Received: 22 February 2018 | Revised: 14 June 2018 | Accepted: 4 July 2018

DOI: 10.1002/jez.2218

RESEARCH PAPER

WILEY JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY

Chronic exposure to environmental stressors enhances production of natural and specific antibodies in rats

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Funding information
Fondo para la Investigación Científica y Tecnológica, Grant/Award Number: PICT 2012-1552; Universidad Nacional del Litoral, Grant/Award Number: CAI + D 2011

Abstract

Although the immunosuppressive effect of chronic stress has been established, a stress response that downregulates the whole immune system does not make biological sense, especially if an animal has to endure difficult times in which there is also increased infection risk. At high animal densities, animals are faced simultaneously with food restriction (FR), social conflict (SC), and greater parasite–pathogen exposure. We hypothesized that the stress response to chronic stressors that covary with infection risk is not entirely immunosuppressive. Our prediction was that a chronically stressed animal would respond by enhancing innate defenses, while reducing investment in acquired immunity. In a laboratory setting, rats were exposed to prolonged FR and/or SC, and natural and specific antibody levels were repeatedly measured. Our prediction was fulfilled only partly, as FR and SC interacted to enhance natural antibodies, but rats exposed to either or both stressors also showed significantly higher levels of specific antibodies. These results suggest that, in the rat, chronic stress results in a prioritization of both innate and acquired humoral defenses, which makes biological sense provided the stressors examined usually signal an increased infection risk.

KEYWORDS
food restriction, humoral defenses, *Rattus norvegicus*, social conflict, wistar rats

1 | INTRODUCTION

The modulatory effect of the stress response on the immune function is widely recognized. While the response to acute stress tends to initially enhance the immune system, prolonged exposure to stressors may result in a depressed immune function (Dhabhar, 2014; Martin, 2009). This downregulation of the immune function resulting from chronic stress has been interpreted to be adaptive, as a depressed immune function would release resources for more critical processes or prevent immunopathological damage (Boonstra, 2013; Derting & Compton, 2003; Lochmiller & Deerenberg, 2000; Sapolsky, Romero, & Munck, 2000). However, as animals (including humans) are exposed to a rich parasite community, a stress response that suppresses the whole immune system might not be a reasonable adaptive strategy to overcome difficult times (Beldomenico & Begon, 2015; Dhabhar, 2014; Martin, 2009). Indeed, recent evidence from rodents (laboratory and wild) and crocodilians showed that chronic exposure to stressors might enhance some components of the immune system (Chester, Bonu, & Demas, 2010; Eberhardt et al., 2013; Moleon et al., 2018). Laboratory experiments with Siberian hamsters (*Phodopus sungorus*) showed that specific immunoglobulin G (IgG) levels were decreased in socially defeated hamsters, but serum bactericidal activity was enhanced (Chester et al., 2010). In turn, capybaras (*Hydrochaerus hydrochaeris*) and broad snouted caimans (*Caiman latirostris*) experimentally exposed to prolonged nutritional stress increased natural antibody (NAb) levels (Eberhardt et al., 2013; Moleon et al., 2018).

Maintaining immune defenses throughout periods of chronic stress is likely to be particularly important to maximize chances of

survival when stressors are accompanied by an increased infection risk. Such is the case at high animal population densities, when there is food shortage, increased social conflict (SC), and concurrent high parasite exposure (Beldomenico & Begon, 2015; Huitu, Jokinen, Korpimäki, Koskela, & Mappes, 2007; May & Anderson, 1979; Nunn, Jordan, McCabe, Verdolin, & Fewell, 2015). For instance, during SC, the contact rate between individuals increases, and so does the exposure to pathogens and the risk of infection (Caillaud, Craft, & Meyers, 2013; May & Anderson, 1979; Nunn et al., 2015); while the change of behavioral patterns in response to food shortage also increases pathogen exposure (Sykes, 1987). In response to stressors that covary with infection risk, we hypothesize that animals favor some components of the immune system over others, as a strategy to maximize fitness and survival in the face of increased infection risk. Taking into account the wide diversity of parasites that infect a host in nature, an optimal strategy during increased infection risk may be to invest in unspecific defenses able to counteract an array of pathogens rather than in a specific immune response against every member of the parasite community (Eberhardt et al., 2013; Martin, Weil, & Nelson, 2007). Thus, we predict that an animal exposed to chronic stressors that covary with infection risk would respond by enhancing innate defenses, while reducing investment in acquired immunity.

We conducted an experiment with laboratory rats (*Rattus norvegicus* var. Wistar) exposed to prolonged food restriction (FR), SC, or both, comparing natural and specific antibodies (components of the innate and acquired immunity, respectively). We assessed antibody levels as they play a pivotal role in the vertebrate immune response and represent an integrated measure of the proficiency of the host's defenses (Murphy & Weaver, 2016). We addressed two questions: (a) Does chronic exposure to environmental stressors modulate the immune function differentially for innate and acquired responses? and (b) Do the stressors evaluated interact to modulate the immune response?

2 | MATERIALS AND METHODS

2.1 | Ethical considerations

All the procedures were performed according to the "*Guide for the care and use of agricultural animals in agricultural research and teaching*" (ILAR, 2010), and the protocol was approved by the Ethics and Safety Committee of the Facultad de Ciencias Veterinarias of the Universidad Nacional del Litoral (Santa Fe, Argentina) under protocol number 135/12.

