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RESEARCH ARTICLE

Net superoxide levels: steeper increase with activity in cooler female and hotter male lizards

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SUMMARY

Ectotherms increase their body temperature in response to ambient heat, thereby elevating their metabolic rate. An often inferred consequence of this is an overall upregulation of gene expression and energetic expenditure, and a concomitant increased production of reactive oxygen species (e.g. superoxide) and, perhaps, a shortened lifespan. However, recent work shows that this may be a superficial interpretation. For example, sometimes a reduced temperature may in fact trigger up-regulation of gene expression. We studied temperature and associated activity effects in male and female Australian painted dragon lizards (*Ctenophorus pictus*) by allowing the lizards to bask for 4 h *versus* 12 h, and scoring their associated activity (inactive *versus* active basking and foraging). As predicted, long-basking lizards (hereafter 'hot') showed heightened activity in both sexes, with a more pronounced effect in females. We then tested for sex-specific effects of basking treatment and activity levels on the increase in net levels of superoxide. In males, short-baskers (hereafter 'cold') had significantly more rapidly decreasing levels of superoxide per unit increasing activity than hot males. In females, however, superoxide levels increased faster with increasing activity in the cold than in the hot basking treatment, and females earlier in the ovarian cycle had lower superoxide levels than females closer to ovulation. In short, males and females differ in how their levels of reactive oxygen species change with temperature-triggered activity.

Key words: reactive oxygen species, thermoregulation, lizard.

INTRODUCTION

In ectothermic animals, ambient and body temperature have a fundamental impact on all aspects of life, from dictating scope for activity *via* active basking to concomitant metabolic rate (Huey and Kingsolver, 2011). This 'metabolic theory of ecology' has grown into a discipline of its own, emanating from the legendary astronomer Harlow Shapley's observation that walking speed in ants increased directly and predictably with temperature to the point that air temperature could be predicted to within 1°C from ant walking speed alone (Huey and Kingsolver, 2011) [see also Shapley (Shapley, 1966), cited in Huey and Kingsolver (Huey and Kingsolver, 2011)]. This close relationship between metabolic physiology, metabolic rate and temperature also applies in ectothermic reptiles (Bennett, 1982; Bennett and Dawson, 1976; Beyer and Spotila, 1994; Zari, 1991). This suggests that with increasing temperature comes an inevitable increase in metabolic rate and mitochondrial activity, and with that an increased production of reactive oxygen species (ROS), perhaps even at the cost of a reduced lifespan (Hollingsworth, 1969; Farmer and Sohal, 1987). However, more recent work suggests that this is an oversimplification. A microarray study of the expression of 15,512 genes in another ectotherm, the zebrafish (*Danio rerio*), at 28 *versus* 18°C, revealed that 'cold fish' upregulated >600 genes more than 1.7-fold and that these genes over-represented two functional gene groups, 'oxygen and reactive oxygen species metabolism' and 'response to oxidative stress' (Malek et al., 2004). Thus, a simplistic relationship between body temperature and genome-wide gene expression level was refuted. To what extent these effects reduce net ROS levels still needs examining though.

In order to investigate sex-specific effects of activity and temperature on net ROS levels, we studied these phenomena in the Australian painted dragon lizard, *Ctenophorus pictus* (Peters 1866), males and females of which have different patterns of behaviour during their activity period August to May. Males engage in overt aggressive behaviour with rising testosterone levels, open exposure to rivals and predators at territory patrolling and mate searching (Olsson et al., 2007). Females are camouflaged and have a more cryptic lifestyle than males, while spending most of their time basking to maintain appropriate incubation temperatures for developing follicles and eggs (Olsson et al., 2007).

Given the conflicting information on the relationships between temperature, metabolic rate and antioxidant production in the recent literature, especially from the fish literature demonstrating that gene expression may increase at a relatively lower temperature, we made no *a priori* predictions but designed an experiment to specifically model net superoxide levels as a result of basking treatment and activity in both sexes.

MATERIALS AND METHODS

Field methods and husbandry

This work was performed under the Animal Ethics permit AE10/11-13 at the University of Wollongong. The lizards were all caught by

noose or by hand at Yathong Nature Reserve, NSW, Australia (145°35′E; 32°35′S) and were brought back to holding facilities at the University of Wollongong a week before the onset of the experiments (27 October, running till 21 December 2008). Lizards were housed individually in cages (330×520×360 mm) and randomly assigned to a light:dark regime for 2 months of either 12 h:12 h (hot) or 4 h:20 h (cold). The lizards were fed crickets and mealworms to satiation at 09:00–10:00 h every second day. The lizards were weighed to the nearest 0.01 g and measured snout to vent to the nearest 1.0 mm on 27 October.

