Reduced resistance to oxidative stress during reproduction as a cost of early-life stress.

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Abstract

Stress exposure during early-life development can have long-term consequences for a variety of biological functions including oxidative stress. The link between early-life stress and oxidative balance is beginning to be explored and previous studies have focused on this link in adult non-breeding or immature individuals. However, as oxidative stress is considered as the main physiological mechanism underlying the trade-off between self-maintenance and investment in reproduction, it is necessary to look at the consequences of early-life stress on oxidative status during reproduction. Here, we investigated the effects of exposure to pre- and/or post-natal stress on oxidative balance during reproduction under benign or stressful environmental conditions in an avian model species, the Japanese quail. We determined total antioxidant status (TAS), total oxidant status (TOS) and resistance to a free-radical attack in individual exposed to pre-natal stress, post-natal stress or both and in control individuals exposed to none of the stressors. TAS levels decreased over time in all females that reproduced under stressful conditions. TOS decreased between the beginning and the end of reproductive period in pre-natal control females. In all females, resistance to a free-radical attack decreased over the reproductive event but this decrease was more pronounced in females from a pre-natally stress development. Our results suggest that pre-natal stress may be associated with a higher cost of reproduction in terms of oxidative stress. These results also confirm that early-life stress can be associated with both benefits and costs depending of the life-history stage or environmental context.

Keywords: antioxidant, developmental programming, early life stress, oxidative stress, post-natal stress, pre-natal stress, reactive oxygen species, reproduction cost

1. Introduction

Adverse environmental conditions during early development can shape individual phenotypes over the long-term in a range of species (Lupien et al., 2009; Monaghan, 2008). This phenomenon, known as 'developmental programming', is well documented and appears to be a conserved mechanism across vertebrates (Cottrell and Seckl, 2009; Love et al., 2013; Lupien et al., 2009; Marasco et al., 2013). Glucocorticoid (GC) stress hormones, released after activation of the hypothalamic-pituitary-adrenal (HPA) axis, appear to be prime candidates for mediating developmental stress programming (Lupien et al., 2009; Welberg and Seckl, 2001). Indeed, stress experienced by the mother can increase embryonic exposure to GCs through the placenta in mammals or through their deposition into the egg in birds, reptiles and fish (Henriksen et al., 2011; Lupien et al., 2009). During post-natal development, environmental stressors can also directly activate individual HPA axis, again resulting in an increased exposure to GCs during a sensitive period for development (e.g. Banerjee et al., 2012; Macrì and Würbel, 2007). GCs exposure during early-life can affect different biological functions including energy metabolism, HPA axis functioning, oxidative stress and behavior (Costantini, 2014; Haussmann et al., 2011; Henriksen et al., 2011; Lupien et al., 2009; Marasco et al., 2013; Monaghan, 2008; Zimmer et al., 2013; Zimmer and Spencer, 2014). Although, developmental programming is generally considered as a constraint (Lupien et al., 2009; Welberg and Seckl, 2001), an alternative hypothesis recognizes the potential for adaptive responses, which enhances fitness if developmental environments match those experienced later in life (Bateson et al., 2004; Monaghan, 2008). In accordance with this 'environmental matching hypothesis', it has been shown that exposure to either pre- or post-natal stress or a combination of both programmed neuro-physiological and behavioral traits in a potentially adaptive way in the Japanese quail (*Coturnix japonica*) (Zimmer et al., 2013; Zimmer and Spencer, 2014). However, early-life stress is likely to have both benefits and

costs that may arise at different life-history stages or may depend on the environmental context (Haussmann et al., 2011; Marasco et al., 2013).