2.2 | Animals

We chose the laboratory rat because it has been extensively used as an animal model in laboratory experiments, in many cases with the perspective of understanding human immunology and disease (e.g., Allison, 2004; Viney, Lazarou, & Abolins, 2015). In addition, the low genotypic and phenotypic variability, and the adaptation to laboratory confinement, grants considerable reduction of the data "noise" due to substantial genetic and environmental variance (Calisi & Bentley, 2009). Also, being a well-developed animal model, it offers a myriad of advantages that make the studies possible and reliable, especially when considering immunological studies.

Male Wistar rats were obtained at 4 weeks of age ($n = 32$) from the Centro de Medicina Comparada (Instituto de Ciencias Veterinarias del Litoral, Santa Fe, Argentina). Males were selected because social interactions influence a wider range of behaviors in males than in females (Blanchard, McKittrick, & Blanchard, 2001). We selected pubertal rats to measure the immune responses while having growth as an additional physiological demand, as multiple demands are expected in real contexts (growth patterns of the same rats were analyzed and published in Cuervo, Racca, and Beldomenico (2016)). The rats were randomly selected and distributed into 11 groups (three animals in each of 10 polysulfona cages of 274 × 443 × 231 mm [+one cage with two rats] and a floor area of 800 cm^2, Type III High Allentown Inc. [Allentown, NJ], with treated softwood as bedding material) in an experimental room with 24-hr light–dark 12:12 cycle. The cages were placed in a commercial cage-rack, with individual HEPA-filtered ventilation (Allentown Inc., Allentown, NJ). Temperature was kept at 21 ± 2°C and relative humidity was maintained at 50 ± 5%. A 2-week acclimation period was used for baseline comparisons, in which commercial rat chow and tap water were available *ad libitum*.

2.3 | Experimental design and procedures

Our hypothesis was tested using a 2 × 2 experimental design with prolonged exposure to FR (FR+) and/or SC (SC+). After the acclimation period, 24 rats were randomly assigned to one of four combinations of treatments, carried out in duplicate (two cages per combination). The combinations of treatments were as follows: (a) No stressor (FR–SC–; $n = 4$; two per cage); (b) FR only (FR+SC–; $n = 4$; two per cage); (c) SC only (FR–SC+; $n = 8$; four per cage); and (d) both stressors (FR+SC+; $n = 8$; four per cage). It should be considered that 12 rats were exposed to FR (FR+), whereas an equal number of individuals was not (FR–). Eight rats were housed at low densities (SC–) while the remaining 16 individuals were exposed to SC (SC+). We chose to use this sample size to convey with the "Reduction" recommendation of the animal welfare considerations, taking into account that the design involved several repeated measures, which boosts the statistical power. The remaining eight animals (defined as "intruders," not included in the analyses) were kept in the same conditions as in the acclimation period, to be later used to impose social instability (see below).

As we evaluated chronic environmental stressors that might affect whole populations for a long period of time, the treatments were implemented for 13 consecutive weeks and started concomitantly at Week 0 (Figure 1a). FR–SC– groups were fed *ad libitum* and the animal density was kept low and stable (two per cage; Figure 1b). Consequently, FR–SC– refers to an absence of FR and an absence of SC. FR was implemented on a daily basis by feeding 60% of the

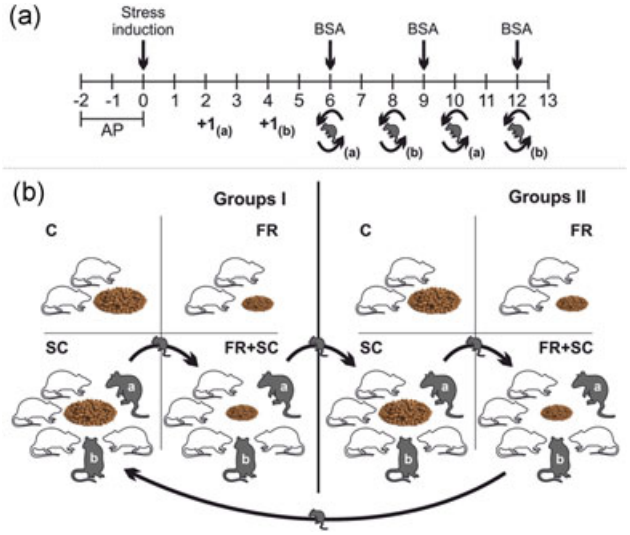

FIGURE 1 Timeline and experimental design. Animals under study (white rats) were exposed to one of four combinations of treatments, and immunized with BSA every 3 weeks, beginning 6 weeks after stress induction. Intruder animals (gray rats) were added ($+1_{(a)}$ and $+1_{(b)}$ indicate the addition of the first and second intruder animals) and exchanged (🔄 every 2 weeks to maintain SC. AP: 2-week acclimation period; BSA: immunization with bovine serum albumin; C: no stressor (FR−SC−); FR: food restriction only (FR+SC−); FR+SC: both stressors (FR+SC+); SC: social conflict only (FR−SC+) [Color figure can be viewed at wileyonlinelibrary.com]

