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## Research paper

## Development of a hyena immunology toolbox

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## ABSTRACT

Animals that hunt and scavenge are often exposed to a broad array of pathogens. Theory predicts the immune systems of animals specialized for scavenging should have been molded by selective pressures associated with surviving microbial assaults from their food. Spotted hyenas (*Crocuta crocuta*) are capable hunters that have recently descended from carrion feeding ancestors. Hyenas have been documented to survive anthrax and rabies infections, and outbreaks of several other viral diseases that decimated populations of sympatric carnivores. In light of the extreme disease resistance manifested by spotted hyenas, our objective was to identify tools available for studying immune function in spotted hyenas and use these tools to document the hyena antibody response to immunization. Domestic cats (*Felis catus*) are the closest phylogenetic relatives of hyenas that have been studied in detail immunologically, and we hypothesized that anti-cat isotype-specific antibodies would cross react with hyena immunoglobulin epitopes. We used ELISA and Western blots to test isotype-specific anti-feline antibodies for specific cross-reaction to hyena Ig epitopes. Molecular weights of heavy (IgA, IgG, IgM) and light chains of hyena immunoglobulins were determined by protein electrophoresis, and as expected, they were found to be similar to feline immunoglobulins. In order to further validate the cross-reactivity of the anti-feline antibodies and document the hyena humoral response, eight spotted hyenas were immunized with dinitrophenol conjugated keyhole limpet hemocyanin (DNP-KLH) and serum anti-DNP responses were monitored by enzyme-linked immunosorbent assay (ELISA) for one year. The full array of isotype-specific antibodies identified here will allow veterinarians and other researchers to thoroughly investigate the hyena antibody response, and can be used in future studies to test hypotheses about pathogen exposure and immune function in this species.

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## 1. Introduction

Wildlife disease outbreaks can have major impacts on conservation efforts and lasting effects on ecosystem processes (Claude, 1996). For example, rabies and canine distemper virus (CDV) epizootics were associated with the

extirpation of wild dogs (*Lycan pictus*) in the Maasai Mara National Reserve (MMNR) in Kenya (Alexander and Appel, 1994; Kat et al., 1995, 1996). Additionally, a CDV outbreak in East Africa killed more than 1000 lions (*Panthera leo*) (Munson et al., 2008; Roelke-Parker et al., 1996).

Animals that hunt and scavenge are likely exposed to a broad array of pathogens (Schulenburg et al., 2009). Although most carnivores, including lions and wild dogs, scavenge to some extent (Houston, 1979), theory predicts that the immune systems of carnivores exhibiting morphological specializations for carrion-feeding should have been molded by selective pressures associated with surviving microbial assaults from their food (Blount et al., 2003; Mendes et al., 2006; Schulenburg et al., 2009). Spotted hyenas (*Crocuta crocuta*) are capable hunters that have descended within the last million years from carrion feeding ancestors (Lewis and Werdelin, 2000; Werdelin, 1989). Despite documented exposure to anthrax, rabies, CDV and several other pathogens, spotted hyenas in East Africa have exhibited extremely low mortality rates due to infectious diseases, even when epizootics decimated sympatric carnivore populations (Alexander et al., 1995; East et al., 2001, 2004; Harrison et al., 2004; Lembo et al., 2011; Watts and Holekamp, 2009). Spotted hyenas are the most abundant large carnivores in Africa, and may play a critical role in the ecology of disease in African wildlife and domestic animals throughout the continent (Hofer, 1998).

In light of the extreme disease resistance manifested by hyenas and their potential importance for overall disease dynamics in African ecosystems, we set out to identify tools available for studying immune function in the spotted hyena. The two specific aims of this study were to identify antibodies that cross-react with hyena immunoglobulins and to assess the dynamics of the hyena humoral immune response to immunization with a non-pathogenic antigen. Domestic cats (*Felis catus*) were the closest phylogenetic relatives of hyenas that had been studied in detail immunologically (Bininda-Emonds et al., 1999; O'Brien and Johnson, 2005), and we hypothesized that anti-cat isotype-specific antibodies would cross react with hyena immunoglobulin (Ig) epitopes.

We used ELISAs to test isotype-specific anti-feline antibodies for cross-reaction to hyena Ig epitopes and to assess temporal dynamics of hyena immunoglobulins in response to immune challenge. We used Western blots to confirm cross-reactivity and to estimate the molecular weight of hyena immunoglobulins. Reverse transcriptase polymerase chain reaction (RT-PCR), serum neutralization tests, western blots, and agglutination tests have been used previously to document pathogen exposure in spotted hyenas (East et al., 2001, 2004; Honer et al., 2006; Speck et al., 2008), but only a few studies have gone beyond documenting exposure and examined the immune response itself (East et al., 2008; Hanley et al., 2005; Honer et al., 2006; van Helden et al., 2008). The current paper represents the first report of antibodies capable of detecting spotted hyena immunoglobulins other than IgG, and the first to document the temporal dynamics of the humoral immune response of the major isotypes with a defined antigen.

