Early swelling response to phytohemagglutinin is lower in older toads

Francisco Javier Zamora-Camacho¹,² and Mar Comas³

¹ Department of Biological Sciences, Dartmouth College, Hanover, NH, United States of America
² Department of Biogeography and Global Change, Museo Nacional de Ciencias Naturales (MNCN), Spanish National Research Council (CSIC), Madrid, Spain
³ Department of Integrative Ecology, Estación Biológica de Doñana (EBD), Spanish National Research Council (CSIC), Sevilla, Spain

ABSTRACT

The effects of age on performance of life-history traits are diverse, but a common outcome is senescence, an irreversible deterioration of physical and physiological capabilities of older individuals. Immune response is potentially bound to senescence. However, little is known about immune response ageing in amphibians. In this work, we test the hypothesis that amphibian early immune response is reduced in older individuals. To this end, we captured adult natterjack toads (Epidalea calamita) and inoculated them with phytohemagglutinin, an innocuous protein that triggers a skin-swelling immune response whose magnitude is directly proportional to the ability of the individual to mount an immune response. We measured early swelling immune response (corresponding to an innate-response stage) hourly, for six hours, and we calculated the area under the curve (AUC) for each individual’s time series, as a measure of immune response magnitude incorporating time. We estimated toad age by means of phalanx skeletochronology. Swelling and AUC decreased with age. Therefore, in accordance with our predictions, early immune response seems subject to senescence in these toads. Reduced ability to get over infections due to senescence of immune response might be—together with a worse functioning of other organs and systems—among the causes of lower survival of older specimens.

INTRODUCTION

Performance of life-history traits shifts with age in most organisms (Stearns, 1992), as physiological and ethological processes change throughout organisms’ lifetime (Kirkwood & Austad, 2000). However, ageing may affect differently various life-history traits in different organisms (Jones et al., 2014), enhancing some while impairing others in the same individual, implying different genetic and physiological trade-offs (Massot et al., 2011; González-Tokman et al., 2013). Also, the effect of ageing on a given trait may depend on the ontogenetic stage of organisms (Partridge & Gems, 2006). Although processes underlying ageing are rather intricate, some common patterns can be recognized. Ageing typically results in senescence, an irreparable decay of physical and physiological conditions.
(Nussey et al., 2013), which compromises performance (MacNulty et al., 2009), health (Møller & De Lope, 1999), and survivorship (Monaghan et al., 2008) in late life stages (Forslund & Part, 1995).

Although the potential genetic basis of senescence remains unclear (Charmantier et al., 2006; Brommer, Wilson & Gustafsson, 2007; Wilson, Charmantier & Hadfield, 2008), some agents involved have been identified (López-Ótín et al., 2013). For instance, deleterious mutations accumulate with age (Kirkwood & Austad, 2000). Also, cumulative oxidative stress promotes senescence (Finkel & Holbrook, 2000). Oxidative stress is defined as the imbalance between pro-oxidant metabolites and anti-oxidant defences, with negative effects on animal health and physiological homeostasis (Halliwell, 2007). Indeed, oxidative stress decreases longevity (Costantini, 2014), and may play a role in reduced sexual attractiveness of older individuals (Torres & Velando, 2007). More generally, senescence could be a consequence of physical deterioration, which has proven to impair performance (Combes, Crall & Mukherjee, 2010).

As senescence happens in late stages of individual lives, many individuals die before senescence, and/or have already reproduced when it happens, so the effects of selection on late life stages are mild (Rose, 1984).

Moreover, senescence often impairs reproductive output (e.g., Møller & De Lope, 1999; Moya-Laraño, 2002; Reed et al., 2008). However, according to the terminal investment hypothesis, reproductive effort might also be greater in older individuals, with expectancy of fewer future reproduction events (Williams, 1966; Clutton-Brock, 1984). Terminal investment hypothesis predicts that older individuals, with lesser residual reproductive value, will attempt optimized fitness by increasing current reproductive effort to the detriment of future reproduction events, as well as other energy-consuming life-history traits, since their probabilities of future reproduction events are low (Pärt, Gustafsson & Moreno, 1992).

Among traits susceptible to ageing, immune response is of particular interest, for several reasons (review in DeVeale, Brummel & Seroude, 2004). Despite its role on soma preservation and survival (Møller & Saino, 2004) by combating pathogens and parasites (Wakelin & Apanius, 1997), immune response entails a number of energetic and physiological costs (Schmid-Hempel, 2011). As a consequence, immune response may diminish reproductive success, growth, and even survival (Uller, Isaksson & Olsson, 2006; Bascuñán-García, Lara & Córdoba-Aguilar, 2010). Some of those costs might therefore be related to ageing. Firstly, immune activity may provoke oxidative damage (Lambeth, 2007; Sorci & Faire, 2009), which could contribute to soma senescence (reviewed in Selman et al., 2012), including immune system itself (De la Fuente, 2002). Also, immune system activation increases energy expenditure (Martin, Scheuerlein & Wikelski, 2003). Indeed, energy-consuming traits are particularly likely to be affected by senescence, as mitochondrial respiration becomes dysfunctional in aged individuals, limiting energy metabolism (Navarro & Boveris, 2007). However, the relationship between age and immune response might be particularly intricate: for example, whilst immune system senescence could impair animal ability to overcome infections and parasites, at the same time older
individuals could acquire immune memory, which may reduce infection rates in older animals (Raffel et al., 2009).

