Oxidative stress in wild European rabbits naturally infected with myxoma virus and rabbit haemorrhagic disease virus

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Abstract

The European rabbit (*Oryctolagus cuniculus*) is one of the most important vertebrate species in the Mediterranean Basin ecosystem. Over the last 60 years, the arrival of two viral diseases, myxomatosis and rabbit haemorrhagic disease, have led to dramatic declines in wild rabbit populations across the Iberian Peninsula. These diseases are currently endemic. Periodic outbreaks occur and have significant impacts on wild populations. Both infection types have diverse physiological effects on their hosts that are rooted in aerobic metabolic processes. To fight off these viruses, rabbits activate their immune systems. However, the production of immune defences generates reactive oxygen species that may consequently damage host tissues. Hypothesising that immune responses increase oxidative stress, we examined whether wild rabbits naturally infected with myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV) had high oxidative stress. Using blood samples, we measured anti-MV and anti-RHDV antibody concentrations and different oxidative stress markers (i.e., glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, and malondialdehyde). Our results show that rabbits that were seropositive for both MV and RHDV had high concentrations of malondialdehyde. Age and body condition were also positively related to dual seropositivity. No significant relationships were observed between serostatus and the concentrations of the other oxidative stress markers. Although we expected infection with MV and RHDV to be correlated with oxidative stress, the influence of external sources of oxidative stress (e.g., climatic conditions) likely made it more difficult to detect such relationships in wild rabbits.

Key words: myxomatosis, *Oryctolagus cuniculus*, oxidative damage, rabbit haemorrhagic disease, ROS, serostatus

1. Introduction

The wild European rabbit (*Oryctolagus cuniculus*) is an important keystone species in the Mediterranean Basin ecosystem. Over the past several decades, its populations have undergone a sharp decline in the Iberian Peninsula, largely due to the impact of two major viral diseases: myxomatosis and rabbit haemorrhagic disease (RHD) (Calvete et al. 2002; García-Bocanegra et al. 2010). Myxomatosis and RHD are currently endemic; significant annual mortality is caused by myxomatosis outbreaks in summer and autumn and RHD outbreaks in spring and winter (Calvete et al. 2002; Villafuerte et al. 2017). Both myxoma virus (MV) and RHD virus (RHDV) can induce significant physiological stress because host metabolic rate climbs during infection (Tuñon et al. 2003; Sanchez-Campos et al. 2004; Lastra 2009; Costantini 2014). In general, immune responses draw upon metabolic processes, increasing immunity and disease resistance at an energetic cost (Lochmiller and Deerenberg 2000). There are also biological fitness costs (Costantini 2008). To fight pathogens, rabbits first activate an innate inflammatory immune response. Inflammation is a non-specific process during which fluids, compounds, and immune cells (e.g., phagocytes) are disseminated through the bloodstream to damaged or infected tissues. Secondly, an acquired immunity is developed when hosts experience repeated exposure to viruses over a long-term period. This is a specific, adaptive response in which a large variety of cells (i.e: lymphocytes) play part in the recognition of specific antigenic configurations of pathogens and respond by triggering cellular (i.e: cytotoxic T-cells) and humoral (i.e: antibodies) effectors (Sorci and Faivre 2009). In a parallel way, viruses develop mechanisms to overcome host immune defences. These strategies allow them to subvert and manipulate key elements of the intracellular and extracellular environment to facilitate their replication, dissemination and transmission (Masters et al. 2001; Johnston et al. 2003). For example, viral proteins secreted from MV suppress lymphocyte and macrophage activation (Cameron et al. 2005a, b) and inflammatory responses (Kerr and McFaden, 2002) and destroy or supress lymphocytes (Kerr 2012). In the case of RHDV, the main strategy is apparently involved in the manipulation of cellular death (Vallejo et al. 2014). Even though, immune system defends against pathogens, it generates reactive oxygen species (ROS). ROS also play an important role since they act as molecular messengers in biological processes (Apel and Hirt 2004; Foyer and Noctor 2005; Gechev et al. 2006); but at high levels they can induce DNA, lipid, and protein damage (Harman 1956; Beckman and Ames 1998; Dowling and Simmons 2009; Selman et al. 2012). Therefore, although ROS can act as highly effective antimicrobial agents, they can also potentially damage host tissues and cells. Such negative side effects can be counteracted by a complex antioxidant (AOX) system that consists of a wide range of endogenous and exogenous compounds (Halliwell and Gutteridge 1999; van de Crommenacker et al. 2010), although any imbalance between ROS and AOXs in favour of the

former gives rise to oxidative stress (OS) (Sies 1991; Halliwell and Gutteridge 2007). OS causes gradual deterioration of organismal function and cell senescence over time and is thus believed to be an important modulator of life-history trade-offs in vertebrates (Costantini 2008; Nussey et al. 2009; Costantini 2014). Therefore, an organism's OS level plays an important role in reproductive performance and aging, which means it is also significantly linked to body condition and biological fitness (Beckman and Ames 1998; Hulbert et al. 2007). Evaluating OS levels is therefore an important part of determining an individual's health status. Since both plasma and serum markers can reveal exposure to stress, biochemical assays that characterise blood oxidative profiles could be used to determine the extent of OS in wild vertebrates (Costantini 2008). According to recent findings, at least one marker of AOX activity and one marker of oxidative damage should be measured to adequately quantify overall OS (Prior and Cao 1999; Clarkson and Thompson 2000; Cohen and McGraw 2009; Costantini and Verhulst 2009; Selman et al. 2012; Christensen et al. 2015). Laboratory and field studies have revealed that mounting an immune response can increase oxidative damage and decrease AOX activity (Tanchev et al. 2003; Costantini and Moller 2009; Marri and Richner 2015; von Schantz et al. 2016). However, despite evidence suggesting that infection risk, OS, and host immune capacity are related, little is known about the temporal consistency of relationships among viral infections, OS markers, and immune function in wild animals. In this study, we tested the hypothesis that immune response increases oxidative stress within three wild populations of the European rabbit that were naturally exposed to MV and RHDV infections. We measured anti-MV and anti-RHDV antibody concentrations as a proxy of contact with the viruses. The presence of MV and RHDV antibodies does only inform on previous exposure to MV and RHDV respectively, therefore we cannot assert that rabbits seropositive for MV and/or seropositive for RHDV were suffering from an infection in that moment. We measured as well markers of AOX activity and oxidative damage in blood samples collected over six sampling periods. The goal of our study was to improve our understanding of immune response dynamics and changes in OS and, more specifically, to determine how animals could physiologically cope with oxidative damage resulting from a natural immune challenge. We wished to clarify any potential harmful effects on body condition and biological fitness that could threaten the viability of natural populations.