## 2. Materials and methods

### 2.1. Captive spotted hyenas, sample collection, and immunization

All captive spotted hyenas were born and housed in the Field Station for Behavioral Research (FSBR) of the University of California, Berkeley (UCB). Berger et al. (1992) describe the husbandry conditions at this facility. Eight healthy adult (4 female, 4 male) spotted hyenas were subjected to an immunization protocol approved by both the University of California, Berkeley (Animal Use Protocol # R091-0609R) and Michigan State University (MSU) Institutional Animal Care and Use Committees (IACUC) (AUF # 07/08-099-00). Captive hyenas ranged from 4 to 17 years of age when immunizations began. Animals were immunized with blow dart-delivered i.m. injections of ketamine (4–6 mg/kg) and xylazine (1 mg/kg) for immunization and blood sampling.

Blood samples for serum analysis were collected on days 0, 14, 28, 180 and 365 from the jugular vein using Vacutainer tubes (BD, Franklin Lakes, NJ, cat# 366430), allowed to clot at ambient temperature, and then centrifuged. Serum was aliquoted into cryovials and stored at  $-80^{\circ}\text{C}$ . Following blood collection on day 0, four animals were immunized with 250  $\mu\text{g}$  of 2,4-dinitrophenol conjugated to keyhole limpet hemocyanin (DNP-KLH) (Calbiochem/EMD Biosciences, Germany, cat# 324121) in sterile water, and four animals were immunized with 250  $\mu\text{g}$  of DNP-KLH emulsified in TiterMax Gold (TiterMax USA, Inc., Norcross, Georgia, cat# G1). Each animal received one subcutaneous 250  $\mu\text{l}$  injection on each side of the neck for a total of 500  $\mu\text{l}$ . A booster, consisting of 250  $\mu\text{g}$  of DNP-KLH in sterile water, was administered on day 14. Blood was collected on day 14 prior to administration of booster injections.

### 2.2. Samples from wild spotted hyenas

Serum samples from wild spotted hyenas were collected as part of the long-term research project in the Maasai Mara National Reserve, Kenya, started in 1988. Wild spotted hyenas were immobilized using tiletamine-zolazepam (6.5 mg/kg Telazol; Fort Dodge Animal Health, Fort Dodge Iowa) in a plastic dart fired from an air rifle (Telinject Inc., Saugus, California) (Holekamp and Sisk, 2003). Whole blood samples from wild hyenas were collected from the jugular vein using Vacutainer tubes and allowed to clot at ambient temperature, then centrifuged, aliquoted into cryovials, and snap frozen in liquid nitrogen. All immobilization protocols were approved by the MSU IACUC (AUF # 07/08-099-00) and the Kenya Wildlife Service.

### 2.3. Purification of spotted hyena immunoglobulins

Pooled serum samples from wild spotted hyenas were used for purification of hyena IgG, IgM, and IgA. Immunoglobulins from a 25 ml serum pool were first precipitated in 50% ammonium sulphate solution (Steward and Petty, 1972), redissolved in phosphate buffered saline (PBS), and then extensively dialyzed against water.

The dialysate was centrifuged and the precipitate was redissolved in PBS as a crude IgM preparation; this was then sized over Sephacryl S-300 using a 50 cm × 2.5 cm column and the peak fraction collected as partially purified hyena IgM. Meanwhile, the clarified dialysate was passed over a DEAE Bio-Gel A column (12 cm × 2.5 cm) equilibrated with 0.1 M Tris pH 8.0, and a semi-purified IgG fraction was eluted using 0.1 M Tris pH 8.0 containing 45 mM sodium chloride; this peak was then sized over Sephacryl S-300 and the appropriate sized IgG fraction was collected. A further fraction was eluted from the DEAE column using 0.1 M Tris pH 8.0 containing 70 mM sodium chloride. This peak was then sized over Sephacryl S-300, and Ig which eluted at a position behind the IgM peak but ahead of the IgG peak, was collected as a hyena IgA enriched fraction. Protein concentrations were determined with a UV spectrophotometer reading at 280 nm. Further details about the purification of immunoglobulins are available in Grant (1995).

#### 2.4. Western blot verification of cross-reactive antibodies and molecular weight of hyena Igs

Purified hyena and feline (*F. catus*) IgG, IgM, and IgA fractions were subjected to SDS-PAGE and Western blot analysis under denaturing conditions to verify cross-reactivity of anti-feline antibodies to hyena Ig epitopes and to compare the molecular weight of Ig heavy and light chains between the two species. Purified hyena immunoglobulins were diluted in Laemli sample buffer (Laemmli, 1970) with 0.1 M dithiothreitol, and were denatured by heating at 95 °C for 5 min. IgG, IgM and IgA samples were then loaded at 2 µg/well, 1 µg/well, and 2.5 µg/well, respectively, into a 10% Tris-HCl polyacrylamide gel. Prestained protein standards were added to each gel to assess molecular weight of the target proteins. Running conditions (150 V for 45 min) and running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) were used in accordance with manufacturer's instructions for the Bio-Rad Mini Protean II System.

Hyena and feline serum samples diluted 1:10 in Laemli sample buffer were used for analysis of cross-reactivity and molecular weight comparison of both heavy and light chains. Initial assays using serum samples exhibited migration patterns that appeared to be influenced by large quantities of albumin. AlbuminOut (GBiosciences, cat# 786–251) was therefore used to remove albumin from the serum samples, and this allowed us to estimate the molecular weight of target proteins more accurately.

