect to the total integral region to generate the data matrix for multivariate analysis.

Fecal Glucocorticoid Analysis

Cattle fecal samples were air-dried to eliminate moisture, and 0.2 g of feces were placed in a 16 x 100 extraction tube. A 5-ml volume of 60:40 phosphate-buffered saline (PBS) and reagent-grade methanol was added to each sample. Capped tubes were vortexed for 1 min, and placed on a tube shaker for 20 min. Samples were centrifuged at 3,000 rpm for 15 min, and 2 ml of clear supernatant was transferred to clean tubes. All samples were assayed with the MP Biomedical corticosterone radioimmunoassay (Solon OH; 07-120002) (Wasser et al. 2000). Prior to assay, cattle fecal sample extracts were further diluted 1:4 in PBS. Samples that were above or below the standard curve were diluted appropriately to ensure values within the range of the standard curve (n = 23 samples). Serum samples were assayed in 100 or 50 µl of plasma, depending on volume collected, and appropriate adjustments for volume were made in the final

Figure 2. O-PLS-DA model built on the 1H-NMR dataset of fecal samples collected from all beef cows during the 3 experimental phases: pre-stress (black triangle), stress (gray dot) and post-stress (grey star). The score plot show the metabolic differences between the three groups ($p < 0.0001$).

calculation of concentrations. Serial dilutions of cattle fecal extracts produced displacement curves that were parallel to the standard curves. A pooled sample of cattle fecal extract was assayed in each assay run ($n = 5$) and quality control parameters revealed an intra-assay coefficient of variation (CV) of 5.22%, and an interassay CV of 4.3%.

Statistical Analysis

Orthogonal projections to latent structures discriminant analysis (OPLS-DA) is a supervised pattern recognition technique widely used in the field of metabolomics to interpret large multivariate data sets describing differences between the groups under study in a straightforward and accurate way. OPLS-DA separates the systematic variation in the matrix X (spectroscopic data) into two parts: one linearly related (variation of interest) to the matrix Y (the classification variables) and one orthogonally related (so called orthogonal variation or structured noise) to the matrix Y . This partitioning of the X -data improved the interpretation of the model (Wiklund et al. 2008). The output from the OPLS-DA analysis consists of score plots, which provide an indication of the differences between the classes in terms of metabolic similarity, and loading plots. SIMPCA P v.12 (Umetrics, Umea, Sweden) was used for all the statistical analysis.

T-test and repeated measure ANOVA were also applied to calculate differences between group of samples under study.

RESULTS

Experimental Study

We collected three blood samples per cow ($n = 15$ total of blood samples) about 2.5 h from the beginning of the stress test to measure the cortisol level in the blood. Table 1 shows the average cortisol level per study animal. Two out of five cows have cortisol levels equivalent to baseline ($\mu\text{g/dl} < 1.0$).

We collected from 111 cow feces a total of 222 sub-samples to perform both metabolomic analysis ($n = 111$ total sub-samples) and GCM analysis ($n = 111$ total sub-samples). During the three experimental phases of pre-stress, stress and post-stress we collected respectively 30, 51, and 30 cow feces ($n = 60, 102$, and 60 total fecal sub-samples collected). We analyzed the metabolomic spectra extracted from cattle fecal samples by using a supervised model, OPLS-DA. Considering the three group of samples (pre-stress, stress and post-stress), we obtained a good predictive model ($Q^2 = 0.61$) with two predictive and three orthogonal latent variables (LVs) explaining 53% and 40% of the total variability, respectively (Figure 2). Applying a t -test to these predictive LV scores, we found significant differences among the three groups of samples (pre-stress, stress and post-stress) on LV1 and LV2 plane ($p < 0.00001$).

Subsequently, to provide a validation of the metabolomics technology as a powerful tool for non-invasive stress monitoring in wildlife research, we measured the GCM concentration in fecal sub-samples collected from the same feces used for metabolomics analysis. The Figure 3 shows the mean concentrations of fecal GCM for each cow during the three experimental phases. Applying a repeated measures ANOVA to these levels, the fecal GCM concentrations was not found to

Figure 3. The mean concentrations of fecal GCM calculated for each cow during the three phases (Before, During, After for cows 1-5) of the stress-induced experiment are not significantly different (Levene's test: F-ratio: 0.87, p-value: 0.44).

significantly differ across the three different experimental phases of the stress-induced experiment (Levene's test F-ratio: 0.87, *p*-value: 0.44).