amount of food that the FR−SC− animals (fed *ad libitum*) consumed the day before. The amount of food to be delivered to FR+ animals was calculated considering the mean food intake per FR−SC− animal and the number of individuals in each FR+ cage. The SC+ treatment accounted both for social instability and for crowding (where stability disruption and proximity were the mechanisms of social stress (Blanchard et al., 2001; Brown & Grunberg, 1995). Before the beginning of the experiment, all rats were fed *ad libitum* and housed at equal densities, three rats per cage. On Day 0, to manipulate density (Baldwin, Wilcox, & Baylosis, 1995; Brown & Grunberg, 1995; Chaby et al., 2016), one individual was transferred from a cage with three rats to another cage with three rats, so that the "donor" cage became a SC− group (two individuals) and the recipient a SC+ group (four individuals), thus immediately achieving a substantial contrast in densities (2-fold difference; Figure 1b). Thereafter, to maintain social instability and gradually increase the cage density, two randomly selected intruder animals (Figure 1b; gray rats) were added to each SC+ group (one every 2 weeks, up to a maximum density allowed of six animals per cage, which doubles the suggested by laboratory animal welfare guidelines). Figure 1a,b, $+1_{(a)}$ and $+1_{(b)}$ indicate the addition of the first and second intruder animals. Once the maximum density was reached and every 2 weeks, the intruder animals were exchanged between SC+ groups to maintain the social instability along the duration of the experiment (Figure 1b). The intruder animals were not sampled and not considered in the statistical analyses.

To evaluate the effect of stressors on specific antibody production, on Weeks 6, 9, and 12 after the beginning of the experiment (Figure 1a), all animals received a dose of 100 μg of bovine serum albumin (BSA; Fundación Universidad Nacional de San Luis, San Luis, Argentina), suspended in aluminum hydroxide gel 15% (Alhydrogel 2%; Rivera-Aguilar et al., 2008). The final volume (200 μl) was divided into two doses and injected subcutaneously into each flank (Diehl et al., 2001). BSA is a T-dependent antigen extensively used for evaluating the humoral immune response (Dearman, Caddick, Basketter, & Kimber, 2000; Dearman, Stone, Caddick, Basketter, & Kimber, 2003; Rivera-Aguilar et al., 2008).

On a weekly basis, blood samples (~100 μl) were collected from the lateral tail vein of each rat into an Eppendorf tube with EDTA. Before any other procedure to be carried out that week, each individual was physically restrained and blood samples obtained within 3 min. Samples were centrifuged, and plasma was then removed and stored at −20°C until processed.

2.4 | Enzyme-linked immunosorbent assay (ELISA)

Levels of NAbs and anti-BSA antibodies (IgM and IgG) were analyzed on plasma samples using an indirect ELISA (specific details in Table 1). NAbs were evaluated from Weeks 0 to 13, while anti-BSA antibodies were evaluated from Weeks 6 to 13.

Briefly, microtiter plates (Greiner Bio-One, Germany) were coated with keyhole limpet hemocyanin (for NAbs) or BSA (for anti-BSA) diluted in coating buffer (pH 9.6; Table 1) and incubated at 37°C during 1 hr, followed by overnight incubation at 4°C. Then, the

TABLE 1 Details of the enzyme-linked immunosorbent assay

	NAbs	Anti-BSA IgM	Anti-BSA IgG
Dilution of antigen	0.017 μg/100 μl KLH (H7017)[a]	0.5 μg/100 μl BSA[b]	0.5 μg/100 μl BSA[b]
Dilution of plasma	1:100	1:200	1:200
Secondary antibody (diluted with PBS)	1:1,000 HRP rabbit anti-rat IgG + IgM + IgA (ab102199)[c]	1:500 HRP goat anti-rat IgM (ab98373)[c]	1:20,000 HRP goat anti-rat IgG (ab97090)[c]
Cutoff and reading time	10 min	15 min	15 min

Note. BSA: bovine serum albumin; HRP: horseradish peroxidase; IgG: immunoglobulin G; IgM: immunoglobulin M; KLH: keyhole limpet hemocyanin; NAb: natural antibody; PBS: phosphate-buffered saline.

[a]Sigma-Aldrich (St. Louis, MO).

[b]Fundación Universidad Nacional de San Luis (San Luis, Argentina).

[c]Abcam (Cambridge, MA).

plates were washed with phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween 20 (PBS-T), blocked with 3% nonfat dry milk in PBS and washed again with PBS-T.

Each plasma dilution was added in duplicate to the wells of the antigen-coated plates. A plasma sample, obtained at Week 13, from a same-age rat (not involved in the experiment, but immunized and housed in the same conditions and time that the FR–SC– animals) was used as internal control. Plates were incubated at 37°C for 1 hr and then washed again with PBS-T.

Diluted secondary antibodies were added to the wells (Table 1). The plates were incubated for 1 hr at 37°C and washed again with PBS-T. A chromogen substrate solution (TMB Single Solution; Life Technologies, Carlsbad, CA) was added to each well, and the reaction was terminated with 100 μl of HCL (1 N) per well. Color development was measured on an absorbance microplate reader (SPECTROstar Nano; BMG Labtech GmbH, Ortenburg, Germany) equipped with a 450-nm wavelength filter. Antibody levels were expressed as optical density (OD). Data analysis was performed on sample OD readings as a proportion of that of the internal control plasma sample on each plate.

