Oxidative stress experienced during early development influences the offspring phenotype

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SUMMARY

Oxidative stress (OS) experienced early in life can affect an individual's phenotype. However, its consequences for the next generation remain largely unexplored. We manipulated the OS level endured by zebra finches (*Taeniopygia guttata*) during their development by transitorily inhibiting the synthesis of the key antioxidant glutathione ('early-high-OS'). The offspring of these birds and control parents were cross-fostered at hatching to enlarge or reduce its brood size. Independently of parents' early-life OS levels, the chicks raised in enlarged broods showed lower erythrocyte glutathione levels, revealing glutathione sensitivity to environmental conditions. Control ("early-low-OS") biological mothers produced females, not males, that attained a higher body mass when raised in a benign environment (i.e. the reduced brood). In contrast, biological mothers exposed to early-life OS produced heavier males, not females, when allocated in reduced broods. Early-life OS also affected the parental rearing capacity because 12d-old nestlings raised by a foster pair with both early-high-OS members grew shorter legs (tarsus) than chicks from other groups. The results indicate that environmental conditions during development can affect early glutathione levels, which may, in turn, influence the next generation through both pre- and postnatal parental effects. The results also demonstrate that early-life OS can constrain the offspring phenotype.

Keywords: oxidative stress hypothesis of life histories, glutathione, early environmental conditions, maternal effects, paternal effects, transgenerational effects.

Introduction

The early developmental period may determine individual life-history trajectories. Favorable or adverse environmental conditions early in life can exert positive or negative long-lasting effects on phenotypes (e.g. Ravelli et al. 1976; Lindstrom 1999; Cooper and Kruuk 2018). The consequences can appear promptly or be detected in adulthood only (Lindstrom 1999; Monaghan 2008). In the latter case, early-life effects often emerge during reproduction because this is one of the most resource-demanding phases of life, with animals dealing with a resource allocation trade-off between self-maintenance and reproductive investment (e.g. Harshman and Zera 2007).

Parents can influence the development of their descendants by genetic or non-genetic mechanisms. Non-genetic effects can occur before or after birth. Thus, they can be exerted during the gamete stage, or during embryo or juvenile development, sometimes generating long-lasting effects on offspring phenotypes (Mousseau and Fox 1998; Badyaev and Uller 2009). Such parental effects may be adaptive, preparing the descendants to perform better under future environmental conditions if they are predictable (Mousseau and Fox 1998; Wells 2007; Bonduriansky et al. 2011). Alternatively, they may be generated by parental phenotypes as a result of physiological or life-history constraints (Monaghan 2008), a process referred to as "passive parental condition transfer" or "parental transmissive effects" (Marshall and Uller 2007; Bonduriansky and Crean 2017). In this case, the offspring of parents in good condition will perform better than those from parents in bad condition, independent of the environment experienced by themselves. Given the important role of early life condition in shaping an individual's performance, the environment experienced by an individual early in life may mediate such condition transfer effects (Burton and Metcalfe 2014).

Although under debate, the oxidative stress experienced by an individual early in life seems to have long-lasting consequences for phenotype expression and life-history (Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010). Oxidative stress (OS) is an imbalance between the production rate of pro-oxidant molecules and the amount/efficiency of the antioxidant defenses, leading to oxidative damage (Halliwell and Gutteridge 2007; Monaghan et al. 2009). It may also alter redox signaling pathways with poorly-understood consequences (e.g. Schieber and Chander 2014). Interestingly, some studies have suggested the reproductive effort a parent makes may affect its OS levels (Alonso-Alvarez et al. 2004; Alonso-Alvarez et al. 2017; but see also e.g. Metcalfe and Monaghan 2013), whereas OS experienced just before reproduction may constrain parental investment (Stier et al. 2012). Similarly, OS may be a cost of or a constraint on the rate of development (meta-analysis in Smith et al. 2016). However, the consequences of OS experienced by parents during early life for the next generation remain largely untested. Such trans-generational effects have so far only been inferred through studies quantifying parameters indirectly related to OS, such as exposure to certain pollutants or hypoxia (e.g. Yauk et al. 2008; Wang et al. 2016; Kishimoto et al. 2017).