Field Study

We recorded a total of 307 proximity events in 26 interactions events in both study areas across the grazing season. We collected 215 fecal samples from 40 cows fit with GPS collars with proximity sensors. Of the 215 samples, 125 were included in the non-interaction group and 90 in the interaction group. We included in the interaction group fecal samples collected from one or more cows for four consecutive days (96 h) after the day of the interaction.

To validate the fecal metabolomic analysis for monitoring acute stress response in free ranging cattle, we compared the results obtained from this 'omics' technology with those of the traditional wide used fecal glucocorticoid assay technique. We analyzed the metabolomic data by using a supervised model, OPLS-DA. Considering the two group of samples (interaction and non-interaction), we obtained a good predictive model ($Q^2 = 0.55$) with one predictive and two orthogonal latent variables (LVs) explaining 27% and 40% of the total variability, respectively (Figure 4). Applying a *t*-test to these predictive LV scores, we found significant differences among the two groups of samples (interaction and non-interaction) on LV1 and LV2 plane ($p < 0.00001$).

Subsequently, we measured the GCM concentration in fecal sub-samples collected from the same feces used for metabolomics analysis. However, the fecal GCM concentrations of the two group of feces (interaction and non-interaction) were not significantly different (*p*-value: 0.49).

DISCUSSION

In the present study, we investigated variations in metabolic profiles and GCM of cattle feces under different environmental stressors (restraint and wolf presence) to prove the efficacy of metabolomic analysis for non-invasive stress monitoring in wildlife research. Comparing the results from the 'omics' technique with those of the traditional GCM assay technique, we show that the metabolic profiles of cattle feces can better predict acute stress response in both the controlled and free-ranging environment.

Experimental Study

The biological validation of fecal metabolomic fingerprinting for assessing cattle stress response involved collecting cattle fecal samples before and after an induced-stressful event. We monitored and compared changes in fecal metabolome and fecal GCM in relation to adrenal activity during three experimental phases: pre-stress, stress, and post-stress phase. Our results clearly showed that while the fecal metabolome extracted from cattle fecal

Figure 4. OPLS-DA model built on 1H-NMR spectra obtained from cow fecal samples collected after an interaction with wolves (open diamond), and non-interaction with wolves (black). The score plot shows the metabolic differences between the two groups ($p < 0.0001$).

samples changed during the three phases of the stress-induced experiment, the fecal GCM did not change. According to our hypothesis we would have expected to see an increase of GCM levels in feces collected during the stress-phase respect to the control (pre-stress) and recovery (post-stress) phases of the experiment. This increase would have reflected the excretion of metabolized cortisol in the feces as a response to stress. There is a species-specific lag-time between the release of cortisol in the blood and the peak of GCM in the feces. In cattle this peak occurs between 8 to 16 hours and defecation starts to diminish after 24 h (Palme et al. 2000, Morrow et al 2002). However, although we extended the stress-phase of our experiment to 48 hours, mostly to monitor changes in fecal metabolome, we did not detect the peak of GCM in the fecal samples of the stress-phase. The levels of GCM were higher in cattle fecal samples collected before we performed the test, during the pre-stress phase (Figure 3), when the cows remained in their home pasture to minimize the study-induced disturbances. Indeed, the magnitude of adrenocortical response depends on both the stressor and the stress experience in the animal (Cook 2012). For our experiment we chose to use a natural (e.g., restraint, handling, transport, etc.) and not an artificial stressor (e.g. exogenous injection of Adreno-corticotrophic hormone) because we think that psychological stress could be more similar to the fear the animals experience in free-ranging situations. Nevertheless, our study animals were perhaps

habituated to restraint and handling, showing a low cortisol level in the blood compared to historical controls (baseline group) (Table 1). Thus, we can conclude that although the GCM measured in fecal samples should reflect adrenocortical activity, it is not always the case (Touma and Palme 2006, Cook 2012).