2. Materials and methods

2.1 Ethics statement

All animal experimentation was carried out in accordance with Spanish and European regulations (Law 32/2007, Council Directive 2010/63/EU, R.D. 53/2013, ECC/566/2015).

2.2 Study site

The study was conducted in Hornachuelos Natural Park, which is located in a hilly area in the southwestern part of the Iberian Peninsula (37°49' N, 5°15' W); altitude in the park ranges from 100 to 700 m. The climate is Mediterranean, characterised by hot, dry summers and mild, wet winters. Historically, rabbits were highly abundant in the Sierra de Hornachuelos Natural Park but in the recent decades wild populations decreased dramatically. In an attempt to boost local rabbit populations and help to a great extent the conservation of rabbit endangered predators, a restocking was carried out in the area in 2008. This programme consisted of the creation of enclosures where rabbits were introduced to obtain high population densities (Rouco et al. 2008). These enclosures (E1: 3.8 ha; E2: 4.1 ha; and E3: 2.9 ha) were built using 2.5-m-high chain link fences to prevent immediate dispersal of rabbits and to prevent non-avian predators from entering. In every enclosure, artificial warrens were built above ground, consisting of piles of stumps and rocks covered with loam and branches to provide animals with shelter and nesting sites. Additionally, near every warren, water and commercial food were supplied *ad libitum* during throughout the study period.

2.3 Sampling

Animals were captured seasonally in the three enclosures: six live-trapping sessions were conducted from autumn 2008 to spring 2010. Live rabbits were caught using cage traps placed around each warren, as described in Bertó-Moran et al. (2013). With this methodology, we captured around 50–60% of rabbits in a given warren on any given night (Rouco et al. 2011). Individuals were marked with numbered ear tags, their sex and mass were recorded, and both ear and tarsus length were measured. Age estimates were based on body mass—females and males weighing more than 750 g and 850 g, respectively, were considered to be adults (Villafuerte 1994; Alves and Moreno 1996). Blood samples (1–2.5 ml) were extracted from the auricular marginal vein and kept cold (at

138 approximately 4 °C [no direct contact with ice]). They were transported to a field laboratory and immediately
139 centrifuged in Eppendorf tubes. Serum and blood cells were obtained and stored separately at -80 °C until further
140 analyses could take place (Evans 2008; Maceda-Veiga et al. 2015; Pacios-Palma et al. 2016).

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142 *2.4 Oxidative stress and immunological analyses*

143 Using blood cellular fraction, we assayed five different OS markers: four AOX enzymes—glutathione peroxidase
144 (GPX), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT)—and one compound that
145 signals oxidative damage—malondialdehyde (MDA; a product of lipid peroxidation). GPX (U/mg of protein) is an
146 enzyme that catalyses the reduction of hydrogen peroxide (H_2O_2) and a wide variety of organic peroxides (R-
147 OOH) into their corresponding stable alcohols (R-OH) and water using cellular glutathione as the reducing
148 reagent. Its concentration was determined by estimating NADPH oxidation as per Carmagnol et al. (1983). GR
149 (mU/mg of protein) is a flavoprotein that catalyses the NADPH-dependent reduction of oxidized glutathione
150 (GSSG) into glutathione (GSH). Its concentration was determined as per Cribb et al. (1989). SOD (U/mg of
151 protein) is a metalloenzyme that catalyses the dismutation of the superoxide anion into either molecular oxygen or
152 hydrogen peroxide; it is thus a crucial part of the cellular antioxidant defense mechanism. Its concentration was
153 determined as per McCord and Fridovich (1969). CAT (U/mg of protein) is an enzyme that catalyses the
154 decomposition of hydrogen peroxide into water and oxygen and is a very important enzyme in oxidative
155 metabolism. Its concentration was determined as per Cohen et al. (1969). Finally, MDA (nmol/ml) is a low-
156 molecular-weight molecule that is the end-product of the peroxidative decomposition of unsaturated lipids. Its
157 concentration was determined using the Buege and Aust method (1978). Every OS markers here measured was
158 referred to the total proteins content expressed in mg/ml.

159 And using the serum samples, we determined the concentrations of anti-MV and anti-RHDV antibodies using
160 commercial enzyme-linked immunosorbent assay (ELISA) kits. Before measuring anti-MV antibody levels, we
161 diluted sera 1:40 and calculated the relative immunity index (RI). The RI was the coefficient relating the optical
162 density of the controls (positive and negative) to that of the samples. Its value ranged from 1 to 10. Rabbits whose
163 samples had an RI > 2 were considered to be antibody positive (CIV TEST CUNI MIXOMATOSIS, HIPRA
164 Laboratories, Girona, Spain). The specificity (proportion of seropositives that are correctly identified as such) and
165 sensitivity (proportion of seronegatives that are correctly identified as such) of the test were 95% and 100%,
166 respectively. To measure anti-RHDV antibody levels, the INGEZIM kit for rabbits (INGENSASA Laboratories,

Madrid, Spain) was used. Sera were screened using dilutions of 1:200, 1:400, 1:800, and 1:1,600. Samples presenting optical densities > 0.3 were considered to be antibody positive, since such antibody concentrations should be adequate to confer protection against the disease (see Bertó-Moran et al. 2013). The test's specificity and sensitivity were 83.1% and 98.5%, respectively, and there was 93% correspondence with the reference technique. All analyses were carried out in the Laboratory of Ecophysiology at the Doñana Biological Station (CSIC, Seville, Spain).