One key component of the antioxidant machinery is glutathione, a tripeptide thiol highly conserved across taxa and often considered the most abundant and important intracellular antioxidant (Jones 2006; Isaksson et al. 2011; Lu 2013). Besides, glutathione has a cornerstone role in cellular redox signaling (Jones 2006; Ghezzi 2013), being able to penetrate the cell nucleus and modify gene expression (Markovic et al. 2010). Glutathione levels in erythrocytes have been found to be highly heritable (e.g. in sheep: Rizzi et al. 1988; humans: Van't Erve et al. 2013; chicken: Matsumoto et al. 1958; pied flycatchers *Ficedula hypoleuca*: Lopez-Arrabe et al. 2016).

Yet its levels can also be influenced by environmental factors such as temperature and pollution (see in birds; Galván and Alonso-Alvarez 2009; Wlostowski et al. 2010; Del Vesco et al. 2014), being some of the most reliable biomarker when assessing environmental contamination (e.g. Isaksson 2010). Glutathione can also be affected by the diet because its production requires specific amino acid precursors (Lu 2013). For example, adult great tits (*Parus major*) foraging in deciduous forests had lower blood glutathione levels than those foraging in evergreen ones, which might be due to habitat differences in cysteine availability (Isaksson 2013). Moreover, chickens supplemented with cysteine and methionine showed higher glutathione concentrations than controls during growth (Enkvetchakul and Bottje 1995; Németh et al. 2004). Therefore, we hypothesize that glutathione levels during development could serve as a cue of future environmental conditions. Accordingly, the glutathione level might "program" individual life history trajectories (Romero-Haro and Alonso-Alvarez 2015; Romero-Haro et al. 2016; see also Isaksson et al. 2011).

Previous work has shown that injecting a blocker of glutathione synthesis (i.e. buthionine sulfoximine; BSO) lowers the erythrocyte glutathione levels in captive zebra finch (*Taeniopygia guttata*) nestlings, which induced OS by reducing blood antioxidant capacity (Romero-Haro and Alonso-Alvarez 2015). The BSO treatment is considered a specific procedure to alter OS in animal studies (see e.g. review in Koch and Hill 2017). At 100 days old, early-BSO-treated birds showed redder bills (a reproductive investment in sexual signaling) but also greater oxidative damage in erythrocytes compared to controls (Romero-Haro and Alonso-Alvarez 2015). We also found that early-BSO-treated females, not males, were heavier at 100 days old but showed a weaker erythrocyte resistance to OS compared to control females (Romero-Haro and Alonso-Alvarez

2015). Interestingly, early-BSO-treated birds of any sex showed a better erythrocyte resistance to OS when forced to breed enlarged broods (Romero-Haro et al. 2016). Our previous findings thus support our hypothesis that early glutathione levels can shape phenotypes and affect individual life-history trade-offs. However, as far as we know, no study has tested whether OS in early life can exert phenotypic effects on the next generations, at least by using techniques specifically designed to alter OS (e.g. Koch and Hill 2017).

Here, we tested this in the same zebra finch population (i.e. Romero-Haro et al. 2016). We quantified the transgenerational impact of the parents' early-life OS (induced by BSO) on offspring phenotype measured in terms of erythrocyte glutathione levels, body mass and size (see Appendix A table A1 for a summary of hypothetical predictions and how they relate to the experimental outcomes). We first hypothesized that low antioxidant (i.e. glutathione) levels early in life should exert a constraining role on the offspring phenotype (Appendix A, table A1, H1). This is supported by recent works showing that BSO administered on adult birds constrains their phenotype (lowering song rates and other reproductive investments; Costantini et al. 2016; Messina et al. 2017). Our BSO administration also affected the parent's adult phenotype (Romero-Haro and Alonso-Alvarez 2015; Romero-Haro et al. 2016; see previous paragraph). However, in the sample studied here and just before the breeding period, early BSO-treated and control zebra finches did not significantly differ in bill redness, body mass, tarsus size and OS-related variables (i.e. Romero-Haro et al. 2016; see also Methods). In any event, by assuming a constraining effect, descendants from early-BSO-treated parents should develop poorer-quality morphological traits and lower glutathione levels derived from passive parental condition-transfer effects (above).