Prior to transfer, the nitrocellulose membranes were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 15 min. Proteins were transferred at 100 V for 75 min at 4 °C. After transfer, membranes were placed directly into 5% non-fat dry milk (NFDm) in Tris buffered saline (TBS) (5% NFDm-TBS; 420 mM Tris-HCl, 80 mM Tris, 1.5 M NaCl, 5 g/L blotting grade NFDm) and incubated overnight at 4 °C. See Table 1 for a comprehensive list of antibodies tested.

Staining was done using a Bio-Rad Multiscreen Apparatus. Primary monoclonal detection antibodies were added at 1 µg/ml in 1% NFDm-TBS and incubated for 90 min at ambient temperature on a shaking platform. The

membrane was then washed 3 times with 0.05% Tween-20 in TBS (TBS-T). Secondary biotin-F(ab')<sub>2</sub> fragment goat anti-mouse IgG (H+L) was used with mAbs and was diluted 1:5000 in 1% NFDm-TBS and incubated on a shaker for 60 min. The membrane was again washed 3 times with TBS-T. Extravidin-peroxidase was diluted 1:1000 in TBS-T and incubated with the membrane for 30 min. Color change was developed using a CN/DAB substrate kit. Color change reaction was stopped by washing with distilled water after approximately 10 min. The staining process used with polyclonal HRP conjugated anti-IgG, anti-IgM, and anti-IgA involved the following process: a blocking step, wash, incubation of antibodies at 1 µg/ml, a final wash step, and followed by color development. Images were captured using the Bio-Rad VersaDoc Molecular Imaging system, and molecular weight was determined using Bio-Rad Quantity One software package.

#### 2.5. Identification of cross-reactive anti-feline antibodies and quantification of humoral response using an enzyme-linked immunosorbent assay (ELISA)

To further examine the cross-reactivity of commercially available anti-feline immunoglobulins with spotted hyena immunoglobulins and to quantify spotted hyena humoral response to immunization, we tested spotted hyena sera using ELISAs. ELISA plates were coated with either 50 µl of bovine serum albumin (BSA) as a control or DNP-BSA at 5 µg/ml in 50 µl of carbonate buffer (0.1 M, pH 9.5) and stored overnight at 4 °C. BSA coated wells were used as a reference for background binding.

Plates were washed with PBS containing 0.05% Tween-20 (PBS-T). Plates were then blocked for 1 h with 5% NFDm-PBS. This and all subsequent incubations were run at ambient temperature on a shaking platform. Plates were then washed with PBS-T. Monoclonal anti-DNP (Silver Lakes Research, cat# CH1911) was subjected to serial two fold dilutions to generate a standard curve. Negative control wells were incubated with PBS only, and pre-immune sera (day 0) also served as an additional control. See Table 2 for dilutions of serum samples and anti-isotype detection antibodies. All serum samples were incubated for 90 min before washing and each sample was tested in duplicate. Plates were then incubated with primary anti-isotype antibodies for 90 min and washed with PBS-T. Next, 50 µl of anti-mouse IgG-HRP was diluted 1:5000 and added to each well with mAbs, including the standard wells. Plates were then incubated for 45 min and washed with PBS-T. Color change was developed using 50 µl/well of 3,3',5,5'-tetramethylbenzidine and reaction was stopped using 50 µl of 0.5 M H<sub>2</sub>SO<sub>4</sub> after 20–60 min, depending on the primary antibody (Ab). Absorbance was then read at 450 nm on a standard plate reader.

#### 2.6. Statistical analysis

All analyses were performed in R (R Development Core Team, 2011). Results from ELISAs are expressed as anti-DNP equivalent concentrations in order to create a relative measure that can be used for all isotypes. The

**Table 1**  
Antibodies tested by ELISA and Western blot (WB).

Target	Catalog #	mAb or pAb	Assays confirmed <sup>a</sup>	Supplier
IgA	IgA5-3B	mono	ELISA (cat, hyena)	CMIC <sup>b</sup>
IgA	CDA2-43	mono	ELISA (cat, hyena)	CMIC
IgA	NB7264	poly	ELISA (cat, hyena), WB (cat, hyena)	Novus Biologicals
IgE	E6-71	mono	ELISA (cat, hyena)	CMIC
IgE	E2-19	mono	ELISA (cat, hyena)	CMIC
IgG	GPB2-2	mono	ELISA (cat, hyena), WB (cat)	CMIC
IgG	04-20-02	poly	ELISA (cat, hyena), WB (cat, hyena)	KPL <sup>c</sup>
IgG(H+L)	102-065-003	poly	ELISA (cat, hyena), WB (cat, hyena)	Jackson ImmunoResearch
IgG(H+L)	OB-680-05	poly	ELISA (cat, hyena), WB (cat, hyena)	Southern Biotechnology
IgM	CM7	mono	ELISA (cat, hyena), WB (cat, hyena)	CMIC
IgM	CM6E	mono	ELISA (cat, hyena), WB (cat, hyena)	CMIC
IgM	04-20-03	poly	ELISA (cat, hyena), WB (cat, hyena)	KPL
$\kappa$ light chain	FIG1-7A	mono	ELISA (cat, hyena), WB (cat, hyena)	CMIC
$\lambda$ light chain	CAG8-7C	mono	ELISA (cat, hyena), WB (cat)	CMIC

<sup>a</sup> Parentheses contain the species for which antibody binding has been confirmed.