2.5 | Measures of growth and stress

Growth pattern results of these same rats and stress induction were previously published (for details, see Cuervo et al., 2016), and a summary provided here with the only purpose of supporting the statements presented.

The animals were weighed and measured on a weekly basis. The measures of growth and body condition considered were: weekly body mass (wBM), and weekly body mass index (wBMI) = log body mass/log body length (Labocha, Schutz, & Hayes, 2014). As a disparate effect of FR due to food monopolization was a plausible situation, homogeneity of variances between groups was assessed with the Bartlett's test.

The induction of stress by the treatments applied was verified by comparing the adrenocortical histoarchitecture, the food intake, and the levels of plasmatic corticosterone (CORT) in a radioimmunoassay (Cuervo et al., 2016). CORT values were obtained in selected weeks of the experiment (0, 3, 6, 9, and 13) from diethyl ether extracted plasma aliquots (15 μl) using a radioimmunoassay, following Jahn, Moya, Jammes, and Rosato (1995) and Valdez, Bonafede, Carreño, Deis, and Jahn (2012).

2.6 | Statistical analysis

Baseline comparisons were used with the analysis of variance test to verify that body mass, body length (measured from nose to base of tail), and body mass index (log body mass/log body length) were similar between groups before the start of the experiment (for these baseline comparisons, $\alpha = 0.1$; for the main analyses, $\alpha = 0.05$).

To compare the effect of treatments, two different sets of analyses were conducted: (a) Longitudinal analyses to determine the impact of treatments along the experiment; (b) a "pre–post immunization analysis" to test if the investment in specific humoral immunity trades-off against investment in NAbs. The response variables were the repeated measures of CORT, NAbs, and anti-BSA (IgM and IgG). The treatments applied (FR and SC) were considered as independent variables, and the interaction term FR × SC was included to consider the potential synergism between them. Repeated measures were analyzed by including the polynomial term "week" (linear + quadratic) as main effect in a three-way interaction with treatments (FR × SC × Week and FR × SC × Week^2). This three-way interaction was included to test the hypothesis that both stressors interact. The polynomial term (Week^2) was included to consider that the relationship between the response variable and the main effects might not be lineal. In the case of NAbs, the longitudinal analysis was divided in two parts: Between the 1st and 6th weeks, to analyze the impact of the treatments during the "stress induction phase"; and between the 7th and 13th weeks, to analyze the effect of treatments during the "stress + immunization phase."

The "pre–post immunization analysis" was based on the evaluation of the difference in NAbs levels between weeks (current week–one previous week) with regard to each immune challenge. To assess the difference in the trajectories of the response variable (NAbs levels) before and after each immune challenge, two "dummy" variables were added to the model to properly indicate 1 week prior and 1 week after ("Pre/Post," 0/1) each immune challenge ("Challenge," 1st/2nd/3rd). These dummy variables were considered as main effects and included in a four-way interaction with treatments (FR × SC × Pre/Post × Challenge).

The relevance of the interaction terms was evaluated with the second-order Akaike Information Criterion (AICc) to account for small sample sizes (Johnson & Omland, 2004). When the inclusion of each interaction did not reduce AICc values in 2 or more units (ΔAICc < 2), it was dropped from the model. The main effects were retained (whether significant or not). The distribution of the residuals from every final model was checked for normality and transformations applied when required.

The analyses were conducted using linear mixed models with the statistical software "R" (R Development Core Team, 2016). Linear mixed effects models are the best option because they allow to simultaneously take into account the lack of independence of observations from the same animal (repeated measures), the same cage and/or the same ELISA plate (Bolker et al., 2009; Paterson & Lello, 2003; Schielzeth & Nakagawa, 2013). Random intercepts were used to account for this lack of independence. The random factors "Cage ID" and "Individual ID" (nested within "Cage ID") were included to take into account that groups of observations belonged to the same cage and to the same individual. In addition, "plate ID" was included to account for interassay variation.

3 | RESULTS

3.1 | Natural antibodies

Every experimental group started with similar levels of NAbs, which gradually increased during the entire experiment (Figure 2

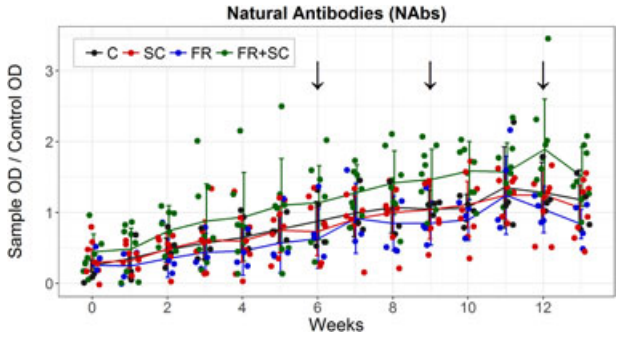

FIGURE 2 Effect of FR and SC on weekly levels of natural antibodies (mean ± *SD*). Black arrows depict when the immune challenges occurred. C: no stressor (FR–SC–); FR: food restriction only (FR+SC–); FR+SC: both stressors (FR+SC+); OD: optical density; SC: social conflict only (FR–SC+) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Linear mixed model describing the effect of treatments on NAbs levels