2.5 Body condition

To estimate rabbit body condition (BC), we calculated a scaled mass index (Peig and Green 2009). This index standardises body mass based on a chosen metric of body length (tarsus length in our case) and is designed to account for allometric changes in scaling that are observed in many species (Gibbs and Chiucchi 2012). BC was used in combination with serostatus to assess rabbit health costs. To calculate BC, mass (in grams) and tarsus length (TL, in mm) were log-transformed, and Model II Regression (lmodel2 package in R v. 3.3.2; Legendre, 2008) was used to calculate the slope (b_{SMA}) of the best-fit line using major axis regression. Mean value of TL based on the entire dataset (mean TL females=71.7 mm; mean TL males=72.8 mm) was calculated (L_o). We calculated separate scaled mass indices for female and male rabbits because, as mentioned above, rabbit age was based on mass and patterns differed between the sexes. Furthermore, gravid females were excluded because they were expected to be heavier relative to their length, which could have biased the calculations.

2.6 Data analysis

We performed generalised linear models (GLMs, stats package in R v. 3.3.2). OS markers (GPX, GR, SOD, CAT, and MDA) were the response variables whereas serostatus, age, sex, enclosure and, capture session were the explanatory variables. Also the interactions of the serostatus with the other four variables were considered within the models. We created a model for each marker that was considered independently to each other. Models here run satisfied assumptions concerning normality of residuals and homoscedasticity. All variables were selected as explanatory variables in the final model since no correlation among them was revealed in previous analyses. Statistical analyses were performed using R software (v. 3.3.2; R Core Team, 2016).

3. Results

We obtained a total of 669 blood samples from 589 adult and 80 juvenile rabbits (264, 203, and 202 rabbits in enclosures E1, E2, and E3, respectively). A total of 221 samples were seropositive for MV alone, 86 samples were seropositive for RHDV alone, 133 samples were seropositive for both MV and RHDV (dual seropositivity), and 229 samples were seronegative.

None of the AOX enzymes were significantly associated with MV or RHDV serostatus. Neither we detected significant relationships between MDA concentrations and serostatus (see Table A in supplementary material). Concentrations of OS markers were quite consistent over time. Overall, markers of AOX activity such as GPX and GR were present in lower concentrations in individuals that were seropositive for MV alone, RHDV alone, and both MV and RHDV. SOD and CAT, appeared generally in lower concentrations in individuals that were seronegative (Fig. 1). Finally, MDA concentrations were higher in seropositive individuals (MV, RHDV, and MV/RHDV) and lower in seronegative individuals (Fig. 1). Table 1 provides the descriptive statistics for the OS marker concentrations for each enclosure and each serostatus group.

4. Discussion

To our knowledge, this study is the first to look at the relationships between MV and RHDV seropositivity and OS in wild European rabbit populations. We found no association between the concentrations of OS markers and serostatus. One possible explanation is that OS may be relatively minor in wild rabbits potentially infected with MV and/or RHDV because other environmental factors (e.g., climatic conditions, food availability, and parasite infections) have a much greater impact on an animal's oxidative status (Gassó et al. 2016). Additionally, we mentioned before the particular physiology of viruses. They manipulate hosts to successfully replicate within their cells by subverting innate and adaptive immunity. Viruses produce a wide array of effectors and immune modulators that alter cellular processes and in turn they can enhance their survival in the host (Kerr 2012). In this regard, the lack of antioxidant responsiveness to immune viral challenge might be feasible in some way. High viral loads would demand high ROS production that would exceed the AOX defences, and they might remain undetected. Nonetheless, we have to point out that we are only looking at some aspects of antioxidant pathways. Therefore, additional in vivo measures of ROS production are necessary to adequately quantitate the overall

oxidative stress status in the organism of study (Selman et al. 2012). This would draw a more accurate interpretation that supports extra robust conclusions. The incorporation of extra markers of oxidative damage (i.e: protein carbonyls (PC)) would provide also reliable information.

We would like to acknowledge some of the limitations of the current study. Assessments of OS levels can be highly variable due to taxonomic differences, different environmental conditions, or the short-term nature of many sampling efforts (e.g., because of logistical constraints). Despite Costantini (2008) described oxidative stress as a cost of immune response, most studies are mainly focused on birds in captivity or in the wild. The number of reports dealing with other wild vertebrate species is quite low (Schneeberger et al. 2013; Gassó et al. 2016), so therefore they may provide mixed evidences and consequently uncertain answers that make difficult to draw general conclusions (Costantini et al. 2009; Norte et al. 2009; van de Crommenacker et al.2011; Raja-aho et al. 2012; Rubolini et al. 2012; Pap et al. 2014). In this regard, caution is called until further studies in wild rabbits are available to support the question we have raised. We thus wish to emphasise the need for experimental studies, which could elucidate mechanisms underlying OS and facilitate the interpretation of field-study results (Costantini 2008; Monaghan et al. 2009; van de Crommenacker 2010). That said, this study is important because it examines the ability of OS to serve as a proxy for the body condition costs that wild rabbits pay when infected with these viral diseases. It is crucial to conduct further research to disentangle the consequences of oxidative damage on the health and biological fitness of wild European rabbits to ensure the viability of natural populations of this keystone species.