In the last decade, there has been considerable interest in the role of oxidative stress in mediating the trade-off between investment in self-maintenance and reproduction (reviewed in Costantini, 2008; Metcalfe and Monaghan, 2013; Monaghan et al., 2009; Selman et al., 2012; Speakman and Garratt, 2014). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defenses to neutralize these highly reactive compounds creating an imbalance between pro- and anti-oxidative compounds which results in the accumulation of oxidative damage. As early-life stress, oxidative stress appears to ultimately contribute to ageing and mortality (Bouwhuis et al., 2010; Costantini, 2014; Hayward et al., 2009; Monaghan et al., 2009; Nussey et al., 2007; Selman et al., 2012; Speakman and Garratt, 2014). Therefore, it is not surprising that the link between early-life stress and oxidative balance is beginning to be explored (Costantini, 2014; Marasco et al., 2013). In this context, it has been shown that pre-natal stress resulted in a higher basal intermediate oxidative damage compounds (ROMs) levels in the chicken (*Gallus domesticus*) (Haussmann et al., 2011). However, in the Japanese quail, exposure to pre- or post-natal stress or a combination of both has been associated with an increase in enzymatic antioxidant activity whereas ROS production was not affected. This up-regulation of antioxidant defenses in birds stressed during early-life may be adaptive to protect biomolecules from oxidative damage (Marasco et al., 2013). Previous studies have focused on the consequences of early-life stress on oxidative stress in adult non-breeding or immature individuals. However, as oxidative stress may be the proximate mediator of the trade-off between investment in reproduction and survival, it is significant to look at the consequences of early-life stress on oxidative status during reproduction to fully understand how developmental experience might affect this important life history trade-off.

In this study, we investigated the effects of exposure to pre- and/or post-natal stress on oxidative balance during reproduction under benign or stressful environmental conditions in the Japanese quail. As it has been shown in the Japanese quail that exposure to early-life stress resulted in up-regulated antioxidant defenses (Marasco et al., 2013), we made the hypothesis that level of antioxidant defenses would be higher in females exposed to one or both of our early-life stresses , at least before reproduction. Consequently, if investment in reproduction imposed oxidative stress costs, we expected that this cost should be buffered in females exposed to early-life stress. Finally, we hypothesized that reproduction under stressful condition should increase the cost of reproduction and thus oxidative stress should be higher compared to reproduction under benign condition. If the environmental matching hypothesis applies in this context, females exposed to early-life stress should be able to better cope with this higher cost of reproduction.

2. Material and methods

2.1 Pre- and post-natal treatments

We used 76 unrelated fertile Japanese quail eggs. After 5 days of incubation, half of these eggs were injected with 10 µl corticosterone (CORT) dissolved in sterile peanut oil at the egg apex under sterile conditions (pre-natal CORT: n= 38). This increased endogenous CORT concentrations in the yolk within 1.8 SD above control yolks (Zimmer et al., 2013). Control eggs were injected with peanut oil alone (pre-natal Ctrl: n = 38). At hatching, chicks (n = 59) were individually marked with a unique pattern of colors using nail polish allowing individual recognition. The day after, chicks of each pre-natal treatment were randomly allocated to two separated pens with *ad libitum* food. When chicks were 4 days old, one pen of each pre-natal treatment (pre-natal CORT or Ctrl) was assigned to one of two post-natal food treatments: either food removal on a random schedule for 3.5h per day (25% of daylight hours) between 8

A.M. and 8 P.M. between the age of 4–20 days (post-natal Food-: n = 28) or *ad libitum* food at all times during the same period (post-natal Ctrl: n = 31) (see Boogert et al., 2013; Zimmer et al., 2013 for details). After this time, all birds were provided with *ad libitum* food until reproduction experiment. We thus created four treatment groups: pre-natal Ctrl/post-natal Ctrl (n = 15); pre-natal Ctrl/post-natal Food- (n = 13), pre-natal CORT/post-natal Ctrl (n = 16) and pre-natal CORT/post-natal Food- (n = 15).