Term	Estimate	SE	*T* value	*p* value	ΔAICc
"Stress induction phase" (≤6th week)					
Intercept	0.250	0.170	1.48	0.142	
SC	0.046	0.212	0.22	0.828	
FR	−0.066	0.241	−0.27	0.785	
Week	0.103	0.017	5.89	<0.001	
SC × FR	0.213	0.301	0.71	0.481	
SC × Week	−0.021	0.021	−1.00	0.317	
FR × Week	−0.027	0.025	−1.08	0.282	
SC × FR × Week	0.071	0.031	2.31	0.022	3.1
"Stress + immunization phase" (≥6th week)					
Intercept	−0.683	0.371	−1.84	0.067	
SC	−0.065	0.209	−0.31	0.755	
FR	−0.201	0.241	−0.83	0.406	
Week	0.334	0.072	4.63	<0.001	18.1
Week^2	−0.014	0.004	−3.8	<0.001	12
SC × FR	0.641	0.296	2.17	0.031	2.3

Note. Model "Immunization phase": lmer (NAbs~SC × FR + Week + Week^2 + (1|Cage ID/Individual ID) + (1|plate ID)). Model "Stress phase": lmer (NAbs~SC × FR × Week + (1|Cage ID/Individual ID) + (1|plate ID)).
FR: food restriction; NAb: natural antibody; SC: social conflict.

and Table 2). The FR+SC+ groups evidenced a significant positive synergistic effect ($p = 0.022$), presenting greater levels of NAbs than the other groups (Table 2). The comparison among phases demonstrated that this difference was built during the stress induction phase (Weeks 1 to 6).

The pre–post immunization analysis did not show a decrease in NAbs levels after each immune challenge, except after the third one ($p < 0.001$). However, these drops in NAbs levels were not different among treatments, indicating that stress does not modulate the trade-offs between the innate and acquired immunity (Table 3).

TABLE 3 Linear mixed model describing the "pre–post immunization analysis" of NAbs levels

Term	Estimate	SE	*T* value	*p* value	ΔAICc
Intercept	0.009	0.074	0.12	0.907	
SC	0.027	0.049	0.53	0.599	−2.1
FR	0.017	0.052	0.34	0.734	−2.3
Pre/Post	0.139	0.085	1.63	0.104	
Challenge 2	−0.008	0.085	−0.10	0.922	
Challenge 3	0.030	0.085	0.35	0.724	
Challenge 2 × Pre/Post	−0.093	0.120	−0.77	0.441	
Challenge 3 × Pre/Post	−0.448	0.120	−3.72	<0.001	10.7

Note. "Challenge 2" and "Challenge 3" refer to the second and third immune challenges (the first immune challenge being the reference level). Model "Pre/Post NAbs": lmer (diff.NAbs~SC + FR + Pre/Post × Challenge + (1|Cage ID/Individual ID) + (1|plate ID)).
FR: food restriction; NAb: natural antibody; SC: social conflict.

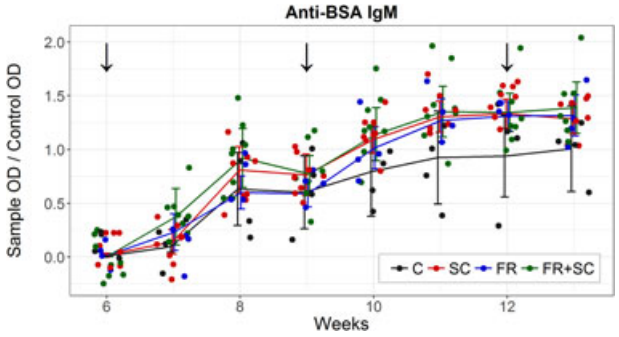

FIGURE 3 Effect of FR and SC on weekly levels of anti-BSA IgM antibodies (mean ± *SD*). Black arrows depict when the immune challenges occurred. C: no stressor (FR–SC–); FR: food restriction only (FR+SC–); FR+SC: both stressors (FR+SC+); OD: optical density; SC: social conflict only (FR–SC+) [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | Anti-BSA antibodies

All experimental groups mounted a detectable immune response of both anti-BSA IgM and IgG. In every group, IgM and IgG levels increased after the first immune challenge and stabilized after the second one (Figures 3 and 4; Table 4). The IgG response was different from typical kinetics, as the secondary response was nearly absent, when the expected according to the literature was a logarithmic increase in the secondary response (Murphy & Weaver, 2016). FR+ and SC+ groups demonstrated higher levels of IgM and IgG than FR–SC–. The three-way interaction "SC × FR × Week" was found significant ($p = 0.004$), but presented coefficients opposite to those of the main effects. This indicates that the concurrent presence of both stressors had no additive nor synergistic effect, meaning that the stressors combined had the same effect than when exposed alone.

3.3 | Measures of growth and stress

A summary of the growth and stress measures is presented in Table 5.