In conclusion, although OS markers appeared to remain unaffected as we did not observe a significant increase or decrease in the values of the markers in rabbits seropositive for MV and/or RHDV. This finding likely suggests that, within wild populations, other factors may have a major impact on OS levels, potentially masking the contribution of viral infections. At present, there is increasing interest in using OS markers to assess animal health status in ecological studies of wild populations. However, to date, they have yielded mixed evidence, perhaps because of methodological variation, taxonomic differences, variable environmental conditions, or short sampling periods. Further research is needed to better understand the influence of pathogens on OS levels in wild animals and to unravel the consequences for individual biological fitness and population viability.

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List of abbreviations

ROS: reactive oxygen species

OS: oxidative stress

AOX: antioxidant

MV: myxoma virus

RHD: rabbit haemorrhagic disease

RHDV: rabbit haemorrhagic disease virus

GPX: glutathione peroxidase

GR: glutathione reductase

SOD: superoxide dismutase

CAT: catalase

MDA: malondialdehyde

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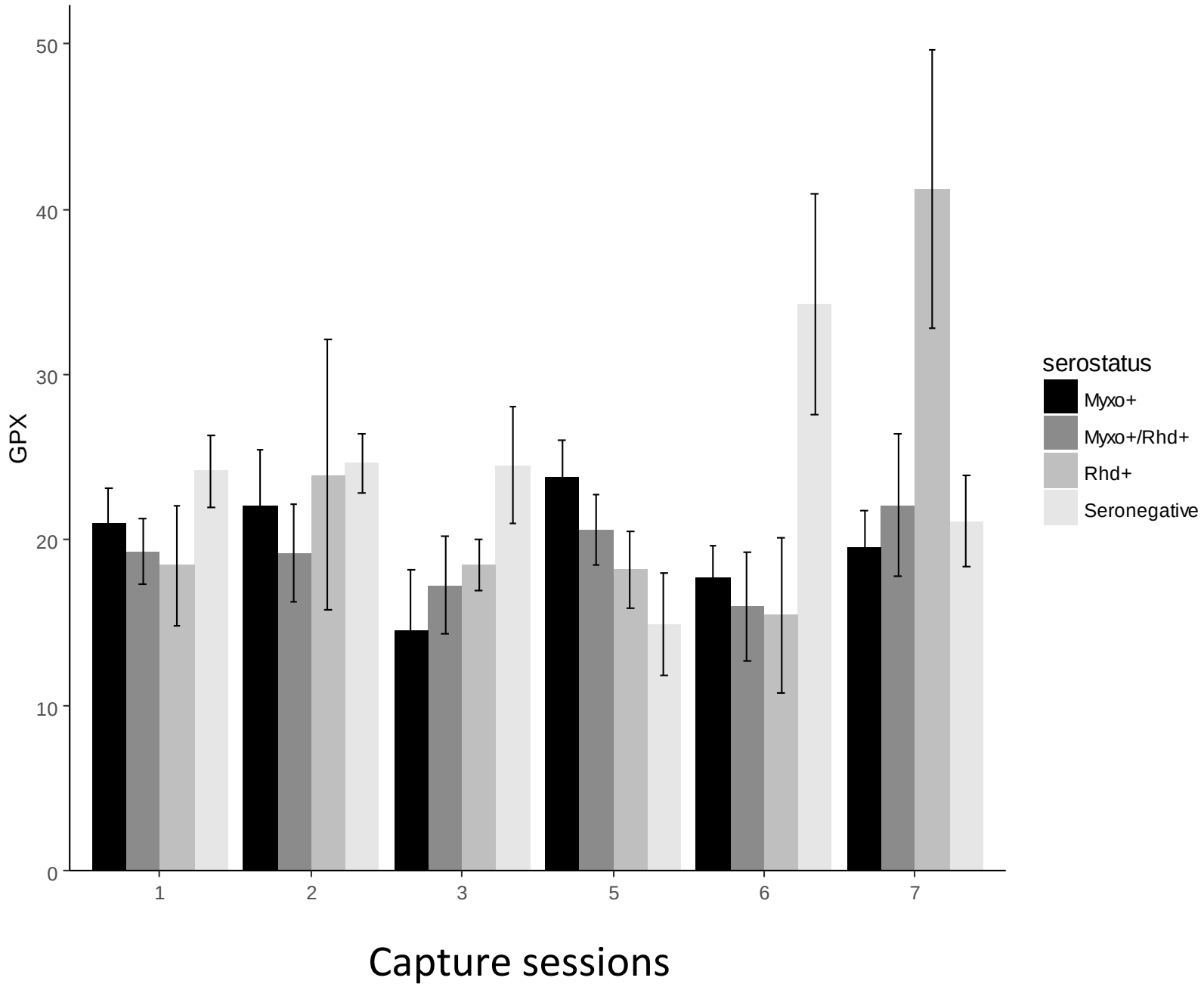
Table 1. Concentrations of oxidative stress (OS) markers in wild European rabbits in three study enclosures (E1, E2, and E3). Results are grouped by rabbit serostatus (seronegative, seropositive for myxoma virus [Myxo+], seropositive for rabbit haemorrhagic disease virus [Rhd+], and seropositive for both viruses [Myxo+/Rhd+]). The values are the mean ± SE.