2.2 Reproduction experiment

Females were mated at 236.5 ± 1.3 days old and were monitored during clutch production. During clutch production, females were placed in individual cages (76 x 48 x 53 cm). For each of the four early-life treatment groups, half of the females experienced food removal conditions for 25% of daylight hours (3.5h) on a random daily schedule for 28 days (reproduction Food-: n = 16) and the other half under *ad libitum* food conditions (reproduction Ctrl: n = 17). It resulted in 8 experimental groups: pre-natal Ctrl/post-natal Ctrl/reproduction Ctrl (n = 5), pre-natal Ctrl/post-natal Food-/reproduction Food- (n = 5), pre-natal Ctrl/post-natal Food-/reproduction Ctrl (n = 3), pre-natal Ctrl/post-natal Food-/reproduction Food-(n = 3), pre-natal CORT/post-natal Ctrl/reproduction Ctrl (n = 6), pre-natal CORT/post-natal Ctrl/reproduction Food- (n = 5), pre-natal CORT/post-natal Food-/reproduction Ctrl (n = 3), pre-natal CORT/post-natal Food-/reproduction Food- (n = 3). During food removal the food bowls were removed from cages and trays were cleaned to remove spilled food. Females within each group were randomly assigned to a reproduction treatment. During this period, a control male was placed in each female cage for 10 minutes once a day. This has been shown to be an effective way to produce fertile eggs whilst minimizing harassment of females from males (Duval et al., 2014). We used eight different

males with each male allocated to four females every day. The order of presentation of females was randomly assigned every day for each male.

2.3 Blood sampling and oxidative stress analyses

The day before the first (initial) and the last day (final) of the breeding treatment, between 9:30 and 11:30 A.M. each female was captured from its home cage and blood (80 µl) was collected by venipuncture of a brachial vein within 2 minutes of the experimenter entering the room. Three experimenters silently entered the room and each caught a quail in its cage and then went to a nearby room where the blood was collected. Additionally, both sides of the rooms were visually divided so birds could not see us entering. Consequently, we were able to catch birds from one side of the room without disturbing birds of the other side. Bleed order was added as a covariate in the analyses and showed no effect. Blood was collected in a heparinized capillary and then transferred into a microtube and kept on ice until centrifugation (within 1 hour after collection). Twenty microlitres of whole blood was immediately mixed with 730 µl of saline buffer (158 mM Na^+, 144 mM Cl^-, 6 mM K^+, 24 mM HCO_3^-, 2 mM Ca^{2+}, 340 mOsm, pH 7.4) and kept at 4°C before analysis of resistance to free-radical attack, which occurred within 6 hours (Alonso-Alvarez et al., 2007). The remaining blood was centrifuged for 5 minutes at 3500 rpm and plasma stored at -20°C for later analysis. All experimental procedures were carried out under Home Office Animals (Scientific) Procedures Act project license 60/4068 and personal license 70/1364 and 60/13261.

From the frozen plasma, we assessed oxidative stress level by determining plasma total antioxidant status (TAS) and plasma total oxidant status (TOS) using commercial kits (TAS assay kit RL00017, TOS assay kit RL00024, Rel Assay Diagnostics, Gaziantep, Turkey). TAS assesses the non-enzymatic antioxidants present in the plasma. TOS assesses both hydrogen peroxide components and lipid hydroperoxides (see Bourgeon et al., 2012) for

166 details). Plasma and reagent volumes were adapted and validated for our species by checking
167 that dilution of standards and pools of plasma are linear and that pools dilution curves are
168 parallel to standard dilution curve. For TAS, 160µl of assay buffer was pipetted in a 96-well
169 microplate (Nunc^{TM}). We added 10 µl of plasma of initial and final sample for each
170 individual, 10µl of standard (1.0 mmol equivalent Trolox.L^{-1}) in 4 wells, 10 µl of deionized
171 water as a second standard in 4 wells and 10 µl of a pool of quail plasma in 4 wells as a
172 quality control. Initial absorbance at 660 nm was read and 25µl of colored ABTS radical
173 solution was added in all wells and the plate was incubated at 37°C for minutes before a
174 second reading at 660 nm. For TOS, 200 µl of assay buffer was pipetted in a 96-well
175 microplate (Nunc^{TM}). We added 30 µl of plasma of initial and final sample for each individual
176 was added, 30 µl of standard (20 µM equivalent H_2O_2.L^{-1}) in 4wells, 30 µl of deionized water
177 as a second standard in 4 wells and 30 µl of a pool of quail plasma in 4 wells as a quality
178 control. Initial absorbance at 540 nm was read and 10 µl of prochromogen solution was added
179 in all wells and the plate was incubated at 37°C for minutes before a second reading at 540
180 nm. All samples were run in duplicate. For both assays, results were calculating following the
181 instruction provided in the protocol. Intra- and inter-assay coefficients of variation were 0.03
182 and 0.03 for TAS and 0.08 and 0.10 for TOS, respectively.
183 Resistance to free-radical attack was assessed as the time needed to haemolyse 50 %
184 of red blood cells following a controlled free-radical attack (see Alonso et al., 2007; Kim et
185 al., 2010) for all females at the beginning and at the end of the reproduction treatment.
186 Briefly, we loaded 80 µl of each whole blood sample in duplicate into a 96-well microplate
187 (Nunc^{TM}). Then, we added 136 µl of a 150 mM solution of 2,2'-azobis-
188 (amidinopropane)hydrocholoride (AAPH) into each well. The microplate was incubated at
189 40°C and read with a microplate reader spectrophotometer every 10 minutes at 540 nm for 3–
190 4 hours until all samples reached their baseline values (Alonso-Alvarez et al., 2007; Kim et