Additionally, to infer strategical (adaptive) parental effects on the offspring, the environment experienced by the nestlings was manipulated early after hatching. We enlarged or decreased the original brood size by a cross-foster manipulation (also Romero-Haro et al. 2016). Offspring raised in enlarged broods should face a poorer environment (stress, intense sibling rivalry, and reduced food availability) than those in reduced broods (reviewed in Griffith and Buchanan 2010). Thus, offspring in enlarged broods should become smaller and lighter at the end of the nestling period (Griffith and Buchanan 2010). They should also show lower glutathione levels if these are constrained by food (amino acid) availability or antioxidant consumption to fight-off OS derived from social stress (Costantini et al. 2011). We predicted a constraining effect on the offspring of the early exposure of parents to OS (above) and/or due to the fact of being raised in enlarged broods. We predicted the sign of the effect (table A1, H1), but we might also predict effect sizes, i.e. additive or multiplicative patterns. Additive constraining effects would be double when both early OS exposure and enlarged brood size simultaneously act on the same individual. Multiplicative effects would, instead, be disproportionally higher in the same case. As an alternative to constraining effects, i.e. hypothesizing an adaptive (programming) strategy, low early glutathione levels of parents could trigger anticipatory mechanisms to improve the capacity of the offspring to endure challenging environmental conditions (i.e. “environmental matching”, Monaghan 2008; table A1, H2). Thus, nestlings enduring stressful conditions (i.e. enlarged broods) should develop a superior phenotype or develop it faster (showing higher mass, larger tarsus and higher glutathione levels) when their parents were early-exposed to high OS.

Our cross-fostering manipulation at post-hatching date also allowed us to discriminate transgenerational effects transmitted via pre- or post-natal mechanisms, i.e. transferred by

biological or foster parents, respectively. Nonetheless, predicting what of these type of effects should prevail on the offspring phenotype, or how they should interact, is complicated. The effects could again be additive or multiplicative. We can only argue that the prenatal period offers more channels to influence the phenotype (i.e. via gametes/embryos/incubation vs. those linked to nestling care), which could favor a stronger transgenerational impact. Moreover, it is often assumed that the earlier an individual's development is disturbed, the stronger are the effects (Royle et al. 2015). We could have also considered sex-specific effects on the offspring, which should theoretically depend on the variation in the reproductive value of each sex (e.g. Trivers 1972; Kokko and Jennions 2008). This factor was tested, but we avoided formulating firm predictions as differences in reproductive value variability between male and female zebra finch nestlings are difficult to infer (see Discussion section).

METHODS

The experiment was carried out at Finca Dehesa Galiana (Ciudad Real, Spain). Eighty randomly formed zebra finch pairs (F0 birds) were housed in indoor facilities (details in Romero-Haro and Alonso-Alvarez 2015) and produced nestlings (F1 generation) whose glutathione levels were manipulated during their postnatal development (6-12d old; Romero-Haro and Alonso-Alvarez 2015). F1 birds were then bred in a fully factorial design and F2 offspring were cross-fostered shortly after hatching. At the same time, the brood size was manipulated by producing enlarged or reduced brood sizes to manipulate both the reproductive investment of F1 adults (i.e. results

in Romero-Haro et al. 2016) and the early-life conditions of F2 nestlings (see Appendix A, figure A1 for a chronogram of the long-lasting and transgenerational experiment).

EARLY DEVELOPMENT IN F1 BIRDS: MANIPULATION OF GLUTATHIONE LEVELS

The early physiology of F1 birds was manipulated by subcutaneously injecting a 50mg/mL solution of BSO (Sigma, ref. B2640.) in sterilized saline serum (n = 206) or, instead, sterilized saline serum only (controls; n = 203). The treatment was randomly assigned (see also Appendix A for more methodological details). We injected 0.06 mL of solution/saline every two days from 6 to 12 days old (i.e., four injections). BSO-treated birds showed lower glutathione levels at 14 days-old compared to controls, but no difference at 100 days-old (see also Appendix A and Romero-Haro and Alonso-Alvarez 2015). The term "glutathione" was used to refer to circulating total glutathione concentration in erythrocytes.