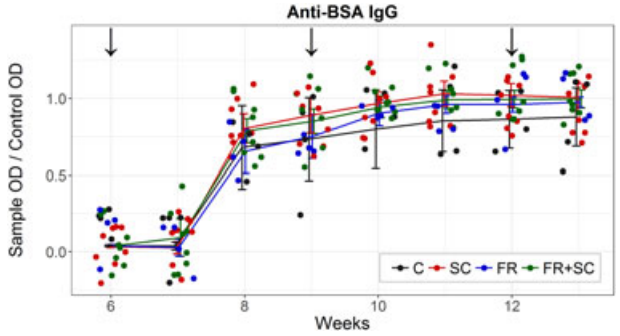

FIGURE 4 Effect of FR and SC on weekly levels of anti-BSA IgG antibodies (mean ± *SD*). Black arrows depict when the immune challenges occurred. C: no stressor (FR–SC–); FR: food restriction only (FR+SC–); FR+SC: both stressors (FR+SC+); IgG: immunoglobulin G; OD: optical density; SC: social conflict only (FR–SC+) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Linear mixed model describing the effect of treatments on anti-BSA (IgM and IgG) levels

Term	Estimate	SE	*T* value	*p* value	ΔAICc
Anti-BSA IgM					
Intercept	−3.315	0.380	−8.72	<0.001	
SC	−0.275	0.181	−1.52	0.130	
FR	−0.355	0.209	−1.70	0.091	
Week	0.710	0.075	9.42	<0.001	
Week^2	−0.029	0.004	−7.85	<0.001	52.1
SC × FR	0.484	0.256	1.89	0.060	
SC × Week	0.053	0.017	3.10	0.002	
FR × Week	0.059	0.020	2.95	0.004	
SC × FR × Week	−0.073	0.025	−2.94	0.004	5.9
Anti-BSA IgG					
Intercept	−18.691	1.221	−15.31	<0.001	
SC	−0.107	0.094	−1.13	0.258	
FR	−0.138	0.109	−1.26	0.208	
Week	5.434	0.381	14.24	<0.001	
Week^2	−0.496	0.039	−12.77	<0.001	98.1
Week^3	0.015	0.001	11.58	<0.001	88.1
SC × FR	0.198	0.133	1.48	0.140	
SC × Week	0.023	0.009	2.62	0.009	
FR × Week	0.024	0.010	2.34	0.020	
SC × FR × Week	−0.035	0.013	−2.63	0.009	4.7

Note. Model "IgG": lmer (IgG~SC × FR × Week + SC × Week^2 + Week^3 + (1|Cage ID/Individual ID) + (1|plate ID)). Model "IgM": lmer (IgM~SC ×FR × Week + Week^2 + (1|Cage ID/Individual ID) + (1|plate ID)).
BSA: bovine serum albumin; FR: food restriction; IgG: immunoglobulin G; IgM: immunoglobulin M; SC: social conflict.

Individuals exposed to FR evidenced a much slower growth than those with free access to food (evidenced by the wBM and the wBMI; Tables 5 and Supporting Information Tables S1 and S2; see Cuervo et al., 2016). The growth rate greatly differed among treatments, with FR+ animals gaining by week around 1/3 of the weight gained by the FR–SC– (Supporting Information Table S1). On the other hand, the animals exposed to SC but fed *ad libitum* (FR–SC+), grew 11% less than those FR–SC– (Supporting Information Table S1). Additionally, we found no evidence of a disparate effect of FR due to food monopolization, as no differences in final body mass (fBM) and fBM index (fBMI) were found between group variances (Bartlett's test: fBM $p = 0.520$; fBMI $p = 0.898$).

TABLE 5 Summary of previous results from these same rats presented in Cuervo et al. (2016) (in comparison to FR–SC– groups)

	Experimental groups		
Variables	FR+SC–	FR–SC+	FR+SC+
Weekly body mass	↓↓	↓	↓↓
Weekly body mass index	↓	=	↓
Relative food intake	↓	↓	↓↓
Total plasma corticosterone	↑	=	↑
Histoarchitecture of the adrenal cortex			
Relative size of the *Zona fasciculata*	↑	=	↑
Relative size of the *Zona glomerulosa*	↑	↓	=

Note. Arrows denote direction of significant changes.

Individuals exposed to FR showed a detectable stress response. The significant FR × Week interaction ($p < 0.001$) indicates that the levels of CORT from these animals differed from those fed *ad libitum*, and consistently increased along the entire experiment (Figure 5 and Table 6). On the other hand, SC+ had no effect on CORT levels (Figure 5 and Table 6).

The stress-related changes observed (increased CORT levels and changes in the histoarchitecture of the adrenal glands) support the notion that FR+ animals perceived the scarcity of resources as threatening and thus elicited a stress response (Figure 5 and Tables 5 and 6 and Supporting Information Table S3; this paper and Cuervo et al., 2016). Despite SC+ groups had CORT levels similar to those of