Overall, senescence occurs in most organisms. However, its rates vary considerably within and among species (Nussey et al., 2013), but this variation is poorly understood (Jones et al., 2014; Hammers et al., 2015). In animals in general, and in amphibians in particular, little is known about the trade-offs involving ageing processes affecting the immune system (Kara, 1994), although some components of it are known to decline with age (review in Torroba & Zapata, 2003). In this work, we test the hypothesis that older natterjack toads (Epidalea calamita) should elicit an early immune response (corresponding to an innate-response stage; Brown, Shilton & Shine, 2011) less intense than younger conspecifics. Moreover, we compare toads from natural habitats with conspecifics from agrosystems, since the latter show delayed early swelling response to PHA (Zamora-Camacho, in press), most likely due to the physiological stress caused by agrosystem conditions (Zamora-Camacho & Comas, 2017). Plus, we compare males and females, because the pattern of early swelling response to PHA is different between sexes in this species (Zamora-Camacho, in press), probably due to the greater reproductive investment of females through egg production (Tejedo, 1992), and/or the immunosuppresser effect of testosterone in males (Folstad & Karter, 1992).

MATERIALS AND METHODS

Study species

Epidalea calamita is a medium-sized (54–75 mm snout-vent length -SVL- in this system) Bufonid toad that inhabits diverse habitats throughout Central and Southwestern Europe (Gómez-Mestre, 2014). Activity takes place mainly during warm, wet nights, and thus phenology varies according to climate: this species hibernates in habitats where winters are cold, but resorts to aestivation instead to avoid summer drought in southern warm locations (Gómez-Mestre, 2014). Similarly, there is variation in reproduction period, which may take place in late winter or in spring (Gómez-Mestre, 2014). Eggs are often laid in very small temporary ponds, and tadpoles can finish metamorphosis in periods as short as 6 weeks (Gómez-Mestre, 2014).

Study area

Toads were captured in natural pine grove Pinares de Cartaya, and agroecosystems nearby, in the Southwest of Spain (37°20′N, 7°09′W). Pine grove was composed of stone pine (Pinus pinea) as the tree stratum, and Pistacia lentiscus, Cistus ladanifer, and Rosmarinus officinalis as an undergrowth. Although some debate exists on the autochthonous or introduced origin of stone pine in this area, their dominance in this ecosystem has been estimated for at least 4,000 years (Martínez & Montero, 2004). Agroecosystems were around 5 km away, in a traditional crop area, which in the recent years has shifted to intensive orange and strawberry plantations, among other crops. Small ponds where E. calamita reproduces were numerous throughout both habitats. Warm winter and arid summer climate in the area makes toads skip hibernation, but they undergo an inactivity period during the summer instead.
Toad collection and management

We collected 18 female and 20 male *B. calamita* by hand during their mating season (January–April) in 2015. Toads were caught while active on rainy nights, or actively searched for under rocks or logs during the day. We distinguished males because their throats are purple-to-pink due to their vocal sacs, and because they show brown or black nuptial pads in their fingers and forelimb tubercles (*Gómez-Mestre, 2014*). For each toad, we measured SVL with a ruler to the nearest mm, body mass with a balance (model CDS-100, precision 0.01 g), and left forelimb sole pad thickness to the nearest 0.01 mm, using a pressure-sensitive micrometer (Mitutoyo). We took three consecutive measures in quick succession, and considered the mean as sole pad thickness (*Brown, Shilton & Shine, 2011*). Afterwards, we subcutaneously injected 0.1 mg of phytohemagglutinin (PHA, Sigma Aldrich L-8754; Sigma Aldrich, St. Louis, MO, USA) diluted in 0.01 ml phosphate buffer saline in left forelimb sole pad. We inoculated the same amount of PHA to all individuals because there were no significant differences in body mass between toads from both habitats ($F_{1,37} = 0.146; P = 0.704$). PHA is a protein that provokes a safe skin-swelling immune response that involves T-cells and other components of the immune system (*Kennedy & Nager, 2006; Martin et al., 2006; Bílková, Vinklerová & Vinkler, 2015*). The magnitude of that swelling is directly proportional to the capacity of the individual to elicit an immune response (*Parmentier, De Vries Reilingh & Nieuwland, 1998; Vinkler, Bainova & Albrecht, 2010; Clulow, Harris & Mahony, 2015*). This method has proved valid in amphibians (*Brown, Shilton & Shine, 2011; Clulow, Harris & Mahony, 2015*). According to *Smits, Bortolotti & Tella* (1999), omitting phosphate buffer saline (PBS) controls in PHA tests has little impact on the results, decreases handling errors, and reduces the coefficient of variation resulting from measurement inaccuracy. For this reason, PHA tests are often simplified by not using PBS controls in amphibians (*Gervasi & Foufopoulos, 2008; Iglesias-Carrasco, Martín & Cabido, 2017; Zamora-Camacho, in press*) as well as in other groups (v.g. *Martin, 2005; Hale & Briskie, 2007; Moreno-Rueda, 2010; Moreno-Rueda & Redondo, 2012*).