<sup>b</sup> Custom Monoclonals International Corp.

<sup>c</sup> Kirkegaard & Perry Laboratories, Inc.

**Table 2**  
Peak anti-DNP equivalent concentration.

	Age	Adjuvant	IgA	IgE	IgG	IgM	$\kappa$ light chain	$\lambda$ light chain	poly IgG	poly IgM
Individual										
A	4.7	YES	0.464	0.561	1.623	2.104	6.2501	0.392	12.500 <sup>a</sup>	0.722
B	6.8	NO	0.139	0.138	0.123	6.361	4.035	0.077	5.365	2.502
C	11.2	YES	0.322	0.350	0.684	2.361	6.2501	0.235	12.500 <sup>a</sup>	1.044
D	11.3	NO	0.042	0.048	0.024	0.356	0.824	0.016	1.365	0.236
E	13.9	YES	0.011	0.024	0.009	0.192	0.371	0.006	0.820	0.101
F	15.1	NO	0.003	0.011	0.002	0.159	0.106	0.001	0.322	0.134
G	15.1	YES	0.034	0.035	0.018	0.307	0.608	0.012	1.183	0.175
H	17.0	NO	0.010	0.006	0.001	0.208	0.055	0.001	0.160	0.198
Detection Ab catalog #			IgA5-3B	E6-71	GPB2-2	CM7	CAG8-7C	FIG1-7A	04-20-02	04-20-03
Detection Ab conc. ( $\mu$ g/ml)			5	10	10	5	10	5	0.5	0.5
Serum dilution			1:100	1:100	1:100	1:100	1:100	1:100	1:4000	1:1000

Age is in years. Concentrations are the mean of duplicate ELISA results. For all assays, the youngest four individuals had higher antibody responses than the four oldest individuals.

<sup>a</sup> Indicates the concentration exceeded the linear range of the standard curve and was assigned the maximum value of the linear range of the standard curve.

Calib package in R was used to create a logistic regression based on the absorbance values of the standards in each plate. The regression was then used to calculate the relative anti-DNP concentration for each sample. The mean of duplicate anti-DNP equivalent concentrations for each time point for each individual were used in all analyses. Using serum diluted 1:1000 with polyclonal IgM detection Ab or serum diluted 1:4000 with polyclonal IgG detection Ab, we calculated a standardized percent of IgG and IgM out of the sum total of IgG and IgM (percent IgG =  $IgG / (IgG + IgM)$ ).

In the ELISAs using polyclonal anti-IgG and monoclonal anti- $\kappa$ , the absorbance values from the two individuals with the highest titers exceeded the linear range of the standard curve. We were unable to further optimize the assay conditions to fall within the linear range of the standard curve because doing so would render undetectable the absorbance values of the two individuals with the lowest concentrations of anti-DNP antibodies. Instead of extrapolating beyond the linear range of the standard curve for the two individuals with the highest absorbance values, we assigned to these two individuals the maximum values within the linear range of the standard curve.

### 3. Results

#### 3.1. Western blot verification of cross-reactive antibodies and molecular weight of hyena immunoglobulins

Hyena  $\gamma$  heavy chain was slightly smaller than feline  $\gamma$  heavy chain based on Western blot analysis (Fig. 1). We estimate feline  $\gamma$  heavy chain to be 55–56 kilodaltons (kDa), whereas hyena  $\gamma$  heavy chain was 53–54 kDa (Table 3).

**Table 3**  
Molecular weight (kDa) of heavy and light chains.

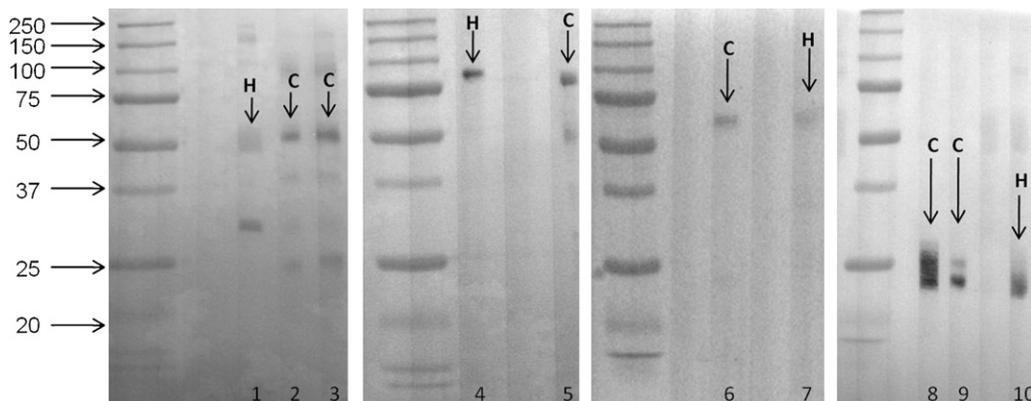
Target	Hyena	Cat
$\alpha$	57–61 <sup>a</sup>	54–60 <sup>a</sup> , 54 <sup>b</sup>
$\epsilon$	Not detected <sup>a</sup>	Not detected <sup>a</sup>
$\gamma$	53–54 <sup>a</sup>	55–56 <sup>a</sup> , 50 <sup>b</sup> , 50 <sup>c</sup> , 59 <sup>d</sup>
$\mu$	77–82 <sup>a</sup>	74–80 <sup>a</sup> , 74 <sup>b</sup> , 72 <sup>c</sup>
$\kappa$ light chain	22–25 <sup>a</sup>	23–25 <sup>a</sup> , 23–29 <sup>b</sup> , 22 <sup>c</sup> , 27 <sup>d</sup>
$\lambda$ light chain	Not detected <sup>a</sup>	23–28 <sup>a</sup> , 24–27 <sup>b</sup> , 22 <sup>c</sup> , 27 <sup>d</sup>