BROOD SIZE MANIPULATION

A random subsample of F1 birds from the BSO experiment was allowed to breed (49 control males, 45 control females, 39 BSO males and 50 BSO females; sex balanced between treatments: χ^2 = 1.26, P = 0.261) in an outdoor aviary (6.20 x 12 x 3 m) that included 194 wooden nest boxes (14 x 12 x 16 cm; see also Appendix A). BSO-treated F1 birds in this subsample also showed lower glutathione levels at 14 d old compared to controls (P < 0.001). All birds received *ad libitum* food, water and material for nest construction (Appendix A).

Birds mated freely and the combinations of treatments among pairs were balanced (Appendix A, table A2 for detailed information about pairs composition). The cross-fostering manipulations were performed over 120 consecutive days on 110 different couples (see also tables A2-3 in Appendix A for details about combinations of treatments between pairs and broods). The original brood size was reduced or enlarged by removing or adding one to four new chicks (see also Appendix A). The different manipulations were clustered into two categories: enlarged or reduced broods. The resulting broods were, on average, two chicks in reduced broods (mean ± SD, range: 1.97 ± 0.57, 1-3 chicks) and four chicks in enlarged broods (4.19 ± 1.28, 2-8 chicks).

A total of 522 chicks were cross-fostered when they were 2-d old (mean: 1.60 ± 1.06 days, range: 0–5). Age at cross-fostering did not differ according to offspring sex, brood size manipulation or parents' BSO treatments (all tests $P > 0.17$; see Appendix A). As a result, 174 and 342 chicks were reared in reduced and enlarged broods, respectively (*n* are shown in figures 1-3). Six chicks were cross-fostered, but their foster brood size was not modified, so these chicks were removed from the statistical analyses. The original clutch or brood size did not differ between the parental early treatments or its interaction (Appendix A). Similarly, the number of removed or added chicks to a brood did not differ with the early treatments of foster parents or its interaction (Appendix A). Parental early treatments were similarly represented in the two brood size manipulation treatments (table A3). Importantly, the proportion of hatched eggs in a brood was not affected by the early BSO treatment of their parents (Binomial GLIMMIX in SAS with couple identity as a random coefficient term: father BSO treatment: $F_{1,66} = 0.05$, $P = 0.829$, mother BSO treatment: $F_{1,66} = 0.02$, $P = 0.876$; interaction: $F_{1,66} = 0.36$, $P = 0.551$), which should mostly discard

biases in the offspring related to differential embryo mortality before the brood size manipulation.

When the chicks were 12 d old (mean 12.25 ± SD: 1.30 d, range: 9-17 d), a blood sample was taken from the brachial vein and the body mass and tarsus length were recorded. Ninety-nine chicks died before this age (19.2 %), but the probability of death did not differ between brood size manipulation treatments or the early treatment of the foster parents (all $P > 0.44$; see Appendix A).

GLUTATHIONE QUANTIFICATION

Glutathione was quantified following Griffith's method (1980) with modifications (Appendix A).

MOLECULAR SEXING

Those nestlings not sexed by their plumage traits were molecularly sexed, using RBC or muscle tissue and primers 002R and 0057F (Round et al. 2007).

PATERNITY GENETIC ANALYSES

All F1 parents released in the aviary and 451 F2 offspring (412 from a blood sample and 39 from bird corpses found before the bleeding date (see Appendix A) were genotyped at 12 microsatellite markers by following Forstmeier et al. (2007) to determine maternity and paternity (see Appendix

A for further details). Five chicks were not bled because they were too light. DNA fragment separation and detection were conducted in a 16 capillary sequencer (ABI PRISM 3130XL, Applied Biosystems) based on fluorescence. Allele sizes were assigned both automatically using GeneMapper 5.0 (Applied Biosystems, Foster City, CA) and manually.