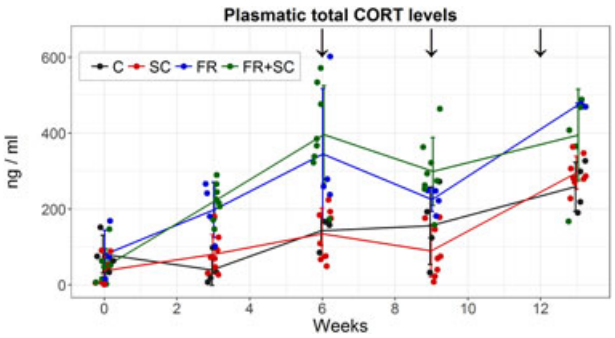

FIGURE 5 Effect of FR and SC on weekly levels of plasmatic CORT (mean ± *SD*). Black arrows depict when the immune challenges occurred. Linear mixed model describing the effect of treatments on CORT levels. C: no stressor (FR–SC–); CORT: corticosterone; FR: food restriction only (FR+SC–); FR+SC: both stressors (FR+SC+); SC: social conflict only (FR–SC+) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 6 Linear mixed model describing the effect of treatments on CORT levels

Term	Estimate	SE	*T* value	*p* value	ΔAICc
Intercept	56.15	28.02	2.00	0.048	
SC	4.51	21.25	0.21	0.832	−2.3
FR	15.01	34.90	0.43	0.668	
Week	−1.28	8.31	−0.15	0.878	
Week^2	1.35	0.61	2.21	0.029	
FR × Week	52.69	11.89	4.43	<0.001	16.5
FR × Week^2	−3.50	0.89	−3.92	<0.001	12.9

Note. Model: lmer (CORT~SC + FR × Week + FR × I(Week^2) + (1|Cage ID/Individual ID)).
CORT: total plasma corticosterone; FR: food restriction; SC: social conflict.

controls (Figure 5 and Tables 5 and 6), their adrenal histoarchitecture was modified as expected under chronic stress (Supporting Information Table S3; see Cuervo et al., 2016; Ulrich-Lai et al., 2006). Additionally, other reliable markers of chronic stress, as reduced food intake and slower growth patterns (reviewed in Dickens & Romero, 2013 and Maniam & Morris, 2012), were reported in these SC+ rats (Supporting Information Table S4 and Cuervo et al., 2016). The evidenced glucocorticoid-independent effects (e.g., reduced food intake and slower growth patterns; Supporting Information Tables S1, S2, and S4), as well as the nearly significant alterations in the adrenocortical histoarchitecture, indicate that the SC+ rats perceived the prolonged social stimulus as stressful.

4 | DISCUSSION

Becoming infected is an everyday risk that may jeopardize the survival of an animal (Raffel, Martin, & Rohr, 2008). Hence, maintaining proficient defenses is vital to maximize fitness in pathogen-rich environments, particularly at times when infection risk increases (e.g., at high host densities; Beldomenico & Begon, 2010, 2015). To evaluate how a prolonged exposure to environmental stressors modulates innate and acquired immune defenses when faced to an antigenic challenge, we used a model of chronic stress that offers the opportunity to investigate whether resource limitation (FR) and SC (social instability and crowding), as well as their interaction, are factors that cause stress-induced alterations in the levels of natural and specific antibodies. We also investigated if there are effects of stress on trade-offs between innate and acquired compartments of the humoral immune system.

Empirical evidence suggests that the response to chronic stress tends to depress the immune system (Dhabhar, 2014; Martin, 2009). For instance, prolonged food scarcity mostly downregulates the immune response in a variety of taxa (e.g., Książek & Konarzewski, 2012; Neuman-Lee et al., 2015) and compromises the immunological memory (Martin, Navara, Weil, & Nelson, 2007). Similarly, social instability and crowding have been recognized to exert mostly a suppressive impact over the immune system (Bartolomucci, 2007; Martin, 2009). However, our findings run counter to these patterns, as both natural and specific antibodies were stimulated in rats chronically exposed to stressors (Figure 6).

NAbs have been recognized to be relevant in the assessment of the immune system, as they are highly cross-reactive against parasite-associated antigens, providing broad protection against infection (Baumgarth, Tung, & Herzenberg, 2005). Its expression has been reported to be influenced by environmental and ecological factors (i.e., species, age, and food availability; Eberhardt et al., 2013; Moleon et al., 2018; Racca, Eberhardt, Moreno, Baldi, & Beldomenico, 2014; Ujvari & Madsen, 2011). Yet, the influence of chronic environmental stressors over the expression of NAbs is still poorly understood.

In agreement with our prediction, we found that there was a positive synergistic effect in the levels of NAbs when both chronic stressors were combined, suggesting that the nutritional status and the social environment interact to enhance the innate humoral immune response of rats. The latter makes biological sense taking into account the life history of *Rattus norvergicus*: As rodent population densities rise, both stressors are increasingly likely to occur simultaneously, which is also associated with increased parasite exposure (Huitu et al., 2007; May & Anderson, 1979; Nunn et al., 2015). Hence, considering the above, it could be interpreted that when experienced together these stressors signal for greater production of NAbs anticipating likely increases in infection risk (Figure 6).