	E1				**E2**				**E3**			
Marker (conc units)	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***
GPX (mU/mg prot)	24.4±2.2	18.3±2.4	20.8±1.5	18.9±2.2	22.9±1.8	20.2±2.4	21.5±1.7	21.3±2.9	26.0±2.5	19.7±2.4	19.8±2.2	19.1±1.4
GR (mU/mg prot)	1.8±0.2	1.5±0.1	1.8±0.1	1.6±0.1	1.9±0.2	1.6±0.1	1.5±0.1	1.5±0.1	1.7±0.1	1.5±0.1	1.7±0.2	1.6±0.1
SOD (U/mg prot)	15.8±0.6	16.4±0.6	15.8±0.4	15.4±0.5	15.1±0.6	14.1±0.8	14.8±0.5	16.4±1.0	13.0±0.5	13.1±0.5	14.7±0.5	14.2±0.5
CAT (U/mg prot)	64.5±3.5	59.6±3.2	61.6±1.7	60.2±2.5	62.3±3.3	56.9±2.8	62.0±2.9	63.9±4.6	57.8±2.3	57.5±2.8	51.9±2.5	58.1±2.1
MDA (nmol/ml)	5.5±0.1	5.6±0.3	5.7±0.1	5.8±0.2	5.4±0.1	5.3±0.2	5.3±0.2	5.9±0.5	5.1±0.2	5.6±0.3	5.3±0.2	5.7±0.2

Figure captions

Fig. 1 Average concentrations of OS markers (mean ± SE) across capture sessions based on rabbit serostatus categories: seronegative, seropositive for myxoma virus [Myxo+], seropositive for rabbit haemorrhagic disease virus [Rhd+], and seropositive for both viruses [Myxo+/Rhd+]. The marker abbreviations are as follows: glutathione peroxidase (GPX), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA).


GPX
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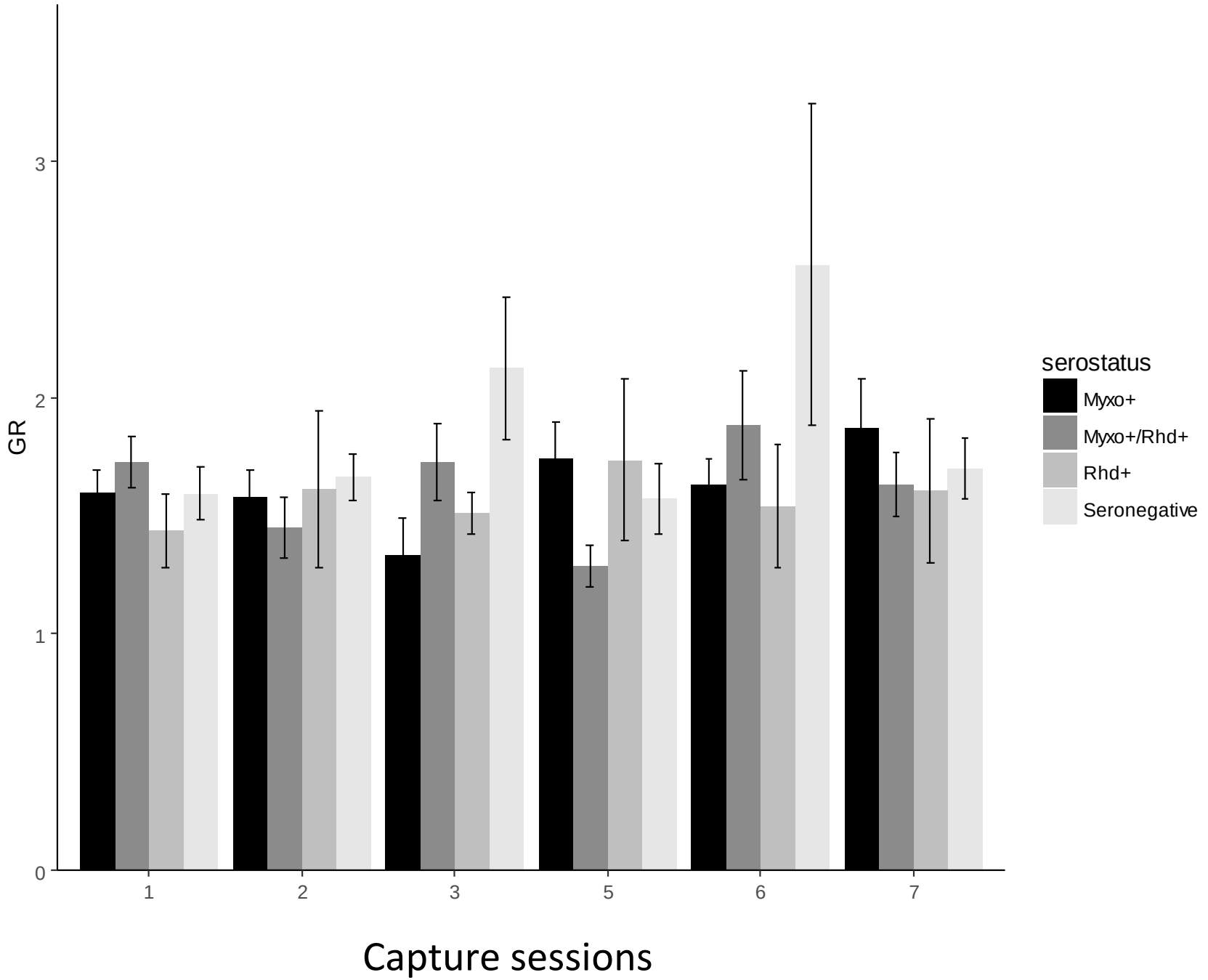
5
6
7
Capture sessions
serostatus
Myxo+
Myxo+/Rhd+
Rhd+
Seronegative