NMR-based metabolomic fingerprinting of cattle samples proved instead to reflect changes in the adrenal activity. The OPLS-DA plot (Figure 2) clearly illustrate the separation of the fecal metabolomes collected during the three experimental phases, even for the two cows with cortisol level below baseline level (Table 1). Particularly, moving along OPLS-DA1, is evident the variations over time between the metabolic profiles of the stress-phase respect to the pre- and post-stress phase together. This separation demonstrates the discrimination power of the fecal metabolome fingerprinting approach to assess stress response. However, although the results show metabolic differences between the three experimental groups, there is variations within groups. Some of the post-stress samples, for example, behaved similarly to those of the stress-phase showing that in some animals the stress response lasted up to 96 hours. So, longer than the 48 hours we chose to detect changes in cattle metabolic profiles. This heterogeneity in the stress response was further analyzed (and partially explained) in terms of microbiome composition variability, but we did not report the results in this study.

Field Study

The discriminating power of the fecal metabolome fingerprinting approach to assess stress response proved to be effective even in free-ranging animals. We conducted a field study in Washington State, using a similar approach of the experimental study that is monitor and compare changes in cattle fecal metabolome and fecal GCM in relation to the presence of wolves. Our approach required a clear definition of wolf presence; however empirical data on interactions between individuals are difficult to obtain, particularly in free-ranging animals (Cross et al. 2009). Moreover, using GPS and radio-telemetry data to document spatial-temporal proximity between individuals, might lack a finer spatial-temporal variation in predation risk (Creel et al. 2005). For this reason, we fit on both wolves and cattle GPS collars with proximity sensors, a relatively new technology to record real-time interactions between the two species. Thus, proximity sensors allowed us to measure directly cattle stress response due to a known interaction with wolves. As we expected, our results show that metabolic profiles of cattle fecal samples collected after an encounter with wolves differed from metabolic profiles of cows far from wolves in space and time (Figure 4). However, these metabolic changes did not reflect changes in the fecal GCM. In fact, in both of our study areas the mean concentration of fecal GCM did not differ in relation to wolf presence.

Once again, metabolomic fingerprinting of cattle fecal samples prove to be a more reliable methodology compared to the fecal GCM analysis to assess stress response in free-ranging animals. Indeed, metabolomic technique uses a holistic approach defining the complete biochemical phenotype (metabolome) of an integral living system. Instead the fecal GCM technique attempts to estimate an animal's physiological state based on single molecular targets (e.g., glucocorticoids). One of the main limits inherent in this reductionist approach consists in the impossibility of interpreting the functioning of an integral living system, based on the characteristics and properties of the individual components of that system (Weiss 1973, Ahn et al. 2006). Moreover, because metabolic finger-printing is an approach in metabolomic technology normally utilized for comparison or discrimination analysis, it resulted very suitable in wildlife research to study animal physiology under different environmental perturbations (e.g., presence or absence of predators).

Our field study represents the first research that utilizes a new cutting-edge technology such as metabolomics for stress monitoring in wildlife research. There is a growing concern about the risk effects of wolf presence on livestock (i.e., behavioral and physiological responses of prey when encountering wolves). However, these effects are often difficult to measure or quantify and thus hard to integrate in applied wildlife management. We believe that NMR-based metabolomic fingerprinting will provide a better understanding of the potential economic conflicts and the costs for successfully implementing recovery programs for endangered species such as wolves in recolonizing areas.

ACKNOWLEDGEMENTS

This study was funded by the Washington Department of Fish and Wildlife. We thank the livestock producers that participated to our study,

and Carter Niemeyer and C. Steven Borrego for wolf captures operations.