Parentage analyses were performed in CERVUS 3.0 using a maximum likelihood method, based on parental and trio LOD scores. All assignments were checked and confirmed manually. At most, only one mismatch between parents and offspring was allowed. We were not able to resolve parent-offspring matching with a strong level of confidence for four out of the 451 chicks (0.89%), and in three nestlings (0.67%) only the mother could be assigned. These seven chicks were not included in the statistical analyses.

TERMINOLOGY OF PARENTS' IDENTITY

We will use the term biological mother/father to refer to parents whose identity was confirmed by genetic markers. In contrast, foster parents were those rearing (feeding, caring) for the chicks in their nests after the cross-fostering event. Biological parents can influence offspring phenotype via genes, egg composition, incubation behavior, and feeding/caring for hatchlings to the cross-fostering date. In some cases, the parental identity established by molecular analyses (here defined as biological parents) differed from the parental identity visually established from color ring codes (observed every other day throughout the study). Here, the biological parent influenced the chick's phenotype via genes and egg composition but not via incubation or hatchling care. The mismatches between genetic and visual identification in mothers were

attributed to egg dumping and occurred in 3.9% of the chicks. In father, the mismatches were due to extra-pair paternity and occurred in 20.8% of the chicks. Nonetheless, alternative statistical models testing the effects of the early-development treatment of visually identified biological parents reported similar results. Anyway, parental identity based on genetics allowed us to address and discuss gene-related mechanisms, which would be unfeasible by only assuming visual identifications.

STATISTICAL ANALYSES

We used SAS version 9.4 (Cary, NC) for statistical analyses. General mixed models (MIXED procedure; Littell 2006) were used to analyze the influence of the BSO treatment of biological and foster parents and brood size manipulation effects on nestling body mass, body size (tarsus length) and erythrocyte glutathione level. The sampling age and hatching date were included as covariates in all the models. The tarsus length was added as a covariate in body mass models to test size-corrected body mass (often referred to as “body condition”; supplementary material, table S1 and figure S2). The identity of the biological parents and the identity of the original and rearing broods were added as random coefficient factors. When analyzing glutathione levels, the identity of the laboratory session was also added as another random term. All the possible combinations of three- or two-way interactions among main fixed factors (sex and F1 and F2 treatments) were included in a saturated model. More than three-level interactions were not tested to avoid overfitting (Forsmeier and Schielzeth 2011) and because no a priori prediction was formulated. Among dependent variables, body mass and glutathione levels were normal

distributed, whereas tarsus length required a Box-Cox transformation (Appendix A). Forward and backward stepwise procedures (from and to saturate models, respectively), as well as the Akaike information criterion (AIC), were always used for model selection, all of them providing similar results. Here, only the results from backward procedures are reported (terms removed at $p > 0.05$) for simplicity. Least square means and 95% confidence intervals (C.I.) from the final best fitted mixed models are provided. Supplementary material also includes alternative figures on the main results (violin plots from model residuals). The Satterthwaite procedure was used to adjust the degrees of freedom. LSD tests were used for pairwise comparisons. The SAS GLIMMIX procedure was used to test for initial biases that could potentially affect the models described above (see Appendix A). Data used in this study are deposited in Dryad (https://doi.org/10.5061/dryad.q83bk3jg1, Romero-Haro and Alonso-Alvarez 2020).

RESULTS

ERYTHROCYTE GLUTATHIONE LEVELS

The brood size manipulation significantly affected erythrocyte glutathione concentration of F2 nestlings (table 1), with those raised in reduced broods having higher glutathione levels than those in enlarged broods (figure 1; Cohen's $d = 0.29$).

BODY MASS

A significant three-way interaction between the biological mother's BSO treatment, the brood size manipulation factor and sex on nestling body mass was detected (table 1, figure 2a). Female chicks reared in reduced broods were significantly heavier than those raised in enlarged broods when their biological mother was a control individual (P = 0.004, d = 0.63; figure 2a, left side). However, when the biological mother was early BSO-treated, it was male offspring raised in reduced broods heavier than males raised in enlarged broods (P = 0.010, d = 0.48; figure 2a, right side). No brood size effect was detected in female nestlings from a BSO mother or in male nestlings from a control mother (both P > 0.29; other pairwise comparisons P > 0.086). Furthermore, included in this three-way interaction, the sex x biological mother interaction reported another significant term (table 1). This interaction was driven by males being heavier than females among nestlings whose biological mothers were early treated with BSO (P = 0.001, d = 0.43; figure S1, supplementary material). Other pairwise comparisons provided P-values > 0.20.