GR
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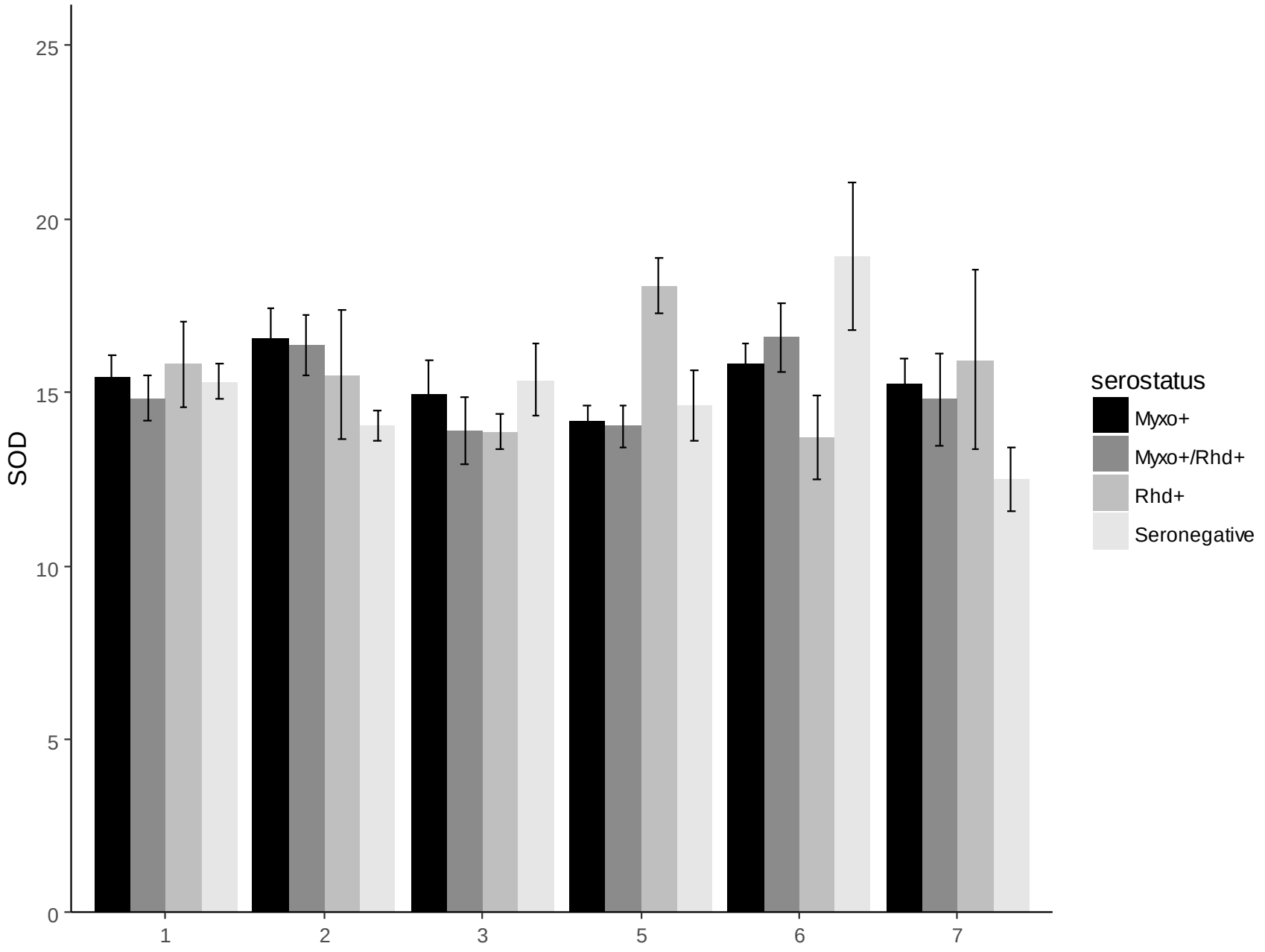
Capture sessions
serostatus
Myxo+
Myxo+/Rhd+
Rhd+
Seronegative



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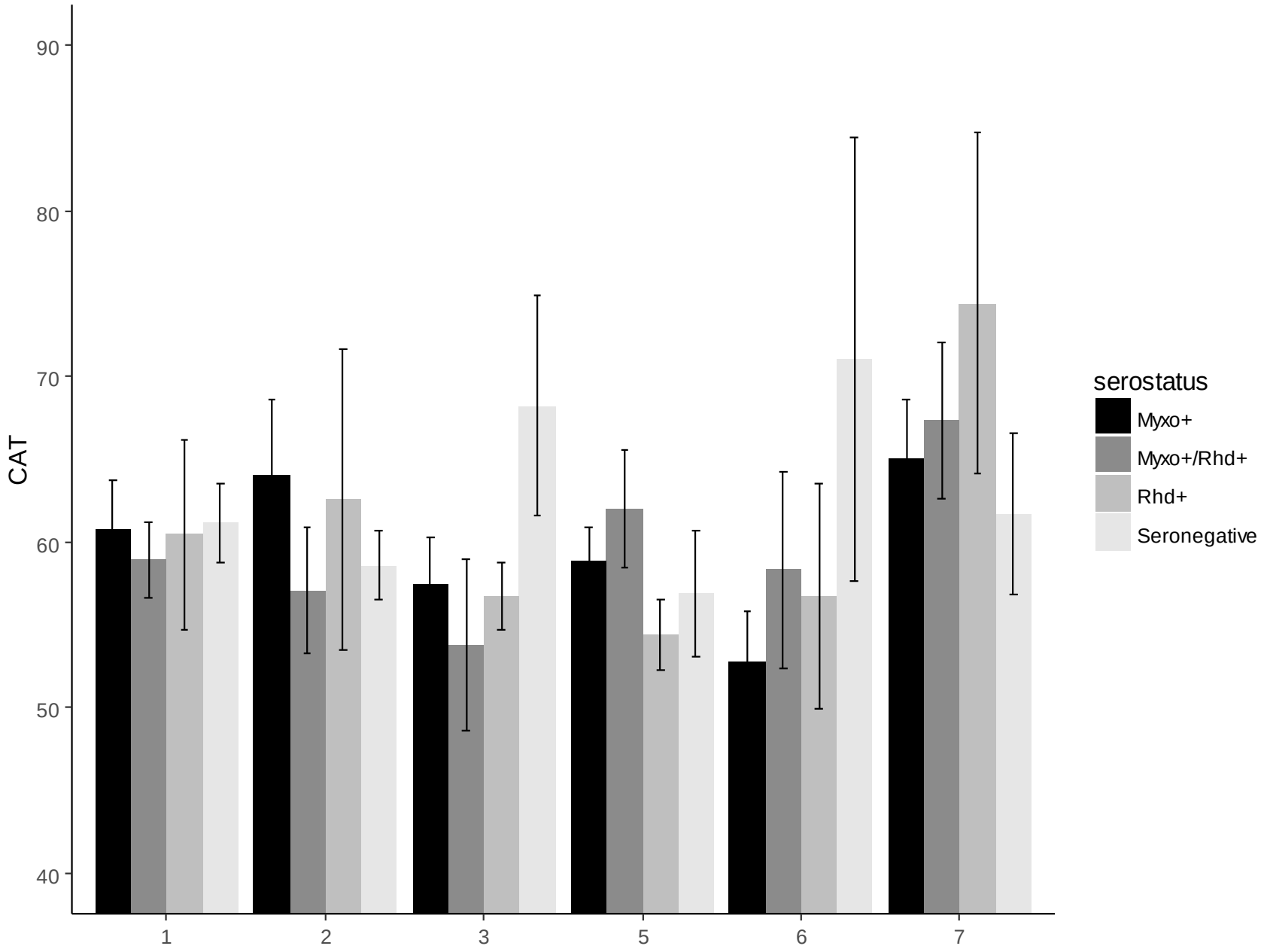