<sup>a</sup> Results from this study.

<sup>b</sup> Grant (1995).

<sup>c</sup> Klotz et al. (1985).

<sup>d</sup> Yamada et al. (2007).



**Fig. 1.** Western blot of heavy and light chains in domestic cats (C) and spotted hyenas (H). Molecular weight standards are indicated with arrows and weights are in kilodaltons. Lane 1. Purified hyena IgG. Hyena  $\gamma$  chain detected with polyclonal anti-IgG (04-20-02). Lane 2. Purified cat IgG. Cat  $\gamma$  chain detected with monoclonal anti-IgG (GPB2-2). Lane 3. Purified cat IgG. Cat  $\gamma$  chain detected with polyclonal anti-IgG (04-20-02). Lane 4. Purified hyena IgM. Hyena  $\mu$  chain detected with monoclonal anti-IgG (CM7). Lane 5. Purified cat IgM. Cat  $\mu$  chain detected with monoclonal anti-IgM (CM7). Lane 6. Purified cat IgA. Cat  $\alpha$  chain detected with polyclonal anti-IgA (NB7264). Lane 7. Purified hyena IgA. Hyena  $\alpha$  chain detected with polyclonal anti-IgA (NB7264). Lane 8. Cat serum with albumin removed. Cat light chain detected with monoclonal anti- $\lambda$  light chain (CAG8-7C). Lane 9. Cat serum with albumin removed. Cat light chain detected with monoclonal anti- $\kappa$  light chain (FIG1-7A). Lane 10. Hyena serum with albumin removed. Hyena light chain detected with monoclonal anti- $\kappa$  light chain (FIG1-7A).

Note that the estimated molecular weight for each heavy or light chain we tested varied slightly depending on whether we used purified Ig or sera, and also based on the serum dilution used; for this reason, molecular weights are presented as ranges rather than as specific values. Also, variations in sample preparation may explain the slight differences between our results and previously published results (Grant, 1995; Klotz et al., 1985; Yamada et al., 2007). Hyena  $\mu$  chain, estimated at 77–82 kDa, was slightly larger than feline  $\mu$  chain, which we estimated at 74–80 kDa. Feline and hyena  $\alpha$  heavy chains were 54–60 kDa and 57–61 kDa, respectively. Neither feline nor hyena  $\epsilon$  heavy chain could be detected in serum by Western blot; we were unable to obtain purified IgE.

Anti- $\kappa$  light chain mAb detected a 23–25 kDa light chain protein band in cat serum samples and a 22–25 kDa band in hyena serum samples. The molecular weight and staining patterns of the anti- $\kappa$  light chain mAb are consistent with the previously reported molecular weight of carnivore light chains (Grant, 1995; Klotz et al., 1985; Yamada et al., 2007). See Table 3 for a complete listing of molecular weights of feline and hyena heavy and light chains. Anti- $\lambda$  light chain mAb detected a 23–28 kDa protein band in cat serum, but no bands were detectable in hyena serum or purified Ig.

### 3.2. Quantification of humoral response using ELISA

During the weeks after experimental immunization of captive hyenas, anti-DNP specific antibodies in serum samples increased from pre-immunization day 0 titers for all isotypes tested (Fig. 2). As expected, day 14 serum samples were elevated above baseline, and peak anti-DNP concentrations in serum samples were attained on day 28 post-immunization for most individuals and isotypes. Individuals with the strongest anti-DNP response still had detectable titers at one year post-immunization.