al., 2010). Rapid lysis of red cells by the AAPH indicates a reduce resistance of their membranes to free radical aggressions. Resistance of red blood cells' membrane to radical attacks depended of the level of membrane lipids peroxidation, past exposure to oxidative attacks and the level of enzymatic and non-enzymatic antioxidant defenses (Brzezinska-Slebodzinska, 2001; Lesgards et al., 2002). Therefore, this test provides both a dynamic assessment of total antioxidant (enzymatic and non-enzymatic) capacity and of oxidative damage suffered by blood cells in a recent past (Alonso-Alvarez et al., 2007; Bize et al., 2008). Moreover, as the average lifespan of erythrocytes in birds is about 30 days (Sturkie and Griminger, 1986), our second measure (30 days from the treatment start) allows assessment of the oxidative damage suffered throughout the experimental treatment.

2.4 Statistical analysis

We used generalized linear mixed models fitted with a gamma law to examine how pre- and post-natal stress and reproduction stress affected the time needed to haemolyse 50 % of red blood cells, TAS, and TOS using the GLIMMIX procedure in SAS 9.2 (SAS Institute Corporation). In each model, we included pre- and post-natal treatment, reproduction treatment and reproduction stage (initial/final) as fixed factors. Individual was added as random factor to account for inter-individual differences. Tukey-Kramer multiple comparison adjustments were applied to obtain corrected p-values. Probability levels < 0.05 were considered as significant. Data presented are mean ± SEM.

3. Results

TAS and TOS were not directly influenced by pre-natal treatment, post-natal treatment, reproduction treatment and reproduction stage (Table 1). Nevertheless, TAS was significantly affected by the interaction between reproduction treatment and reproduction stage ($F_{1,33}$ =

7.36, p = 0.009). At the end of reproductive period, TAS was lower in reproduction Food- females than in reproduction Ctrl females (t_{35} = 2.69, p= 0.045, Table 2). TOS was influenced by the interaction between pre-natal stress and reproduction stage ($F_{1,31.8}$ = 10.89, p = 0.003). TOS decreased between the beginning and the end of reproductive period in pre-natal Ctrl females (t_{25} = 2.85, p = 0.04) but did not change in pre-natal CORT females (Figure 1).

Resistance to free-radical attack was also not directly influenced by pre-natal treatment, post-natal treatment and reproduction treatment but by reproduction stage (Table 1). Resistance to a free-radical attack was higher at the beginning (140 ± 2 min) than at the end (79 ± 2 min) of laying period. Resistance to a free-radical attack was also influenced by the interaction between female exposure to pre-natal stress and reproductive stage ($F_{1,33}$ = 6.5, p = 0.014). Before clutch laying, there was no difference between pre-natal Ctrl and pre-natal CORT females (t_{34} = 0.52, p = 0.95; Figure 2). After clutch laying, resistance in both groups was lower than before (t_{54} < -14.50, p < 0.0001; Figure 1) and resistance in pre-natal CORT was significantly lower than in pre-natal Ctrl females (t_{34} = -2.62, p = 0.01; Figure 2).