LITERATURE CITED

- Ahn, C. A., M. Tewari, C. S. Poon, and R. S. Phillips. 2006. The limits of reductionism in medicine: could systems biology offer an alternative? *PLoS Medicine* 3(6):e208.
- Brindle, J. T., H. Antti, E. Holmes, G. Tranter, J. K. Nicholson, H. W. L. Bethell, S. Clarke, P. M. Schofield, E. McKilligin, D. E. Mosedale, and D. J. Grainger. 2002. Rapid and non-invasive diagnosis of the presence and severity of coronary heart disease using H-1 NMR-based metabolomics. *Natural Medicine* 8:1439-1444.
- Broom, D. M., and K. G. Johnson. 1993. Stress and animal welfare. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Coen, M., M. O'Sullivan, W. A. Bubb, P. W. Kuchel, and T. Sorrell. 2005. Proton nuclear magnetic resonance-based metabolomics for rapid diagnosis of meningitis and ventriculitis. *Clinical Infectious Diseases* 41:1582-1590.
- Cook, N. J. 2012. Review: Minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. *Canadian Journal of Animal Science* 92:227-259.
- Creel, S., J. Winnie, Jr., B. Maxwell, K. Hamlin, and M. Creel. 2005. Elk alter habitat selection as an antipredator response to wolves. *Ecology* 86:3387-3397.
- Cross, P. C., J. A. Drewe, V. Patrek, G. P. Pearce, and M. D. Samuel. 2009. Wildlife population structure and parasite transmission: implications for disease management. Pages 9-29 in R. J. Delahay, M. R. Hutchings, G. C. Smith, editors. *Management of disease in wild mammals*. Springer Publishing, Tokyo, Japan.
- Drewe, J. A., N. Weber, S. P. Carter, S. Bearhop, X. A. Harrison, S. R. X. Dall, R. A. McDonald, and R. J. Delahay. 2012. Performance of proximity loggers in recording intra- and inter-species interactions: a laboratory and field-based validation study. *PLoS ONE* 7(6):e39068.
- Lindon, J. C., E. Holmes, and J. K. Nicholson. 2007. Metabonomics in pharmaceutical R & D. *The FEBS Journal* 274:1140-1151.
- Möstl, E., J. L. Maggs, G. Schrötter, U. Besenfelder, and R. Palme. 2002. Measurement of cortisol metabolites in faeces of ruminants. *Veterinary Research Communications* 26:127-139.
- Oliver, S. G., M. K. Winson, D. B. Kell, and F. Baganz. 1998. Systematic functional analysis of the yeast genome. *Trends in Biotechnology* 16:373-378.
- Palme, R., C. Robia, W. Baumgartner, and E. Möstl. 2000. Transport stress in cattle as reflected by an increase in faecal cortisol metabolites. *Veterinary Record* 146:108-109.
- Plumb, R. S., J. H. Granger, C. L. Stumpf, K. A. Johnson, B. W. Smith, S. Gaulitz, I. D. Wilson, and J. Castro-Perez. 2005. A rapid screening approach to metabolomics using UPLC and oa-TOF mass spectrometry: application to age, gender and diurnal variation in normal/Zucker obese rats and black, white and nude mice. *Analyst* 130:844-849.
- Rosenblum, E. S., R. S. Tjeerdema, and M. R. Viant. 2006. Effects of temperature on host-pathogen-drug interactions in red abalone, *Haliotis rufescens*, determined by ¹H NMR metabolomics. *Environmental Science and Technology* 40:7077-7084.

- Stanley, E. G., N. J. Bailey, M. E. Bollard, J. N. Haselden, C. J. Waterfield, E. Holmes, and J. K. Nicholson. 2005. Sexual dimorphism in urinary metabolite profiles of Han Wistar rats revealed by nuclear-magnetic-resonance-based metabonomics. *Analytical Biochemistry* 343:195-202.
- Touma, C., and R. Palme. 2006. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Annals of the New York Academy of Sciences* 1046:54-74.
- Wasser, S. K., K. E. Hunt, J. L. Brown, K. Cooper, C. M. Crockett, U. Bechert, J. J. Millspaugh, S. Larson, and S. L. Monfort. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. *General and Comparative Endocrinology* 120:260-275.
- Weiss, P. 1973. *The science of life*. Futura Publishing Company, New York, NY.
- Wiklund, S., E. Johansson, L. Sjöström, E. J. Mellerowicz, U. Edlund, J. P. Shockcor, J. Gottfries, T. Moritz, and J. Trygg. 2008. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. *Analytical Chemistry* 80:115-122.