After inoculations, we measured sole pad thickness as described, once per hour, for the six hours subsequent to inoculations. Early swelling response to PHA in this species peaks before six hours from inoculation (*Zamora-Camacho, in press*). This procedure allowed us to calculate swelling immune response on an hourly basis, by subtracting sole pad thickness prior to inoculations to sole pad thickness at each hourly measure after inoculation for each individual. At these early stages, swelling response to PHA is directly proportional to recruitment of macrophages, eosinophils and neutrophils, which corresponds to an innate response in closely-related *Rhinella marina* toads (*Brown, Shilton & Shine, 2011*). With that information, for each individual, we calculated area under the curve (AUC, in h*mm) resulting from swelling-response progression during the six hours measured, as a directly proportional measurement of swelling response magnitude incorporating time (*Fekedulegn et al., 2007*). We obtained AUC with software GraphPad Prism 7.0, which applies the trapezoidal formula. During the whole procedure, we kept toads in individual plastic terraria, with humid peat as a substrate and an opaque piece of plastic as a shelter. Toads were released at their capture sites after the last measure.
Skeletochronology technique

Prior to their release, we clipped two toes from each toad, disinfecting the wounds with chlorhexidine and stanching them with a tissue adhesive glue (Dermabond). Toes were used for age estimates, as previously described in Zamora-Camacho & Comas (2017). Specifically, age was estimated by means of phalanx skeletochronology (Comas et al., 2016) using one phalanx per toad. This method does not involve killing specimens. Phalanx skeletochronology is based on the growth pattern of bones of indeterminate-growth ectotherms. Cross-sections of the bones appear surrounded by a line (lines of arrested growth, hereafter LAGs) delimiting areas of rapid osteogenesis when growth is slow or does not happen, typically during hibernation and/or aestivation. Age can be estimated by counting these LAGs: each LAG correspond to one period of inactivity. Since toads in this area only stop activity, and therefore growing, once a year during aestivation, each LAG corresponds to one year. We confirmed the number of LAGs corresponding to one year by calibrating the technique with six subadults whose age was known, which we did not use in the analyses.

We preserved phalanges in 70% ethanol. We performed several trials to estimate the time needed for decalcification. Based on those trials, we decalcified bones by immersing them in 3% nitric acid for 150 min. A solution of phosphate-buffered saline solution with sucrose was used to preserve decalcified samples for at least 48 h at 4 °C. Then, we sectioned them at 16 µm using a freezing microtome (HM500, MICROM) at the Estación Biológica de Doñana (EBD-CSIC), Seville (Spain). We stained cross-sections for 20 min with Harris hematoxylin. After that, we washed the slides in tap water for 5 min to rinse stain. Finally, we used an alcohol chain to dehydrate sections, which we mounted on slides and fixed with DPX (a common medium for mounting histological samples). Afterwards, we counted the number of LAGs in each cross-section with a light microscope (Leitz Dialux20) at magnifications from 50 to 125X. To do so, we used several representative cross-sections among those in which LAGs were clearly visible, and photographed them several times with a ProgRes C3 camera. For better observation of LAGs, we only photographed bone diaphyses. More specifically, we focused on sections were medullar cavity was at its minimum size, while that of the periosteal area was at its maximum size (Comas et al., 2016). Next, the same researcher counted the amount of LAGs in the periosteal bone, on two independent occasions; in no case did the researcher know the identification of the toad studied (Comas et al., 2016). Readings matched in all cases. Because we captured toads during winter and spring, LAGs formed during the preceding summer inactivity period could be discerned from the exterior border of the bones, and were not counted.

Statistics

Since data met the criteria of residual homoscedasticity and normality, we conducted parametric statistics (Quinn & Keough, 2002). Firstly, we performed two simple regressions with AUC as a dependent variable, and age in the first case and SVL in the second case as independent variables. Then, we performed a multiple regression with AUC as a dependent variable, and SVL and age as independent variables. Finally, we conducted a series of
Figure 1 Relationship between age and area under the curve of Epidalea calamita toads. Black figures represent agrosystem toads, and empty figures represent pine grove toads. Circles represent females, and squares represent males.

ANCOVAs to test the effects of SVL and age on AUC and each hourly measurement independently with sex and habitat introduced as factors—since both affect swelling immune response of these toads (Zamora-Camacho, in press)—without non-significant habitat*sex interactions. Statistical analyses were performed with the software Statistica 8.0.

RESULTS

AUC showed a significant negative correlation with age ($F_{1,36} = 4.867; \beta = -0.345; r^2 = 0.119; P = 0.034; \text{Fig. 1}$). Relationship between the immune response and SVL was not significant ($F_{1,36} = 0.184; \beta = -0.071; r^2 = 0.005; P = 0.670$). Moreover, when age and SVL were simultaneously analysed in a multiple regression on AUC (marginally non-significant: $F_{2,35} = 3.203; r^2 = 0.155; P = 0.053$), we detected a significant relationship of AUC with age ($F_{1,35} = 6.195; \beta = -0.498; P = 0.018$), but not with SVL ($F_{1,35} = 1.474; \beta = 0.243; P = 0.233$). Lastly, we found no effect of habitat ($F_{1,33} = 0.001; P = 0.979$), sex ($F_{1,33} = 0.533; P = 0.470$), and SVL ($F_{1,33} = 1.189; P = 0.283$) on AUC, while the effect of age on AUC was significant ($F_{1,33} = 4.219; P = 0.048$). Swelling one and six hours after inoculations showed a significant negative relationship with age (Table 1).

DISCUSSION

Our results show lower early swelling response to PHA in older individuals. Matching with our predictions, these findings suggest that ageing might impair toad immune capacity, aligned with a senescence pattern, at least during the early stages of the response, which
correspond to an innate response. Supporting senescence of immune system in toads, some studies have found deteriorated immune organs and reduced immune-cell counts in older *Bufo toads* (*Saad et al., 1994*).