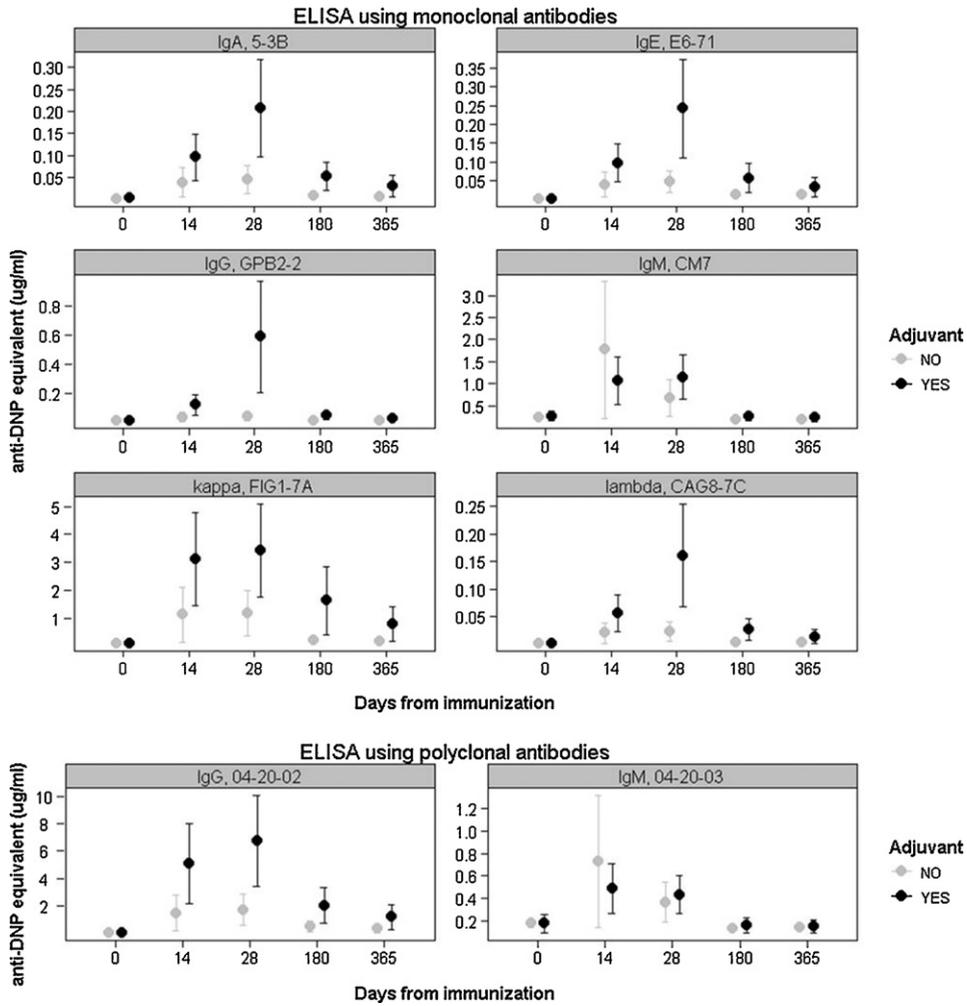
The anti-DNP temporal dynamics for IgG and IgM were found to be similar regardless of whether we used

polyclonal Abs or mAbs as anti-isotype probes. Individuals that had the highest IgG or IgM titers using mAb detection antibodies also had the highest titers using polyclonal antibodies for detection, and peak titers were reached on day 28 in most cases. Anti-IgM had the highest anti-DNP equivalent concentration for all individuals on day 0, suggesting that natural IgM antibodies to DNP were present prior to immunization (Ochsenbein and Zinkernagel, 2000). Among the anti-Ig mAbs tested, the anti- $\kappa$  light chain detection antibody produced the highest anti-DNP equivalent concentrations; light chains are associated with all isotypes, so light chains are represented in higher concentrations than heavy chains.

Not surprisingly, use of adjuvant tended to elevate anti-DNP equivalent concentrations over those obtained with no adjuvant, although no statistical tests on adjuvant effect were performed due to small sample sizes. Interestingly, however, the four youngest individuals immunized attained the four highest anti-DNP titers across all isotypes tested, regardless of whether or not they received adjuvant with the initial immunization (Table 2). The oldest hyena, individual H, was 17 years old at the initial immunization and did not receive adjuvant. Individual H did not produce a detectable response at a 1:100 serum dilution using mAb detection antibodies for IgG and  $\lambda$ , but did produce detectable responses for all other isotypes tested.

### 3.3. Temporal dynamics of the IgG and IgM relationship

Using serum diluted 1:1000 with polyclonal IgM detection Ab or serum diluted 1:4000 with polyclonal IgG detection Ab, we calculated a standardized percent of IgG and IgM out of the sum total of IgG and IgM (percent IgG =  $IgG / (IgG + IgM)$ ). On day 0, anti-DNP IgG accounted for roughly 10% of the sum of IgG and IgM (Fig. 3). However, by days 14 and 28, IgG accounted for approximately 70% and 80% of the total, respectively. At days 180 and 365,



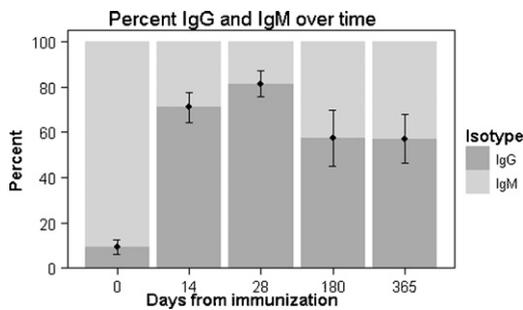
**Fig. 2.** ELISA results from DNP-KLH immunization. Each point corresponds to the mean anti-DNP equivalent concentration ( $\mu\text{g/ml}$ ) and error bars represent the SEM ( $n=4$ ). Black points are the means from individuals immunized with adjuvant and gray points are from individuals immunized without adjuvant. Day 0 represents pre-immune sera and baseline anti-DNP concentration found in serum samples.

IgG continued to account for more than 50% of the total anti-DNP IgG and IgM.