Heating lamps (spotlights; the only heat source) for the hot lizards were turned on at 06:00 h and off at 18:00 h. Those for the cold lizards were turned on at 09:00 h and off at 13:00 h. Average ambient temperature in the animal room was ~25°C and did not differ between the two treatment groups after the cold treatment group's spotlights had been turned off. The spotlights created an ambient heat gradient between ~27 and 45°C in the cages, in which the lizards could thermoregulate to their preferred body temperature observed in the wild (36°C; M.O., unpublished data obtained from cloacal temperature readings in the wild). The lizards were monitored for activity daily at 11:30 h and 14:30 h. We assigned individuals a value of '0' or '1' corresponding to 'inactive' or 'active', respectively. Lizards were considered inactive if they were beneath a shelter at the cooler end of the cage and not visible to the observer, and were considered active if they were moving or basking in their cages. 'Active' lizards were not always moving, but 'active' here represents the natural foraging behaviour in these territorial, sit-and-wait predators, going from basking to foraging and *vice versa*. Thus, we have no ambition to distinguish the effects of some forced activity (e.g. constant running on a treadmill) to ROS production but quantify the effects of a behaviour that is biologically meaningful in the lizards' natural, free-ranging state. The purpose of the monitoring procedure was to test the effects of the treatments on activity and increase the resolution of basking treatment with the rationale that elevated activity also contributes to superoxide production, independent of temperature (Alessio et al., 2000) (i.e. monitored twice daily for 56 days, 27 October to 21 December). Three activity indices were constructed based on these activity scores, taking the mean across individual observations in the morning, afternoon or both. The rationale for these different mean indices (a reviewer is gratefully acknowledged for this suggestion) was to provide a basis for discussing shorter and longer term treatment effects on superoxide production. This yields the following three mean activity indices, with their differences stated: (1) mean daily activity scores incorporating both morning and afternoon observations; (2) mean morning activity scores, i.e. with all lizards having the possibility of basking, with heating lamps having been on for 5.5 *versus* 2.5 h in the hot and cold treatments, respectively); and (3) mean afternoon activity, with hot lizards being able to thermoregulate, whereas cold lizards had their spotlights turned off. Thus, the difference in mean activity was expected to be largest in the mean afternoon scores (when, largely, only hot lizards were active), and smallest in the mean morning scores (when all lizards could thermoregulate to their preferred body temperature and become active foragers and mate searchers – although held in the absence of partners).

Two reproductive parameters in females known to influence ROS levels were also considered, cumulative fecundity (residuals from a regression of total egg mass produced before blood sampling regressed on female mass, range –5.7 to 7.6) (M.O., M.T., M.H., C. Perrin and M.W., submitted), and days to oviposition (as a proxy of days to ovulation, range 5–51 days) (M.O., M.T., M.H., C. Perrin and M.W., submitted). The first of these estimates has been found to increase DNA damage (M.O., M.T., M.H., C. Perrin and M.W., submitted) and we therefore suspected that this might be a result of oxidative stress from ROS exposure, which provided the rationale for including this trait in our first analysis of female ROS patterns. The second of these traits, days to egg laying (as a proxy of ovarian stage), shows tight negative covariance with ROS levels in females, peaking when the least vitellogenin is circulating in the plasma (M.O., M.T., M.H., C. Perrin and M.W., submitted). We therefore also included this trait in our initial analysis.