Eliciting an immune response is indeed energetically costly (*Demas et al., 2012*), and such energy challenge can be harder to afford by older individuals, whose energy metabolism is impaired (*Green, Galluzzi & Kroemer, 2011*). However, a fit physiological state and favourable ecological circumstances may reverse that trend (*Palacios et al., 2011*). For instance, *Massot et al. (2011)* found that immune response to PHA was higher in older than in younger female common lizards, *Zootoca vivipara*. Thus, immune response might be optimised (*Graham, Allen & Read, 2005*) in older individuals, in which reducing innate response could be compensated with a bolstered adaptive response as a consequence of accumulated antigen experiences (*Franceschi et al., 2007; Janeway et al., 1999*). However, other studies have found negative effects of age also on adaptive response (*Solana, Pawelec & Tarazona, 2006; Weng, 2006*), so a general pattern remains unclear. On the other hand, older individuals could have difficulties in facing oxidative stress caused by immune response (*Constantini & Møller, 2009*), so a milder immune response could reduce oxidative unbalance. In fact, oxidative stress induces senescence and favours age-related disorders (*Hosokawa, 2002*).

An alternative, but not mutually exclusive explanation implies a trade-off between immune capacity and reproduction. Limitations in energy-metabolism outcome could be on the basis of reduced immune response in older individuals (*Hipkiss, 2008*). According to the terminal investment hypothesis, older individuals could increase energy investment in current reproduction, since their chances of future reproduction events are low (*Poizat, Rosecchi & Crivelli, 1999*). In that context, the energy-limiting situation due to deteriorated metabolism in older individuals (*Balaban, Nemoto & Finkel, 2005*) could lead to trade-offs favouring reproduction to the detriment of other energy-consuming life-history traits (*González-Tokman, González-Santoyo & Córdoba-Aguilar, 2013*), such as immune response. Accordingly, immune-system activity has proven reduced as a consequence of terminal investment resource reallocation into reproduction (*Krams et al., 2011*). Conversely, an immune challenge can trigger terminal investment (*Bonneaud et al., 2004;
suggestions complex relationships between immune system and reproduction in the context of ageing. Indeed, reproduction and immune activity are under a trade-off in great tit (Parus major) females (Ots & Horak, 1996), as could also be happening in this system, where some indicators of reproductive investment are higher in older individuals in E. calamita toads (Zamora-Camacho & Comas, 2017).

Interestingly, the negative effect of age on immune response affected similarly males and females, so we found no evidence of a sex-dependent mechanism underlying immune response senescence. Although we detected no sex differences in the relationship between immune response and age, they were expectable. On the one hand, the investment of female anurans in reproduction is particularly high (Yu et al., 2017), so the energy trade-off, likely more intense in older, energy-limited individuals (Navarro & Boveris, 2007; Green, Galluzzi & Kroemer, 2011), could be greater in females. Nonetheless, in agreement with our results, reproductive investment of Hyla intermedia female frogs shows no trade-off with age (Cadeddu & Castellano, 2012), and Rana temporaria female frogs do not trade off reproductive investment and growth (Lardner & Loman, 2003). On the other hand, the immunocompetence handicap hypothesis predicts lower immunocompetence in males due to the immunosuppressive effect of testosterone (Folstad & Karter, 1992). Nevertheless, we found no evidence for such handicap, nor a relationship with age. Accordingly, Hyla arborea male frogs showed no negative effect of testosterone injection on swelling response to PHA (Desprat et al., 2015). Indeed, immune responsiveness to PHA has often been found similar in male and female anurans (Zhang et al., 2017), also in this species (Zamora-Camacho & Comas, 2017).

Furthermore, we detected no effect of habitat on the negative relationship between age and early immune response. These agrosystem toads live shorter but grow larger and invest more in reproduction (Zamora-Camacho & Comas, 2017), and at the same time they show a delayed early swelling response to PHA in comparison with natural-habitat toads (Zamora-Camacho, in press). Therefore, we might expect accelerated reduction of early immune response in agrosystem toads due to agrosystem stressors. However, the tendency of early immune response with age was similar in both habitats.

In any case, reduced immune capacity in older individuals, at least in early stages according to our results, could be a consequence of senescence through mechanisms that remain obscure (reviewed in Torroba & Zapata, 2003), and at the same time play a role on increased mortality in older individuals (Hillyer et al., 2005). Older toads in this system showed reduced early immune response to PHA, which might suggest impaired ability to overcome infections (Hawley & Altizer, 2011) and parasites (Campião, Da Silva & Ferreira, 2009), as detected in other species. Therefore, deterioration of early swelling response, corresponding to innate immunity may be at least one of the causes of compromised survival in older adults (Zamora-Camacho & Comas, 2017), as immune response is directly related to survival (Møller & Saino, 2004). However, we cannot completely discard a cohort effect (potential effects caused by inter-annual differences in factors such as temperature, precipitation regimes, food availability, or pathogen exposure), as we measured age with skeletochronology—which implies a snapshot of the age structure at the time when animals were sampled—not with a longitudinal study. The experimental design described here is...
cross-sectional, since it explores characteristics of a population at a given moment (Nussey et al., 2008). On the other hand, longitudinal studies measure the same characteristics of the same individuals at different points of their lives, and are valuable for studying ageing processes, as they avoid biases by differential mortality (Nussey et al., 2008). However, we conducted a cross-sectional study instead because an immune challenge could have an effect on subsequent ones, which would reduce the reliability of repeated-measures results (Boughton, Joop & Armitage, 2011).