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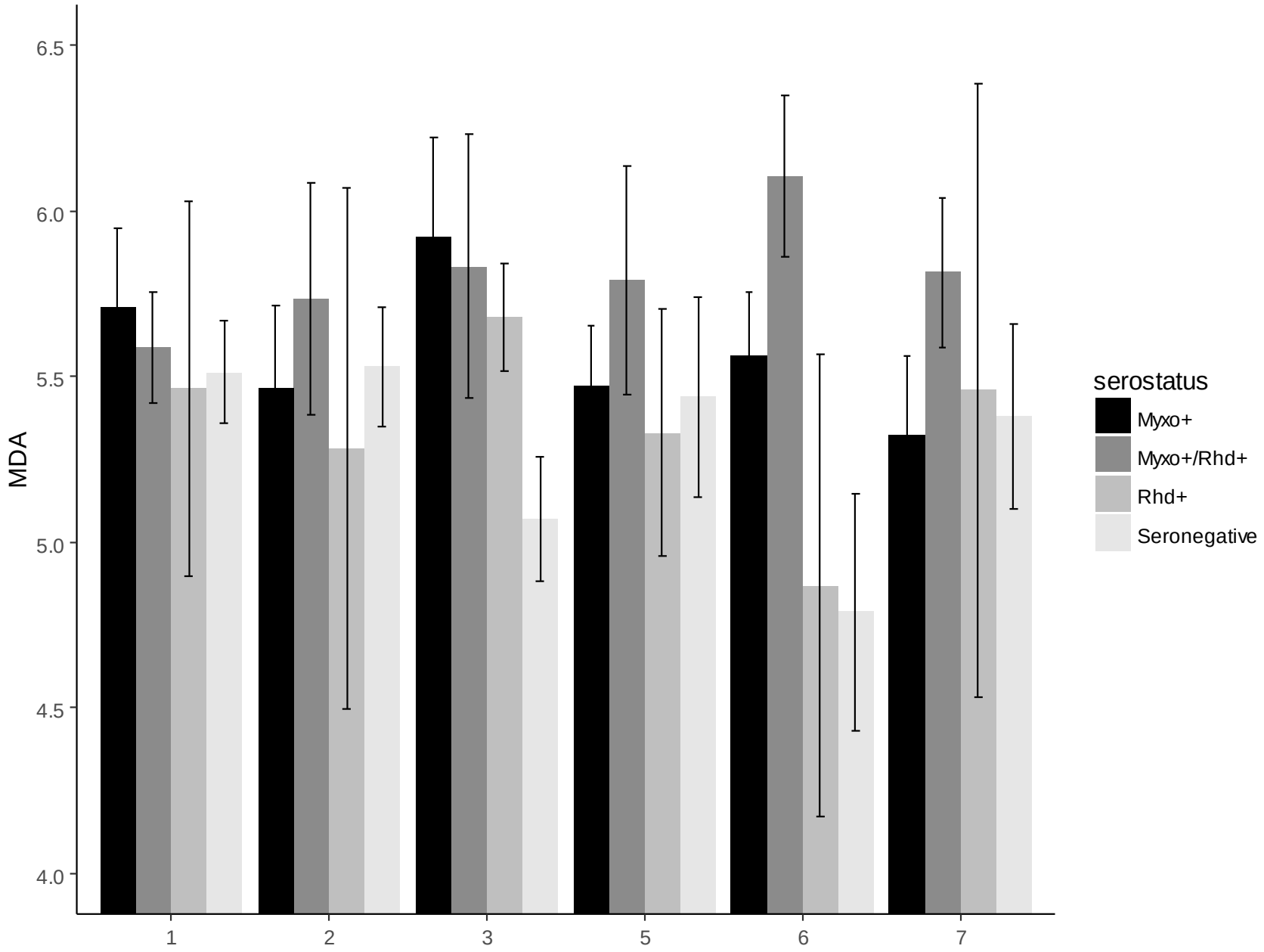