Quantifying superoxide

To analyse ROS, we used flow cytometry in combination with MitoSOX, which freely diffuses into cells, accumulates within mitochondria and becomes fluorescent when oxidised by superoxide. Before and after the experimental treatment (27 October and 21 December 2008), a single sample of peripheral blood (25 µl) was obtained from the vena angularis (in the corner of the mouth) from each animal in random order in the morning when the animals had basked to their preferred body temperature, ~36°C (no blood can be drawn from cold lizards). It was diluted immediately with 9 volumes of phosphate-buffered saline (PBS; 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4, 8 mmol l^{-1} Na_2HPO_4, pH 7.4) and stored on ice prior to analyses, which were completed within 4 h of sampling. Prior to staining, diluted blood was diluted a further 50-fold with PBS and then centrifuged (300 ***g*** for 5 min) to pellet cells; each cell pellet corresponded to 10 µl of whole blood. Cells were resuspended in 100 µl of PBS containing 5 µmol l^{-1} MitoSOX Red (MR; Molecular Probes, Invitrogen, Carlsbad, CA, USA). MR was added from stock solutions in dimethylsulphoxide (DMSO); the final concentration of DMSO was ≤0.2% (v/v). Cells were subsequently incubated at 37°C for 30 min, then washed with PBS by centrifugation as described above and held on ice until analysed by flow cytometry; 50,000 events were acquired for all samples. Flow cytometry was performed using a Becton Dickinson LSR II, with excitation at 488 nm and emitted fluorescence collected using bandpass filters of 575±13 nm. Data were acquired and analysed using FACSDiva v4.0.1 (Becton Dickinson, Sydney, Australia) and FloJo v8.8.7 (TreeStar Inc., Ashland, OR, USA) software. On the basis of forward angle laser scatter and side angle laser scatter, a number of blood cell populations were discerned; the results obtained were similar for all these populations. For each sample, the arithmetic mean fluorescence for all 50,000 cells acquired was determined using FloJo software and used to compare between samples and treatments. The accuracy of flow cytometry results from two samples from the same individuals has been measured in a separate experiment (Olsson et al., 2008), involving 14 males with a correlation coefficient between samples of r=0.97, P<0.0001. Thus, our flow cytometry technique can be argued to be highly consistent.

Statistical analysis

We used SAS 9.1 for all analyses. Our statistical analyses followed conventional procedures in that full models with interactions were first analysed and covariates or other predictors were then removed with a P value to enter of 0.25 (i.e. parameters were backwards eliminated when P>0.25) (Quinn and Keough, 2002). We also report the Akaike information criterion (AIC) scores for these models, which were consistently very low. We used Proc GLM in our analyses of variance and homogeneity of slopes tests. AIC scores were checked by running the same models in Proc Mixed (as GLM does not automatically produce an AIC output). The fixed factors in our models were sex and basking treatment. The reproductive parameters were entered as covariates in the female-specific models. The mean daily activity scores are reported in full detail whereas

the mean morning and afternoon activity scores are reported in contrast to the mean daily scores.

RESULTS

Lizards with more basking opportunities were (not surprisingly) more active (based on mean daily scores), with males having activity scores of 0.73 (±0.047, mean ± s.e.m., N=10) in the hot treatment and 0.53 (±0.022, N=9) in the cold (Satterthwaite's t=3.03, P=0.010; as variances were unequal, F-test, F=5.27, P=0.028, Satterthwaite's algorithm was used for calculating d.f.=12.6). In females, the corresponding comparison was 0.92 (±0.016, N=10) and 0.58 (±0.011, N=9), in the hot and cold basking treatments, respectively. This difference was also significant (pooled d.f.=17, t=16.9, P<0.0001). A two-factor ANOVA confirmed significant effects of sex, basking treatment and their interaction (model $F_{3,34}$=32.0, P<0.0001, R^2=0.74, effect of treatment, d.f.=1, F=75.3, P<0.0001, effect of sex, d.f.=1, F=9.32, P=0.004, treatment × sex interaction, d.f.=1, F=10.3, P=0.003).

Superoxide effects

Mean daily activity scores

There was no significant difference between the sexes in the level of superoxide (two-factor ANOVA with sex and basking treatment as factors, model $F_{4,32}$=0.16, P=0.96). Nor was there a significant sex × treatment interaction (F=0.20, P=0.66).

In males, both predictors and their interaction significantly influenced the level of superoxide throughout the experiment (model R^2=0.32, AIC=–2.6; Type III SS, 5.31<F<5.89, 0.029<P<0.037). Superoxide was higher in the cold basking treatment (model parameter estimate$_{cold}$=2.51±1.09, mean ± s.e.m., F=2.30, P=0.037, compared with the hot basking treatment, the latter set to zero by Proc GLM, SAS). However, quite remarkably, superoxide levels decreased faster with increasing activity in the cold basking treatment whereas in the hot treatment superoxide levels increased with activity (Fig. 1; treatment × activity interaction; β regression coefficients, β_{cold} basking=–4.31±1.78, mean ± s.e.m., P=0.029, β_{hot} basking set to zero; Fig. 1).