4. Discussion

In this study, we showed that pre-natal stress, according to one measure, led to a decrease in resistance to oxidative stress but this decrease was only apparent during investment in reproduction which suggests that pre-natal stress may be associated with a higher cost of reproduction. We also revealed that reproduction under stressful conditions did not increase the oxidative cost of reproduction.

Contrary to our prediction, at the beginning of the reproduction period antioxidant defenses were not higher in females exposed to early-life stress. Exposure to early-life stress did not affect females ROS production and resistance to free-radical attack. These results are not in accordance with those previously obtained in birds. In the Japanese quail, it has been

241 shown that early-life stress exposure had tissue specific effects with notably an up-regulation
242 of enzymatic antioxidant defenses and a decrease in non-enzymatic antioxidant capacity in
243 red blood cells (Marasco et al., 2013). In chicken, pre-natal stress resulted in a basal higher
244 level of intermediate oxidative damage compounds (Haussmann et al., 2011). This
245 discrepancy could arise from the difference in the methods and tissues used to measure the
246 oxidative balance. Another possibility is the age difference between our sexually mature adult
247 individuals (9 months) and juveniles chickens (25 days) and young adult quail (64 days), as
248 oxidative status can differ across the lifespan (Selman et al., 2012). At reproduction level, our
249 TOS and TAS results suggest that there was no oxidative stress cost associated with
250 investment in reproduction in our females. On the contrary, the dramatic decrease (almost 50
251 %) in the resistance to a free-radical attack over the clutch laying period highlights this cost.
252 This oxidative stress cost is apparent at least during egg laying that is the most costly part of
253 reproduction in precocial birds as eggs in these species are extremely energy rich with a large
254 yolk (Moran, 2007; Nelson et al., 2010). However, to ensure that this decrease of resistance to
255 oxidative stress is a cost associated with reproduction it will be necessary to determine if it
256 results in a decrease in investment in reproduction or in lifespan (Metcalfe and Monaghan,
257 2013). Contrary to our prediction, this decrease in the resistance to a free-radical attack was
258 steeper in pre-natally stressed females, and associated with the reduction of ROS production
259 in pre-natal control females, it suggests that pre-natally stressed females were less able to
260 cope with the oxidative insult imposed by reproduction suggesting a higher cost of
261 reproduction, certainly in terms of oxidative damage. It has been already shown that pre-
262 natally stressed quail exhibit an attenuated acute physiological stress response and increased
263 exploration behavior in a stressful novel environment. This work led to the conclusion that
264 pre-natal stress can program an individual in a way that may increase their fitness when adult
265 conditions match those experienced during development (Zimmer et al., 2013). However, as

266 early-life stress probably results in both benefits and costs which may be expressed at
267 different life-history stages or in different environments (Costantini, 2014; Haussmann et al.,
268 2011; Marasco et al., 2013), we suggest that developmental programming may be associated
269 with adaptive stress copying phenotypic traits that may be costly in a different environmental
270 context or at a different life-history stage due to physiological constraints or trade-offs. In our
271 case, pre-natal stress may be adaptive in terms of immediate survival and finding food when
272 the individual faces stressful events during adulthood but this is balanced against significant
273 costs in the context of reproduction through a higher oxidative stress level. However, it is
274 worth noting that the difference in the decrease in resistance to oxidative between controls
275 and pre-natally stressed individuals over the breeding period was significant but remained
276 small. It is possible that female can cope with this little higher oxidative stress level and that it
277 did not really represent a cost for individual fitness or long-term performance. A potential
278 caveat in this study that may explain the lack of direct effect of our different treatments is the
279 lower sample size in each group as the power of our analyses was not very high for the factors
280 that were far from significance level (Table 1). However, for factors that were closer from
281 significance, the power for those factors is acceptable (Table 1).
282 Females that reproduced under our food removal treatment showed a lower TAS level
283 at the end of clutch compared to controls. Unpredictable food access should increase the
284 release of GCs (Buchanan et al., 2003) and this increase in GCs over our 28 days of food
285 manipulation may have an inhibitory effect on antioxidant machinery (Costantini et al., 2011)
286 leading to the observed reduced level of non-enzymatic antioxidants. However, this decrease
287 in antioxidant level did not seem to result in a higher ROS production over the laying period.
288 Therefore, the decrease of antioxidant may be rather due to their use against a higher level of
289 ROS attack in order to maintain the homeostatic balance and avoiding oxidative stress
290 (Costantini and Verhulst, 2009; Metcalfe and Monaghan, 2013) and not to their down-