#### 4. Discussion

Here we identified cross-reactive antibodies that specifically bind hyena epitopes of the four major secreted heavy chain isotypes found in carnivores, as well as two cross-reactive anti-light chain antibodies. The specific cross-reactivity of anti-cat Ig isotypes suggests a high level of homology between cats and hyenas. These antibodies can potentially be used for broad, cost effective monitoring of pathogen infections in spotted hyenas, and to establish baseline parameters of health and disease among wild members of this species throughout sub-Saharan Africa and among captive hyenas at zoos and research facilities. Additionally, these antibodies can likely be used to detect immunoglobulins in other species in the Hyaenidae family.

We confirmed binding of the antibodies to hyena immunoglobulins here with Western blots, and molecular



**Fig. 3.** Percent of total anti-DNP IgG and anti-DNP IgM. Percent IgG =  $\text{IgG}/(\text{IgG} + \text{IgM})$ . Percent IgM =  $\text{IgM}/(\text{IgG} + \text{IgM})$ . Serum was diluted 1:4000 for IgG and 1:1000 for IgM. Polyclonal detection antibodies were used at  $0.5 \mu\text{g}/\mu\text{l}$  for both IgG and IgM. Error bars represent the SEM of the percents for each day in the time course ( $n=8$ ). Individuals were included regardless of adjuvant status.

weights were found to be close to that of cat immunoglobulins for each isotype examined. When we then used the antibodies to monitor the magnitude and temporal dynamics of humoral responses directed against the hapten-carrier complex DNP-KLH in immunized captive spotted hyenas, we observed the common pattern of an increased Ig concentration for all isotypes by day 14, with IgM concentration tapering off quickly and a gradual decrease in other isotypes over the course of one year. Thus, insofar as neither the magnitude nor temporal dynamics of the humoral response were unusual in spotted hyenas, these responses are unlikely to account for the unusual disease resistance observed in this species.

We were only able to identify one anti-IgG mAb that cross-reacted with hyena epitopes, despite identifying several cross-reacting polyclonal anti-IgG antibodies. Furthermore, this anti-IgG mAb showed either weak affinity for hyena IgG or was specific for an IgG subclass that is less abundant in hyenas than in cats. However, it will now be possible to develop anti-hyena IgG subclass-specific mAbs using the purified hyena IgG. Variation in Ab structure among species is often most pronounced in subclasses of the major isotypes (Grant, 1995), and differentiation between IgG<sub>2a</sub> and IgG<sub>1</sub> would aid in the study of Th1 and Th2 subsets (Mosmann and Coffman, 1989; Steinman, 2007).

Interestingly, both ELISA and Western blot detected  $\kappa$  light chains at much higher concentrations than  $\lambda$  light chains in hyena sera. Generally, carnivores have approximately 90%  $\lambda$  and 10%  $\kappa$  light chains (Tizard, 2009); more specifically, cats have been reported to have a 3:1 ratio of  $\lambda$ : $\kappa$  using the same antibodies we used in this study (Grant, 1995). It is possible that the reversed light chain ratio we observed in hyenas in this study is due to low affinity of the anti- $\lambda$  light chain cat antibody for hyena immunoglobulins. However, the alternative possibility, that hyenas have a reversed light chain ratio compared to that found in most carnivores, merits further investigation.

To our knowledge, only three previous studies have reported cross-reacting antibodies that recognize immunoglobulins from any hyena species. First, anti-human IgA and IgM were found to cross-react with IgA and IgM from striped hyenas (*Hyaena hyaena*) using a gel diffusion assay (Neoh et al., 1973). Second, a polyclonal anti-cat IgG was used in an indirect immunofluorescence assay for diagnosis of coronavirus infection in spotted hyenas in Tanzania (East et al., 2004). Finally, polyclonal anti-cat IgG was used for Western blot detection of feline immunodeficiency virus (FIV) in spotted hyenas (Troyer et al., 2005). Two previous studies also used commercially available, Ab based test kits for confirmatory diagnosis of coronavirus and FIV infections (East et al., 2004; Harrison et al., 2004).

There are significant advantages of using hyena specific antibodies over other existing techniques for evaluating pathogen-specific responses. Most studies of pathogens infecting hyenas have relied on serum neutralization tests, agglutination assays, RT-PCR, or competitive ELISAs that do not require species-specific antibodies (Alexander et al., 1995; Cronwright-Snoeren, 2010; East et al., 2001; Harrison et al., 2004). Each of these existing techniques

is limited in its usefulness by various factors, and use of the newly discovered antibodies described here would avoid many of these limitations. Serum neutralization tests rely on cell culture, which can be labor- and resource-intensive (Wellehan et al., 2009). Positive RT-PCR results for pathogens provide clear evidence of infection, but offer little information about duration of the infection or the nature of the immune response. Additionally, RT-PCR can only detect active infections. Agglutination tests are rapid and cost-effective, but are most sensitive to pentameric IgM and less sensitive to other isotypes (Cohen et al., 1967). Competitive ELISAs and serum neutralization tests requiring no species-specific antibodies are limited because they permit no analysis of specific immunoglobulin isotypes. Rapid diagnosis using ELISA techniques can aid wildlife managers and veterinarians by permitting them to quickly diagnose disease outbreaks, and allowing them to develop better-informed responses to such outbreaks.