In females, our analysis explained 83% of the variation in superoxide among individuals (R^2=0.83), with significant effects of all predictor variables in the model except cumulative fecundity ($F_{fecundity}$=1.11, P=0.32). Cumulative fecundity was therefore eliminated from the model and the data reanalysed. This analysis had almost as high a coefficient of determination (R^2=0.81, AIC=–15.6) and showed significant effects of treatment with steeper increasing levels of superoxide in colder females (Fig. 2; F=4.80, P=0.051, model parameter estimate for cold basking=3.69±1.7, mean ± s.e.m., long basking set to zero), activity (F=13.90, P=0.003), their interaction (F=9.19, P=0.011), and time to ovulation (F=27.0, P=0.0003; all predictor d.f.=1, d.f.$_{error}$=11). See further statistical confirmation of these results in the Appendix.

Mean morning activity scores

In males, all significant differences between treatment groups vanished when both cold and hot males could thermoregulate (model F=0.97, P=0.42), with all treatment and mean activity effects and their interaction having P>0.13. Mean activity in the morning did not differ between hot and cold males (mean activity ± s.d., 0.97±0.03 and 0.98±0.03, respectively; t-test, d.f.=17, t=0.62, P=0.54).

In females, the only significant effect remaining when mean morning activity alone was analysed was time to ovulation (F=14.54, P=0.0029; P>0.28 for all remaining predictors). Mean activity in the morning did not differ between hot and cold females (mean activity ± s.d., 0.95±0.02 and 0.88±0.04, respectively; t-test, d.f.=17, t=1.55, P=0.14).

Mean afternoon activity scores

In males, there were significant effects of mean afternoon activity on superoxide (F=5.58, P=0.033) and its interaction with treatment (F=5.17, P=0.039), whereas the effect of treatment *per se* was non-significant (P=0.609). Mean activity in the afternoon differed significantly between hot and cold males (mean activity ± s.d., 0.55±0.22 and 0.09±0.08, respectively; t-test, d.f.=17, t=–5.92, P<0.0001).

In females, there were significant effects of mean activity on superoxide (F=13.14, P=0.004) and its interaction with treatment (F=7.28, P=0.021), whereas the effect of treatment *per se* was non-significant (P=0.396). The effect of time to ovulation was also significant in this analysis (F=20.5, P=0.0009). Mean activity in the afternoon differed significantly between hot and cold females (mean activity ± s.d., 0.84±0.22 and 0.03±0.06, respectively; t-test, d.f.=17, t=–21.4, P<0.0001).

DISCUSSION

We set out with the pragmatic expectation of finding that longer hours of basking would elevate body temperature, activity and, *via* metabolic rate, superoxide production. Our results, however, were

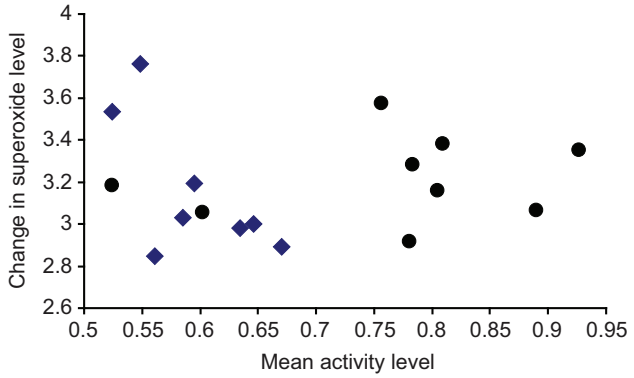

Fig. 1. The relationship between our mean activity score (range 0–1) and flow cytometry-assessed level of superoxide (relative fluorescence) in males from two treatment groups, 4 h basking (cold, diamonds) and 12 h basking (hot, circles). R^2=0.32, Akaike information criterion (AIC)=–2.6.

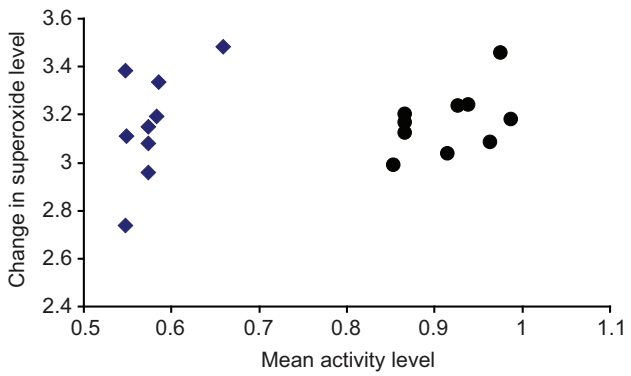

Fig. 2. The relationship between our mean activity score (range 0–1) and flow cytometry-assessed level of superoxide (relative fluorescence) in females from two treatment groups, 4 h basking (cold, diamonds) and 12 h basking (hot, circles). R^2=0.83, AIC=–15.6.

more complex than that, demonstrating sex-specific differences in how basking treatment influences activity patterns in the two sexes and how these impact on net superoxide levels. Both sexes showed significant interaction effects between treatment and mean daily activity on superoxide level. This makes the interpretation of main effects (treatment) complicated and, in principle, we cannot say that there was a significant main effect of the hot and cold basking treatment. This agrees well with the plotted observations in Figs 1 and 2, with the centers of the plotted clusters sitting at the same superoxide mean (on the *y*-axis). This also agrees with our analysis of no difference in superoxide between the sexes.