regulation. This indicates that females reproducing under stressful conditions are able to maintain their oxidative balance as the same level than females under control conditions. Moreover, this result reinforces the idea that it is necessary to determine the two sides of the oxidative balance to measure oxidative stress and to use multiple measures (Costantini, 2008, 2014; Metcalfe and Monaghan, 2013; Monaghan et al., 2009; Selman et al., 2012).

To conclude, in this study, we showed that pre-natal stress increased the cost of reproduction in term of oxidative stress. On the contrary, pre-natal stress appears to promote traits beneficial for survival when facing stressful environments (Zimmer et al., 2013; Zimmer and Spencer, 2014). Therefore, these results confirm that early-life stress can have both benefits and costs arising in different contexts. It also emphasizes the importance of the context in which the consequences of early-life are determined and the need of long-term studies following individuals throughout their lifespan.

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402

Figure 1: Total oxidant status changes over the reproductive period (initial/final) in pre-natal control (Ctrl) and pre-natally stressed (CORT) females. Different letters indicate significant differences.

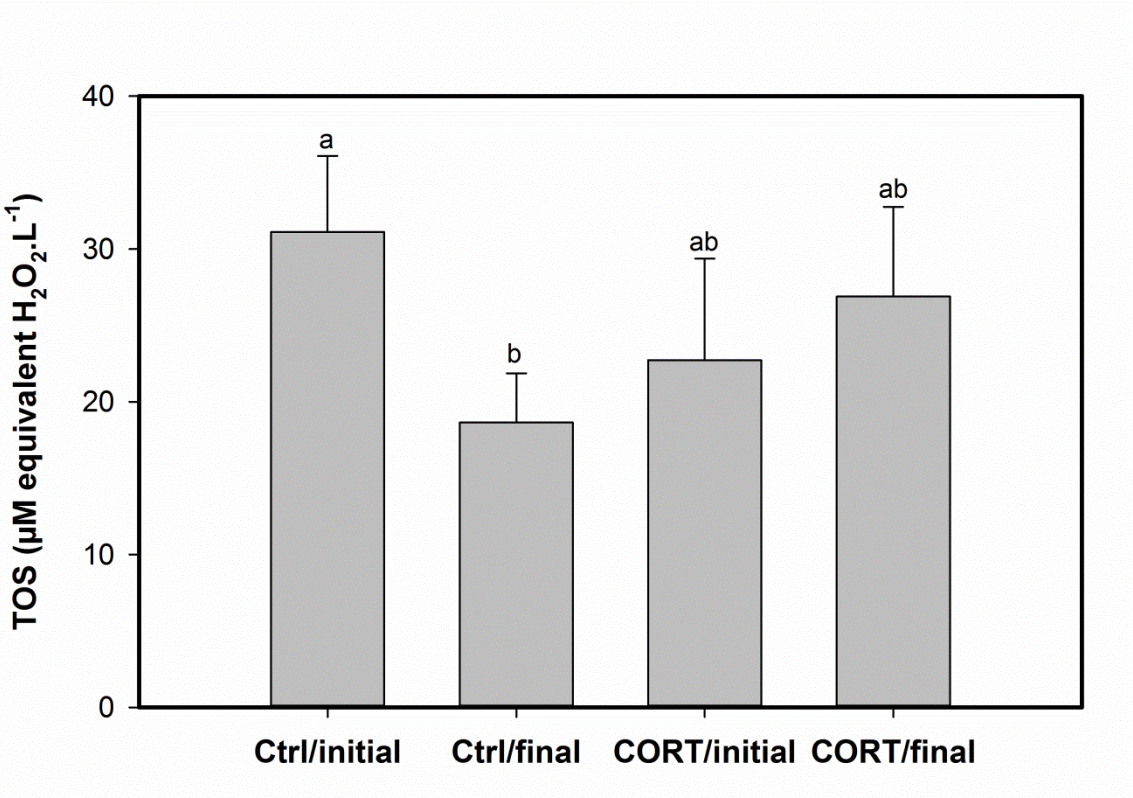
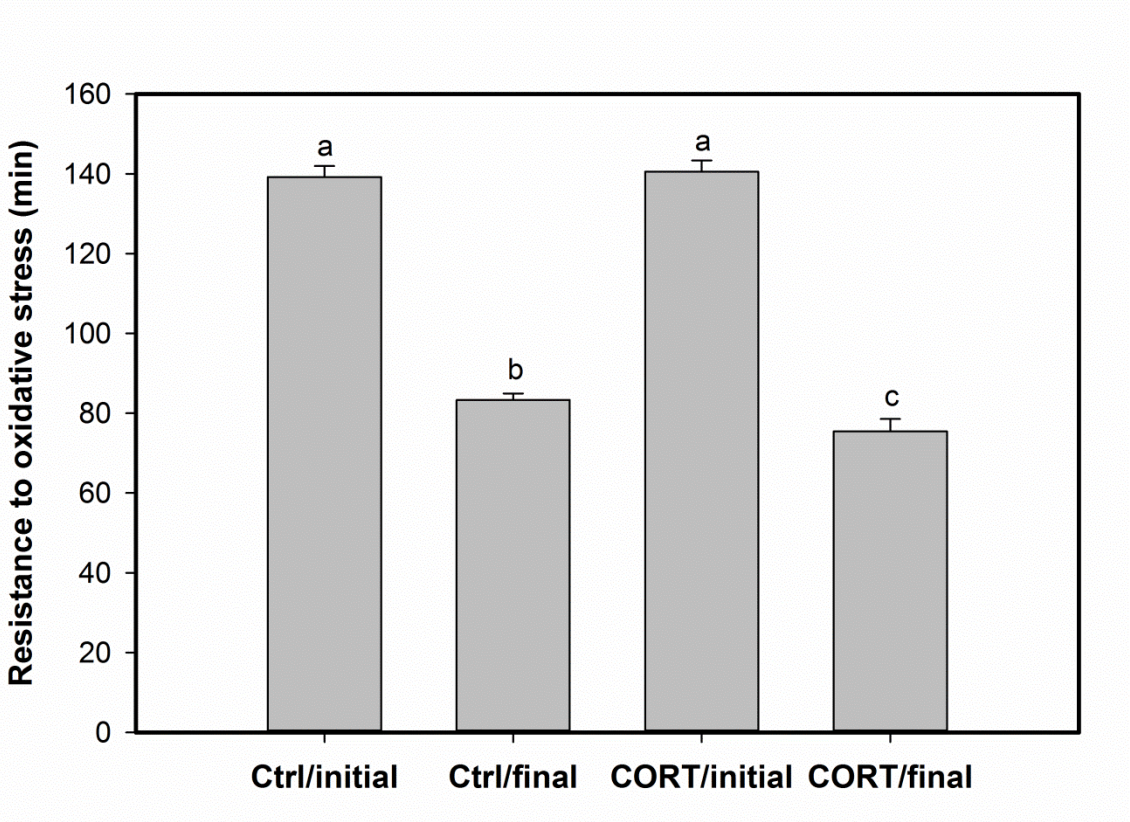


Figure 2: Resistance to oxidative stress (minutes) changes between the beginning and the end of the reproductive period in pre-natal control (Ctrl) and pre-natally stressed (CORT) females. Different letters indicate significant differences.


Resistance to oxidative stress (min)
160
140
120
100
80
60
40
20
0
a
b
a
c
Ctrl/initial
Ctrl/final
CORT/initial
CORT/final

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