CONCLUSIONS

In conclusion, early swelling response to PHA, corresponding to a preliminary innate response, is reduced in older individuals, which might suggest a senescence pattern. Along with deterioration of other organs and systems, senescence of immune response could play a role in reduced survival of older adults, by impairing their ability to overcome infections and parasites.

ACKNOWLEDGEMENTS

We thank Daniel Pinto-Navarro for his logistic support. Comments by Michal Vinkler, Katie Duryea, and one anonymous reviewer improved the manuscript.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding
The authors received no funding for this work.

Competing Interests
The authors declare there are no competing interests.

Author Contributions
• Francisco Javier Zamora-Camacho conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
• Mar Comas performed the experiments, analyzed the data, contributed reagents/-materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):
Toads were captured in accordance with permissions from Bioethics Committee of the University of Granada (18/07/2017/098).
Field Study Permissions
The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Junta de Andalucía granted permission for this study (AWG/MGD/MGM/CB).

Data Availability
The following information was supplied regarding data availability:

The raw data are provided in the Supplementary File.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.6104#supplemental-information.

REFERENCES

Balaban RS, Nemoto S, Finkel T. 2005. Mitochondria, oxidants, and aging. Cell 4:483–495.

Bascuñán-García AP, Lara C, Córdoba-Aguilar A. 2010. Immune investment impairs growth, female reproduction and survival in the house cricket, Acheta domestica. Journal of Insect Physiology 56:204–211 DOI 10.1016/j.jinsphys.2009.10.005.

Bílková B, Vinklerová J, Vinkler M. 2015. The relationship between health and cell-mediated immunity measured in ecology: phytohaemagglutinin skin-swelling test mirrors blood cellular composition. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology 323:767–777 DOI 10.1002/jez.1990.

Bonneaud C, Mazuc J, Chastel O, Westerdahl H, Sorci G. 2004. Terminal investment induced by immune challenge and fitness traits associated with major histocompatibility complex in the house sparrow. Evolution 58:2823–2830 DOI 10.1111/j.0014-3820.2004.tb01633.x.

Boughton RK, Joop G, Armitage SAO. 2011. Outdoor immunology: methodological considerations for ecologists. Functional Ecology 25:81–100 DOI 10.1111/j.1365-2435.2010.01817.x.

Brommer JE, Wilson AJ, Gustafsson L. 2007. Exploring the genetics of aging in a wild passerine bird. The American Naturalist 170:643–650 DOI 10.1086/521241.

Brown GP, Shilton CM, Shine R. 2011. Measuring amphibian immunocompetence: validation of the phytohaemagglutinin skin-swelling assay in the cane toad, Rhinella marina. Methods in Ecology and Evolution 2:341–348 DOI 10.1111/j.2041-210X.2011.00090.x.

Cadeddu G, Castellano S. 2012. Factors affecting variation in the reproductive investment of female treefrogs, Hyla intermedia. Zoology 115:372–378 DOI 10.1016/j.zool.2012.04.006.

Campion KM, Da Silva RJ, Ferreira VL. 2009. Helminth parasites of Leptodactylus podicipinus (Anura: Leptodactylidae) from south-eastern Pantanal, state of Mato Grosso do Sul, Brazil. Journal of Helminthology 83:345–349 DOI 10.1017/S0022149X09289358.
Charmantier A, Perrins C, McCleery RH, Sheldon BC. 2006. Quantitative genetics of age at reproduction in wild swans: support for antagonistic pleiotropy models of senescence. *Proceedings of the National Academy of Sciences of the United States of America* 103:6587–6592 DOI 10.1073/pnas.0511123103.

Clulow S, Harris M, Mahony MJ. 2015. Optimization, validation and efficacy of the phytohaemagglutinin inflammation assay for use in ecomimmunological studies of amphibians. *Conservation Physiology* 3:Article cov042 DOI 10.1093/conphys/cov042.

Clutton-Brock TH. 1984. Reproductive effort and terminal investment in iteroparous animals. *The American Naturalist* 123:212–229 DOI 10.1086/284198.

Comas M, Reguera S, Zamora-Camacho FJ, Salvadó H, Moreno-Rueda G. 2016. Comparison of the effectiveness of phalanges vs. humeri and femurs to estimate lizard age with skeletochronology. *Animal Biodiversity and Conservation* 39:237–240.

Combes SA, Crall JD, Mukherjee S. 2010. Dynamics of animal movement in an ecological context: dragonfly wing damage reduces flight performance and predation success. *Biology Letters* 6:426–429 DOI 10.1098/rsbl.2009.0915.

Constantini D, Möller AP. 2009. Does immune response cause oxidative stress in birds? A meta-analysis. *Comparative Biochemistry and Physiology. A: Comparative Physiology* 153:339–344 DOI 10.1016/j.cbpa.2009.03.010.

Constantini D. 2014. Oxidative stress and hormesis in evolutionary ecology and physiology. *A marriage between mechanistic and evolutionary approaches*. Berlin: Springer.

De la Fuente M. 2002. Effects of antioxidants on immune system ageing. *European Journal of Clinical Nutrition* 56:S5–S80 DOI 10.1038/sj.ejcn.1601476.

Demas G, Greives T, Chester E, French S. 2012. The energetics of immunity. In: Demas G, Nelson R, eds. *Ecoimmunology*. Oxford: Oxford University Press.

Desprat JL, Lengagne T, Dumet A, Desouhant E, Mondy N. 2015. Immunocompetence handicap hypothesis in tree frog: trade-off between sexual signals and immunity? *Behavioral Ecology* 26:1138–1146 DOI 10.1093/beheco/arv057.