#### 4.1. Potential uses of the new antibodies

We found that hyena antibodies can be detected in serum at least one year after exposure, long after many pathogens would be cleared from the host. Testing for both IgG and IgM has been used previously to stage infections; this method was used to assess whether infections are in earlier or later stages for tularemia (Carlsson et al., 1979), dengue (Innis et al., 1989), Rift Valley Fever (Pepin et al., 2010), myxomatosis in rabbits (*Oryctolagus cuniculus*) (Kerr, 1997), and West Nile virus in equids (Durand et al., 2002). Pathogens to which wild spotted hyenas are known to be exposed include CDV, FIV, feline panleukopenia virus/canine parvovirus, feline coronavirus/feline infectious peritonitis virus, feline calicivirus, rabies, and bluetongue (Alexander et al., 1994; East et al., 2001; Harrison et al., 2004; Troyer et al., 2005). The temporal dynamics apparent in the changing Ig ratios (Fig. 3) in our hyena subjects might be carefully exploited to stage infections. However, we emphasize that samples positive for IgM, but negative for IgG, must be interpreted cautiously, as this might indicate either an early stage infection or simply the presence of natural antibodies (Ochsenbein and Zinkernagel, 2000). The results of our experimental immunizations of captive hyenas show that all individuals had readily detectable anti-DNP IgM in pre-immune sera. Pre-infection sera are seldom available in serological studies of wildlife, so natural IgM might be misinterpreted as an active infection based solely on agglutination tests. Thus, ELISA or Western blots for IgG, IgA, and/or IgE in addition to assays for IgM are more effective for serological studies than agglutination tests alone.

In addition to facilitating the monitoring of pathogen exposure in spotted hyenas, the cross-reacting antibodies identified in our study will also provide researchers with tools for assessing maternal immunoglobulins in milk. Hyenas cubs are weaned roughly 14 months after birth, a lactation interval far longer than that of most other carnivores (Hofer and East, 1995; Watts et al., 2009). Within 2–5 weeks of birth, spotted hyena cubs are transferred from an isolated natal den to a communal den where they

live with up to 20 other cubs, and frequently engage in playful and aggressive interactions (Holekamp and Smale, 1998; Kruuk, 1972; Tanner et al., 2007). The frequent social interactions at communal dens create a situation that can lead to high rates of transmission of infectious pathogens (Altizer et al., 2003). Maternal IgG and IgA transferred from mother to offspring in milk could provide critical protection from pathogens during early life stages (Bourne and Curtis, 1973; Brambell, 1966; Claus et al., 2006; Mason et al., 1930).

IgG and IgM are commonly the focus of immunological studies, however, IgA is the primary Ab on mucosal surfaces, and in humans more IgA is produced than all other isotypes combined (Brandtzaeg et al., 1999; Fagarasan and Honjo, 2003; Kerr, 1990; van Egmond et al., 2001). Rabies virus has been detected in saliva of spotted hyenas expressing no clinical signs of disease (East et al., 2001); salivary IgA might play an important role in both intra- and inter-specific transmission of rabies by neutralizing the virus in saliva before the virus has a chance to infect new individuals. Furthermore, fecal samples are the most readily available and least invasive samples available in most studies of free-living carnivores, and these typically contain large quantities of IgA. Analysis of fecal IgA can thus potentially provide a glimpse into the gut and mucosal immune systems of wild hyenas.

Despite the common use of fecal parasite counts in wildlife studies (e.g. Engh et al., 2003; Gompper et al., 2003; Patton et al., 1986; Watve and Sukumar, 1995), few studies have complemented these counts with analysis of fecal IgA concentrations or serum IgE concentrations (Devalapalli et al., 2006; Gompper et al., 2003). IgE is the primary isotype involved in defense against parasitic worms. Our study has identified two cross-reacting mAb against hyena IgE in ELISAs (Table 1) that might be used to assess effects of parasites on Ig concentrations and ratios. However, the specific binding of the anti-IgE mAbs could not be confirmed here by Western blot, possibly due to the low concentration and short half-life of IgE in serum. IgE and IgG<sub>1</sub> are associated with Th2 response (Mosmann and Coffman, 1989; Mosmann and Sad, 1996; Romagnani, 1997), a topic that is ripe for a comparative study. For example, it would be interesting to compare Th1 and Th2 markers in captive hyenas living in relatively clean environments with wild hyenas that face a continual assault from food-borne and socially transmitted pathogens.

In summary, we have identified Ig-class specific antibodies that specifically recognize hyena epitopes. The antibodies identified here can be used for monitoring the health of both wild and captive hyenas. We have shown that the cross-reacting antibodies can be used to assess specific antibody titer by ELISA; it is probable that these antibodies can also be used in Western blots for detecting antibodies against specific pathogens, which is commonly done to confirm ELISA or RT-PCR results. Finally, these basic immunology tools open the door to more advanced studies of immune function in a species that has demonstrated the remarkable ability to survive disease outbreaks that have decimated wild populations of other carnivore species living sympatrically with spotted hyenas.

## Conflict of interest statement

All monoclonal antibodies were provided by Custom Monoclonals International Corp. Co-author Chris K. Grant is President of Custom Monoclonals International Corp. and he contributed in both design and performance of the study.

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