Our experiment was designed to capture the effects of long-term differences in basking and activity regimes on superoxide production (over 56 days) and we therefore took the difference for each individual in superoxide levels at the start and end of the experiment in order to account for inter-individual differences at the start of the experiment. That said, any differences in metabolic activity at the time of blood sampling could potentially eliminate or exaggerate such effects (e.g. if all lizards are hot and active at the blood sampling event, this may mask the long-term treatment effects and prediction of lower superoxide production in overall less active or colder lizards). We therefore followed the advice of a reviewer and calculated three different mean activity indices for all day *versus* morning and afternoon. Interestingly, when we analysed the morning and afternoon activity scores separately, the treatment × activity interactions we identified with the daily activity remained qualitatively unaltered in the afternoon data but vanished in the morning data for both sexes. Thus, this agrees with the conclusion that our long-term effects are, at least partly, masked by the temperature and activity regime at the time of blood sampling (otherwise we would still see differences in superoxide shift from onset levels in the activity index based on morning activity alone).

More interestingly, the significant interaction effects (slope differences) between superoxide and mean daily activity in the two sexes and temperature treatment groups beg an explanation. We focus on these below.

For females, the results are generally unremarkable. First, in our experience, a statistical analysis explaining 83% of the variance in the net levels of ROS is rare and partly based on the fact that we know from previous work that female superoxide levels vary through the ovarian cycle. Thus, we find these results robust and with high predictive power. We have in previous work demonstrated a negative relationship between female instantaneous clutch size (i.e. clutch size resulting from one ovarian cycle, not cumulative through the mating season) and superoxide levels (Olsson et al., 2009a). Furthermore, we have shown in another study that females with more time to ovulation have lower levels of superoxide (Olsson et al., 2009a). In short, high levels of vitellogenin may be a short-term advantage in terms of antioxidation and/or may increase as part of cell signalling at ovulation *per se* (Schkolnik et al., 2011).

Links between reproductive traits and oxidative stress, and sometimes a lack thereof, have been demonstrated in a number of recent reviews. Constantini and colleagues review evidence showing that oxidative stress may damage oocytes and embryos (Constantini et al., 2010), and in Soay sheep (Nussey et al., 2009) rapid growth rate in lambs induces similar effects. Dowling and Simmons (Dowling and Simmons, 2009), Monaghan and colleagues (Monaghan et al., 2009), and Metcalfe and Alonso-Alvarez (Metcalfe and Alonso-Alvarez, 2010) also review the literature for links between superoxide and the cost of reproduction and conclude that investment in reproduction may result in oxidative stress. A case study on Alpine swifts (*Apus melba*) by Bize and colleagues agrees with this, too, demonstrating that male survival from one season to the next is positively influenced by the ability to withstand oxidative stress, and females with higher resistance to oxidative stress lay larger clutches (Bize et al., 2008). Thus, there may well be effects of reproductive investment on levels of superoxide, with resulting effects on oxidative stress.