An alternative explanation might be that the increase in NAbs represents a response to self-antigens resulting from stress-induced damage. There are two distinct types of NAbs with different activities (Lutz, Binder, & Kaveri, 2009). The first type is directed

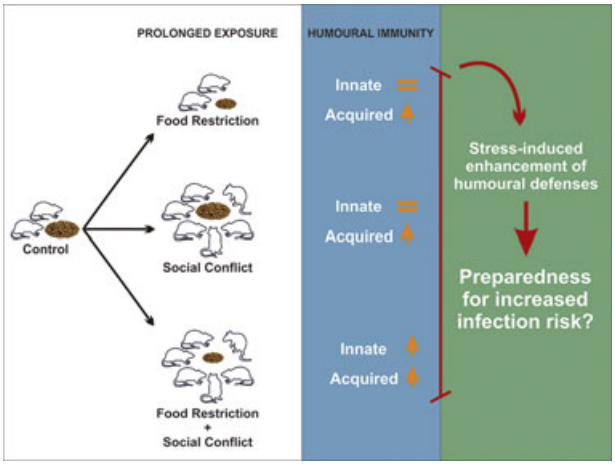

FIGURE 6 Representation of the impact of chronic exposure to environmental stressors on innate and acquired humoral responses. Prolonged exposure to stressors commonly faced in combination raised both natural and specific antibody levels. These results suggest a stressed-induced prioritization of humoral defenses, which makes biological sense when faced to stressors that are prevalent at high population densities and are associated with high infection risk [Color figure can be viewed at wileyonlinelibrary.com]

to nonself antigens, playing a role in infectious disease prevention. The second type, generally called cryptic or natural autoantibodies, is directed to self-antigens or to altered self-antigens, formed as a result of cell damage or inflammation (Cecchini, Rossetti, Tomaso, & Caputo, 2016). We consider this alternative explanation less likely, as the existing literature does not support that the type and magnitude of the stressors assessed here may cause significant cell damage. Further studies should aim at differentiating among NAb types.

Turning to the acquired arm of the immune system, data on chronic stress and specific antibody production are limited. A few studies that examined exposure to FR in rodents and the adaptive humoral response failed to find an effect (Kristan, 2007, 2008; Xu, Liu, & Wang, 2011; Zysling, Garst, & Demas, 2009). As for SC, the few existing studies present evidence in favor of the immunosuppression hypothesis. Social defeat was found to decrease specific IgG levels in rats (Fleshner, Laudenslager, Simons, & Maier, 1989). Similar results were observed in hamsters and mice (Bartolomucci et al., 2001; Chester et al., 2010; Demas, Johnson, & Polacek, 2004; Jasnow, Drazen, Huhman, Nelson, & Demas, 2001). Other studies failed to find an effect of social stress on immunogen-induced antibody levels in laboratory rodents (Karp, Moynihan, & Ader, 1993; Klein et al., 1992).

Due to the arguments posited above, we expected to observe investment in NAbs at the expense of lower specific antibody production. Yet, contrary to our prediction, animals chronically exposed to any or both of the stressors showed higher levels of specific IgM and IgG antibodies compared to FR–SC– individuals. These findings provide evidence that chronic stress may enhance both acquired and innate humoral immunity in rats, and reinforce that the immunosuppressive effect of chronic stress is not a general pattern.

As immune responses are costly in terms of energy demands and immunopathological effects (Graham, Allen, & Read, 2005), this enhancement of the humoral immunity might be interpreted as a dysregulation of the immune system and hence be maladaptive (Martin, Kidd, Liebl, & Coon, 2011). However, provided that an increased immunity can enhance the ability to clear infections, an adaptive explanation becomes reasonable (Bailey, Engler, Powell, Padgett, & Sheridan, 2007; Bailey, Kinsey, Padgett, Sheridan, & Leblebicioglu, 2009). Since the stressors hereby assessed are expected to covary with infection risk, the enhancement of the humoral immunity might prove to be advantageous. In any case, to be truly adaptive a stress-induced enhancement of the immune system needs to be rewarded by benefits to the individual's fitness. Indeed, Bailey et al. (2009) suggested that natural selection would favor those animals that are able to control infections, but also those that can cope with the physiological insult resultant from the immune response. It is noteworthy that the results presented here originate from male pubertal animals, and thus the effects observed should be confirmed in females and adult animals. Additionally, since laboratory rats have been under artificial selection pressure for a long time, our findings should be confirmed in wild *R. norvergicus*.

Summarizing, in the rat, prolonged exposure to stressors commonly faced in combination raises both natural and specific antibody levels. We interpret these results as an indication of a stressed-induced prioritization of humoral defenses (Figure 6) over other physiological processes, such as growth (Cuervo et al., 2016). As the stress response is shaped to maximize the chances of survival, such prioritization of the host's immunity makes biological sense when faced to stressors that are prevalent at high population densities and are associated with high infection risk. Our results shed new light on our understanding of the consequences for the susceptibility of the host and the dynamics of the parasites in a world facing increasing exposure to anthropogenic stressors. Further studies are required to evaluate this phenomenon in natural systems and contexts, as well as the proficiency of these immune strategies.

ACKNOWLEDGMENTS

We thank Carolina Panzani, Leandro Neme, and Enrique Rebelindo for assistance with experimental procedures. We also acknowledge the valuable contribution of two anonymous reviewers.

This study was funded by Agencia Nacional de Promoción Científica y Tecnológica (PICT 2012-1552) and Universidad Nacional del Litoral (CAI + D 2011). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Cuervo PF, Beldomenico PM, Sánchez A, Pietrobon E, Valdez SR, Racca AL. Chronic exposure to environmental stressors enhances production of natural and specific antibodies in rats. *J. Exp. Zool.* 2018;1–10. https://doi.org/10.1002/jez.2218