Capture sessions

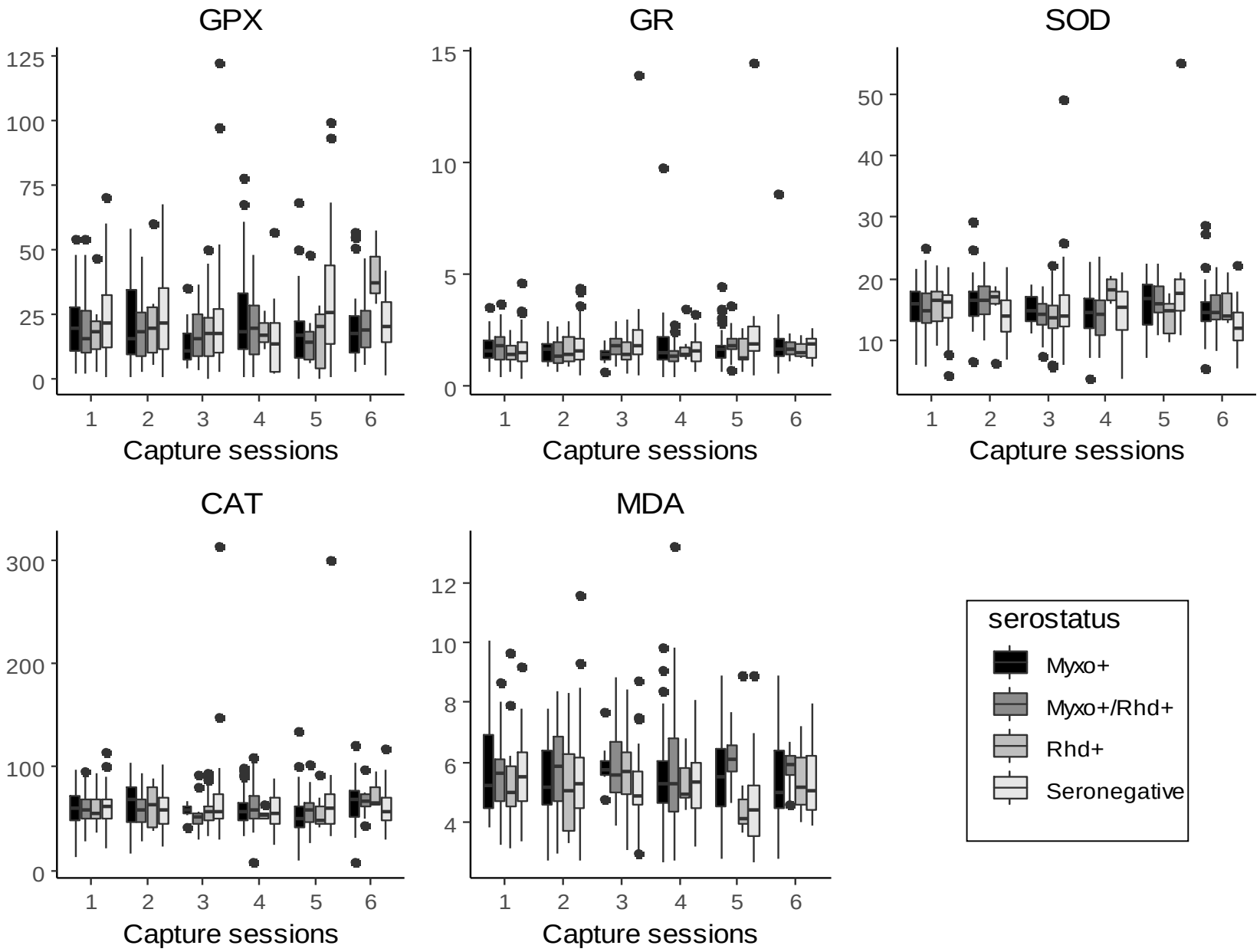
Supplementary material

Table A. Results of the different generalised models in which OS markers were the response variable (i.e. GPX, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; CAT, catalase; MDA, Malondialdehyde). Coefficient estimates (β), estimated standard errors (SE), for explanatory variables (i.e. BC (Body condition Index), serostatus, age, and, sex) are provided. Significance codes are listed below.

	serostatus	age	BC	sex	enclosure	capture session	serostatus*age	serostatus*sex	serostatus*enclosure	serostatus*capture
	β±SE	β±SE	β±SE	β±SE	β±SE	β±SE	β±SE	β±SE	β±SE	β±SE
Model 1										
GPX~serostatus+age+BC+sex+enclosure+capture session+serostatus*age+ serostatus*sex+serostatus*enclosure+serostatus*capture	9.70±17.28	0.36±2.95	0.001±0.003	3.17±2.25	0.22±0.26	-0.08±0.72	-10.30±17.21	-7.33±3.75`	-0,32±0.37	0.36±1.15
Model 2										
GR~serostatus+age+BC+sex+enclosure+capture session+serostatus*age+ serostatus*sex+serostatus*enclosure+serostatus*capture	-0.45±1.22	-0.07±0.20	0.0001±0.0002	-0.27±0.16`	-0.01±0.02	0.09±0.05`	0.46±1.21	0.20±0.26	0.01±0.02	-0.13±0.08
Model 3										
SOD~serostatus+age+BC+sex+enclosure+capture session+serostatus*age+ serostatus*sex+serostatus*enclosure+serostatus*capture	-0.05±4.99	-0.73±0.85	0.0005±0.0009	-0.20±0.65	-0.25±0.07**	0,03±0.07	0.32±4.97	0.26±1.08	0.12±0.10	-0.14±0.33
Model 4										
CAT~serostatus+age+BC+sex+enclosure+capture session+serostatus*age+ serostatus*sex+serostatus*enclosure+serostatus*capture	-9.64±26.94	-4.33±4.60	-0.0009±0.0048	-0.90±3.51	-0.67±0.41	0.97±1.12	7.04±26.82	0.25±5.86	0.43±0.57	-0.06±1.79
Model 5										
MDA~serostatus+age+BC+sex+enclosure+capture session+serostatus*age+ serostatus*sex+serostatus*enclosure+serostatus*capture	0.90±1.44	0.06±0.25	0.00008±0.00026	0.12±0.18	-0.03±0.02	-0.09±0.06	-1.29±1.43	0.27±0.31	0.03±0.03	0.16±0.09

447

(Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1)


GPX
GR
SOD
CAT
MDA
Capture sessions
serostatus
Myxo+
Myxo+/Rhd+
Rhd+
Seronegative