DeVeale B, Brummel T, Seroude L. 2004. Immunity and aging: the enemy within? *Aging Cell* 2004:195–208.

Fekedulegn DB, Andrew ME, Burchfiel CM, Violanti JM, Hartley TA, Charles LE, Miller DB. 2007. Area under the curve and other summary indicators of repeated waking cortisol measurements. *Psychosomatic Medicine* 69:651–659 DOI 10.1097/PSY.0b013e318184c405c.

Finkel T, Holbrook NJ. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408:239–247 DOI 10.1038/35041687.

Folstad I, Karter AJ. 1992. Parasites, bright males, and the immunocompetence handicap. *The American Naturalist* 139:603–622 DOI 10.1086/285346.

Forstlund P, Part T. 1995. Age and reproduction in birds—hypotheses and tests. *Trends in Ecology & Evolution* 10:374–378 DOI 10.1016/S0169-5347(95)80914-7.

Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, Panourgia MP, Invidia L, Celani L, Scurti M, Cevenini E, Castellani GC, Salvioli S. 2007. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged...
from studies in humans. *Mechanisms of Ageing and Development* **128**:92–105 DOI 10.1016/j.mad.2006.11.016.

**Gerassi SS, Foufopoulos J. 2008.** Costs of plasticity: responses to desiccation decrease post-metamorphic immune function in a pond-breeding amphibian. *Functional Ecology* **22**:100–108.

**Gómez-Mestre I. 2014.** Sapo corredor—*Epidalea calamita* (Laurenti, 1768). In: Salvador A, Marco A, eds. *Enciclopedia virtual de los vertebrados españoles*. Madrid: Museo Nacional de Ciencias Naturales Available at http://www.vertebradosibericos.org.

**González-Tokman DM, González-Santoyo I, Córdoba-Aguilar A. 2013.** Mating success and energetic condition effects driven by terminal investment in territorial males of a short-lived invertebrate. *Functional Ecology* **27**:739–747 DOI 10.1111/1365-2435.12072.

**González-Tokman D, González-Santoyo I, Munguia-Steyer R, Córdoba-Aguilar A. 2013.** Effect of juvenile hormone on senescence in males with terminal investment. *Journal of Evolutionary Biology* **26**:2458–2466 DOI 10.1111/jeb.12241.

**Graham AL, Allen JE, Read AF. 2005.** Evolutionary causes and consequences of immunopathology. *Annual Review of Ecology, Evolution, and Systematics* **36**:373–397 DOI 10.1146/annurev.ecolsys.36.102003.152622.

**Green DR, Galluzzi L, Kroemer G. 2011.** Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. *Science* **333**:1109–1112 DOI 10.1126/science.1201940.

**Hale KA, Briskie JV. 2007.** Decreased immunocompetence in a severely bottlenecked population of an endemic New Zealand bird. *Animal Conservation* **10**:2–10 DOI 10.1111/j.1469-1795.2006.00059.x.

**Halliwell B. 2007.** Biochemistry of oxidative stress. *Biochemical Society Transactions* **35**:1147–1150 DOI 10.1042/BST0351147.

**Hammers M, Kingma SA, Bebbington K, Van de Crommenacker J, Spurgin LG, Richardson DS, Burke T, Dugdale HL, Komdeur J. 2015.** Senescence in the wild: insights from a long-term study on Seychelles warblers. *Experimental Gerontology* **71**:69–79 DOI 10.1016/j.exger.2015.08.019.

**Hawley DM, Altizer SM. 2011.** Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. *Functional Ecology* **25**:48–60 DOI 10.1111/j.1365-2435.2010.01753.x.

**Hillyer JF, Schmidt SL, Fuchs JF, Boyle JP, Christensen BM. 2005.** Age-associated mortality in immune challenged mosquitoes (*Aedes aegypti*) correlates with a decrease in haemocyte numbers. *Cellular Microbiology* **7**:39–51.

**Hipkiss AR. 2008.** Energy metabolism, altered proteins, sirtuins and ageing: converging mechanisms? *Biogerontology* **1**:49–55.

**Hosokawa M. 2002.** A higher oxidative status accelerates senescence and aggravates age-dependent disorders in SAMP strains of mice. *Mechanisms of Ageing and Development* **123**:1553–1561 DOI 10.1016/S0047-6374(02)00091-X.
Iglesias-Carrasco M, Martin J, Cabido C. 2017. Urban habitat can affect body size and body condition but not immune response in amphibians. *Urban Ecosystems* 20:1331–1338 DOI 10.1007/s11252-017-0685-y.

Janeway CA, Travers P, Walport M, Capra JD. 1999. *Immunobiology: the immune system in health and disease*. New York: Garland Publishing.

Jones OR, Scheuerlein A, Salguero-Gómez R, Giovanni Camarda C, Schaible R, Casper BB, Dahlgren JP, Ehrlén J, García MB, Menges ES, Quintana-Ascencio PF, Caswell H, Baudisch A, Vaupel JW. 2014. Diversity of ageing across the tree of life. *Nature* 505:169–173 DOI 10.1038/nature12789.

Kara TC. 1994. Ageing in Amphibians. *Gerontology* 40:161–173 DOI 10.1159/000213585.

Kennedy MW, Nager RG. 2006. The perils and prospects of using phytohaemagglutinin in evolutionary ecology. *Trends in Ecology & Evolution* 21:653–655 DOI 10.1016/j.tree.2006.09.017.