Net superoxide level increased faster in more active cold females but we saw the opposite results in males, with cold males having a reduction in superoxide with activity and faster rising superoxide in hotter males that were more active. This suggests that two groups of factors warrant discussion: (i) proximate factors that regulate net levels of superoxide, and (ii) ultimate factors that may explain differences in evolutionary history between males and females. One proximate explanation, realising that net superoxide is the summed effect of its production and antioxidation, is that these two processes may have different relationships to temperature. For example, if the production of innate antioxidation enzymes (e.g. superoxide dismutase) is more compromised at suboptimal temperatures than is superoxide as a by-product from the electron transport chain, the net outcome will be higher superoxide levels at lower temperatures. To the best of our knowledge, no one has explicitly tested these relationships *in situ*. Alternatively, ingested antioxidants (e.g. carotenoids) may be differently metabolised, with their superoxide-scavenging effect influenced accordingly, at different body temperatures. We doubt this has any major explanatory power in this system, as our previous carotenoid experiments reveal no effect of carotenoid treatment on circulating superoxide levels (Olsson et al., 2008). A third potential explanation is that the mitochondrial membrane potential changes with temperature and that superoxide production is showing associated covariation, while superoxide dismutase production is less affected. This has not been tested in any system we are aware of. A fourth option is that there are threshold temperatures at which gene expression is drastically altered [as in the zebrafish study cited in the Introduction (Malek et al., 2004)], and that genes for antioxidant enzyme production have been 'flicked' on or off for different durations in our different basking groups. Again, this remains an untested possibility in this system but has very strong and detailed support in the cited zebrafish work (Malek et al., 2004). To what extent this resulted in changes in ROS levels in the zebrafish study remains unknown, as ROS was not explicitly measured, but a ROS reduction seems highly likely given the increased production of ROS-reducing innate antioxidant enzymes. Fifth, data from sports medicine show that aerobic and isometric activity do indeed increase oxidative stress [e.g. protein damage increased by 67% and lipid peroxidation by 24% following aerobic exercise (Alessio et al., 2000)]. In the present study of ectothermic lizards, activity and body temperature are likely to covary and both contribute to superoxide production. Thus, the higher rate of superoxide production in cold females can perhaps be explained by two phenomena: these females are likely to show a greater difference in temperature (and activity) within their treatment group than is the case for hot females, as hot females are foraging and basking under a warm spotlight at both activity measurement points per day (11:30 h and 14:30 h), whereas cold females only have their spotlights turned on at 09:00 h, and turned off at 13:00 h. Thus, the body temperature interval over which superoxide is measured is likely to be greater in cold than in hot females, which may perhaps facilitate its detection and steeper increase per unit activity and degree of body temperature. As we could not measure body temperature *per se*, only its manifestation in activity, we cannot test this prediction explicitly.

The more rapid decline of superoxide with activity in cold than in hot males requires a different explanation from the reversed outcome in females: we obviously do not know the underlying mechanism for this but suggest that effects of one or more of the suggested proximate mechanisms listed above have been under different selection pressures for net superoxide maintenance in males and females. So, how can these patterns be reconciled from a perspective of selection history and ongoing evolution? Our first reflection is on the differences in natural behaviour between males and females, with males being the much more active sex and probably more often forced to extreme temperatures during territorial disputes and mate search than females. This would suggest that males are better able to cope with extreme temperatures (with better antioxidation of superoxide at these extremes), which agrees with our observations. Furthermore, females are reproducing during the majority of their 1 year lifecycle, producing ~3–5 clutches (Olsson et al., 2007; Olsson et al., 2009b), and, hence, being under strong selection pressure to maintain embryonic development at an optimal level while maintaining a cryptic, sedentary lifestyle. Thus, females are likely to have been under strong selection to tolerate a less extreme and maintain a more precise body temperature ideally suited for embryonic development (e.g. Angiletta et al., 2002). This may result in less strong selection for superoxide antioxidation at higher temperatures.

In summary, our results show no differences in the mean net level of male and female superoxide level but, rather, extreme sex differences in the patterns underlying these levels in the two sexes. We conclude that the most likely explanation for this is differences in selection pressure for precision in thermoregulation in these ectotherms and how this impacts factors such as metabolic rate and sex-specific gene expression associated with the production of antioxidation enzymes like superoxide dismutase.

APPENDIX

The significant effect of activity on superoxide in the cold female treatment, and the significant interaction between treatment and activity, can be argued to be driven by a borderline activity outlier that becomes apparent when the clusters are plotted in separate figures. This outlier deviated approximately two standard deviations from the remaining sampling mean (0.58) (i.e. 2×0.06). We therefore followed Barnett and Lewis (Barnett and Lewis, 1984) and Winsorized the data (i.e. replaced the outlier with the next lower observation; that is, with activity index=0.585) and re-performed the analysis. This still revealed a very high coefficient of determination (R^2=0.71), a borderline effect of treatment (F=4.48, P=0.058), and significant effects of activity (F=7.38, P=0.020), their interaction (F=5.85, P=0.034), and time to ovulation (F=16.33, P=0.002). This analysis also showed that females in the cold treatment had a higher per unit increase in superoxide with mean daily activity than females in the hot treatment (β_{cold}=6.96±2.87, contrasted against β_{hot} basking set to zero, as revealed by their significant interaction, F=5.85, P=0.034; Fig. 2).

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