Kirkwood TBL, Austad SN. 2000. Why do we age? *Nature* 408:233–238 DOI 10.1038/35041682.

Krams I, Daukšte J, Kivleniece I, Krama T, Rantala MJ, Ramey G, Šauša L. 2011. Female choice reveals terminal investment in male mealworm beetles, *Tenebrio molitor*, after repeated activation of the immune system. *Journal of Insect Science* 11:Article 56.

Lambeth JD. 2007. NOX enzymes, ROS, and chronic disease: an example of antagonistic pleiotropy. *Free Radical Biology & Medicine* 43:332–347 DOI 10.1016/j.freeradbiomed.2007.03.027.

Lardner B, Loman J. 2003. Growth or reproduction? Resource allocation by female frogs *Rana temporaria*. *Oecologia* 137:541–546 DOI 10.1007/s00442-003-1390-5.

Lee WS, Monaghan P, Metcalfe NB. 2016. Perturbations in growth trajectory due to early diet affect age-related deterioration in performance. *Functional Ecology* 30:625–635 DOI 10.1111/1365-2435.12538.

López-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. 2013. The hallmarks of ageing. *Cell* 153:1194–1217 DOI 10.1016/j.cell.2013.05.039.

MacNulty DR, Smith DW, Vucetich JA, Mech LD, Stahler DR, Packer C. 2009. Predatory senescence in ageing wolves. *Ecology Letters* 12:1347–1356 DOI 10.1111/j.1461-0248.2009.01385.x.

Martin LB. 2005. Trade-offs between molt and immune activity in two populations of house sparrows (*Passer domesticus*). *Canadian Journal of Zoology* 83:780–787 DOI 10.1139/z05-062.

Martin LB, Han P, Lewittes J, Kuhlman JR, Klasing KC, Wikelski M. 2006. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoeological technique. *Functional Ecology* 20:290–299 DOI 10.1111/j.1365-2435.2006.01094.x.

Martin LB, Scheuerlein A, Wikelski M. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proceedings of the Royal Society B* 270:153–158 DOI 10.1098/rspb.2002.2185.
Martínez F, Montero G. 2004. The Pinus pinea L. woodlands along the coast of South-western Spain: data for a new geobotanical interpretation. Plant Ecology 175:1–18 DOI 10.1023/B:VEGE.0000048087.73092.6a.

Massot M, Clobert J, Montes-Poloni L, Haussy C, Cubo J, Meylan S. 2011. An integrative study of ageing in a wild population of common lizards. Functional Ecology 25:848–858 DOI 10.1111/j.1365-2435.2011.01837.x.

Møller AP, De Lope F. 1999. Senescence in a short-lived migratory bird: age-dependent morphology, migration, reproduction and parasitism. Journal of Animal Ecology 68:163–171 DOI 10.1046/j.1365-2656.1999.00274.x.

Møller AP, Saino N. 2004. Immune response and survival. Oikos 104:299–304 DOI 10.1111/j.0030-1299.2004.12844.x.

Monaghan P, Charmantier A, Nussey DH, Ricklefs R. 2008. The evolutionary ecology of senescence. Functional Ecology 22:371–378 DOI 10.1111/j.1365-2435.2008.01418.x.

Moreno-Rueda G. 2010. An immunological cost of begging in house sparrow nestlings. Proceedings of the Royal Society B 277:2083–2088 DOI 10.1098/rspb.2010.0109.

Moreno-Rueda G, Redondo T. 2012. Benefits of extra begging fail to compensate for immunological costs in southern strike (Lanius meridionalis) nestlings. PLOS ONE 7:e44647 DOI 10.1371/journal.pone.0044647.

Moya-Laraño J. 2002. Senescence and food limitation in a slowly ageing spider. Functional Ecology 16:734–741 DOI 10.1046/j.1365-2435.2002.00685.x.

Navarro A, Boveris A. 2007. The mitochondrial energy transduction system and the aging process. American Journal of Physiology 292:C670–C686 DOI 10.1152/ajpcell.00213.2006.

Nielsen ML, Hokman L. 2012. Terminal investment in multiple sexual signals: immune-challenged males produce more attractive pheromones. Functional Ecology 26:20–28 DOI 10.1111/j.1365-2435.2011.01914.x.

Nussey DH, Coulson T, Festa-Bianchet M, Gaillard JM. 2008. Measuring senescence in wild animal populations: towards a longitudinal approach. Functional Ecology 22:393–406 DOI 10.1111/j.1365-2435.2008.01408.x.

Nussey DH, Froy H, LeMaitre JF, Gaillard JM, Austad SN. 2013. Senescence in natural populations of animals: widespread evidence and its implications for biogerontology. Ageing Research Reviews 12:214–225 DOI 10.1016/j.arr.2012.07.004.

Ots I, Horak P. 1996. Great tits Parus major trade health for reproduction. Proceedings of the Royal Society B 263:1443–1447 DOI 10.1098/rspb.1996.0210.

Palacios MG, Winkler DW, Klasing KC, Hasselquist D, Vleck CM. 2011. Consequences of immune system aging in nature: a study of immunosenescence costs in free-living tree swallows. Ecology 92:952–966 DOI 10.1890/10-0662.1.

Parmentier HK, De Vries Reilingh G, Nieuwland MGB. 1998. Kinetic and immunohistochemical characteristic of mitogen-induced cutaneous hypersensitivity in chickens selected for antibody responsiveness. Veterinary Immunology and Immunopathology 66:367–376 DOI 10.1016/S0165-2427(98)00200-1.
Part T, Gustafsson L, Moreno J. 1992. “Terminal investment” and a sexual conflict in the collared flycatcher (Ficedula albicollis). The American Naturalist 140:868–882 DOI 10.1086/285445.

Partridge L, Gems D. 2006. Beyond the evolutionary theory of ageing, from functional ecology to evo-gero. Trends in Ecology & Evolution 21:334–340 DOI 10.1016/j.tree.2006.02.008.

Poizat G, Rosecchi E, Crivelli AJ. 1999. Empirical evidence of a trade-off between reproductive effort and expectation of future reproduction in female three-spined sticklebacks. Proceedings of the Royal Society B 266:1543–1548 DOI 10.1098/rspb.1999.0813.

Quinn GP, Keough MJ. 2002. Experimental design and data analysis for biologists. Cambridge: Cambridge University Press.

Raffel TR, LeGros RP, Love BC, Rohr JR, Hudson PJ. 2009. Parasite age-intensity relationships in red-spotted newts: does immune memory influence salamander disease dynamics? International Journal for Parasitology 39:231–241 DOI 10.1016/j.ijpara.2008.06.011.

Reed TE, Kruuk LEB, Wanless S, Frederiksen M, Cunningham EJA, Harris MP. 2008. Reproductive senescence in a long-lived seabird: rates of decline in late-life performance are associated with varying costs of early reproduction. The American Naturalist 171:E89–E101.

Rose MR. 1984. The evolution of animal senescence. Canadian Journal of Zoology 62:1661–1667 DOI 10.1139/z84-243.

Saad AH, Mansour MH, Dorgham V, Badir N. 1994. Age-related changes in the immune response of Bufo viridis. Developmental and Comparative Immunology 18:597.

Schmid-Hempel P. 2011. Evolutionary parasitology: the integrated study of infections, immunology, ecology, and genetics. Oxford: Oxford University Press.

Selman C, Blount JD, Nussey DH, Speakman JR. 2012. Oxidative damage, ageing, and life-history evolution: where now? Trends in Ecology & Evolution 10:570–577.

Smits JE, Bortolotti GR, Tella JL. 1999. Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. Functional Ecology 13:567–572 DOI 10.1046/j.1365-2435.1999.00338.x.

Solana R, Pawelec G, Tarazona R. 2006. Aging and innate immunity. Immunity 24:491–494 DOI 10.1016/j.immuni.2006.05.003.

Sorci G, Faivre B. 2009. Inflammation and oxidative stress in vertebrate host-parasite systems. Philosophical Transactions of the Royal Society B 364:71–83 DOI 10.1098/rstb.2008.0151.

Stearns SC. 1992. The evolution of life histories. Oxford: Oxford University Press.

Tejedo M. 1992. Effects of body size and timing of reproduction on reproductive success on female natterjack toad (Bufo calamita). Journal of Zoology 228:545–555 DOI 10.1111/j.1469-7989.1992.tb04454.x.

Torres R, Velando A. 2007. Male reproductive senescence: the price of immune-induced oxidative damage on sexual attractiveness in the blue-footed booby. Journal of Animal Ecology 76:1161–1168 DOI 10.1111/j.1365-2656.2007.01282.x.
Torroba M, Zapata AG. 2003. Aging of the vertebrate immune system. *Microscopy Research and Technique* 62:477–481 DOI 10.1002/jemt.10409.

Uller T, Isaksson C, Olsson M. 2006. Immune challenge reduces reproductive output and growth in a lizard. *Functional Ecology* 20:873–879 DOI 10.1111/j.1365-2435.2006.01163.x.

Vinkler M, Bainova H, Albrecht T. 2010. Functional analysis of the skin-swelling response to phytohaemagglutinin. *Functional Ecology* 24:1081–1086 DOI 10.1111/j.1365-2435.2010.01711.x.

Wakelin D, Apanius V. 1997. Immune defence: genetic control. In: Clayton DH, Moore J, eds. *Host-parasite evolution: general principles and avian models*. Oxford: Oxford University Press.

Weng NP. 2006. Aging of the immune system: how much can the adaptive immune system adapt? *Immunity* 24:495–499 DOI 10.1016/j.immuni.2006.05.001.

Williams GC. 1966. Natural selection, the costs of reproduction, and a refinement of Lack’s principle. *The American Naturalist* 100:687–690 DOI 10.1086/282461.

Wilson AJ, Charmantier A, Hadfield JD. 2008. Evolutionary genetics of ageing in the wild: empirical patterns and future perspectives. *Functional Ecology* 22:431–442 DOI 10.1111/j.1365-2435.2008.01412.x.

Yu TL, Xu Y, Busam M, Deng YH. 2017. Maternal investment decreases under stressful environments in 11 plateau brown frog (*Rana kukunoris*) populations. *Ethology Ecology & Evolution* 30:168–177.

Zamora-Camacho FJ. 2018. Integrating time progression in ecoimmunology studies: beyond immune response intensity. *Current Zoology* In Press.

Zamora-Camacho FJ, Comas M. 2017. Greater reproductive investment, but shorter lifespan, in agrosystem than in natural-habitat toads. *PeerJ* 5:e3791 DOI 10.7717/peerj.3791.

Zhang Z, Jin C, Qu K, Caviedes-Vidal E. 2017. Immune responsiveness to phytohemagglutinin displays species but not sex differences in three anuran species. *PeerJ* 5:e3181 DOI 10.7717/peerj.3181.