A significant interaction between the early treatment of the foster mother and nestling sex was also found (table 1; figure 2b). Male nestlings reared by a control foster mother were heavier than males reared by a BSO foster mother (P = 0.009, d = 0.36) as well as compared to female nestlings of any foster mother group (both P < 0.012, d = 0.49 and 0.37 for control and BSO mothers respectively; figure 2b). Other pairwise reported P > 0.68.

BODY SIZE (TARSUS LENGTH)

A significant interaction effect between the early life BSO treatments of foster parents on offspring tarsus length was found (table 1 and figure 3). Nestlings raised by two BSO parents had a shorter tarsus than those raised by two control birds (LSD test: $P = 0.013$; $d = 0.32$) and also compared to nestlings reared by a mixed pair (control mother + BSO father: $P = 0.015$, $d = 0.38$; BSO mother + control father: $P = 0.010$, $d = 0.34$; also figure S6 in supplementary material).

DISCUSSION

Here we show that OS experienced by parents during their development can exert a transgenerational effect. We first demonstrate that erythrocyte glutathione levels can be affected by the rearing environment (i.e. brood size) during the nestling period. Moreover, early life glutathione levels influenced the phenotype of the descendants. A constraining effect on tarsus length, probably due to reduced nestling care (only via both foster parents), was detected. Besides, an unknown maternal prenatal mechanism affected offspring body mass depending on the offspring sex and quality of the nest environment. Such a hypothetical mechanism might, ultimately, imply some adaptive programming strategy.

Lower glutathione levels in nestlings raised in enlarged broods indicate that environmental conditions can influence the concentration of this antioxidant, although studies in other species have also reported some genetic control (e.g. Rizzi et al. 1988; Van't Erve et al. 2013; Lopez-Arrabe et al. 2016). The impact of the brood size manipulation on nestling glutathione levels suggests that low glutathione values might have been due to reduced availability of nutritional resources, particularly, those amino acids needed for glutathione synthesis (Lu 2013;

Németh et al. 2004). In support of this, brood size manipulation has broadly been used in birds as a way to alter sibling competition and, consequently, the amount of food that each nestling receives (see in zebra finches: Griffith and Buchanan 2010). Nonetheless, two alternative mechanisms can also be proposed, i.e. the influence of stress hormones (i.e. corticosteroids) or nest temperature variation. In the first case, sibling rivalry in enlarged broods can lead to increased corticosteroid levels in birds (e.g. Hardt et al. 2018 and references therein), and corticosteroid levels have been positively associated with OS in vertebrates, likely due to increased free radical production (Costantini et al. 2011). Thus, lower glutathione values in birds raised in enlarged broods might be due to glutathione being spent in fighting-off OS or inhibition of glutathione synthesis by free radicals (e.g. Sun et al. 2018). Regarding nest temperature, we should assume that it is higher in enlarged broods (e.g. in passerines: Andreasson et al. 2016; Nilsson and Nord 2017), and we know that adult poultry exposed to heat stress (34ºC) decreased glutathione levels in different tissues (e.g. Ma et al. 2014; Luo et al. 2018). However, a high temperature in a brood does not mean heat stress as parents should probably be able to regulate this by brooding.

In any event, whatever the physiological mechanism, the finding supports a key assumption of our main hypothesis on the role of this antioxidant as life history organizer, i.e. that the cell glutathione concentration is plastic and easily affected by environmental variability. This would allow glutathione levels to be used as an indicator of environmental conditions programming environment x phenotype adjustments (Monaghan 2008). Moreover, the result also suggests that our BSO-induced manipulation of early glutathione concentrations might

resemble a natural scenario (see also supplementary material for a full comparison between the effects on glutathione levels of F1 and F2 manipulations).

Regarding the transgenerational effects of sustaining low glutathione levels in early life, these were transmitted through both biological and foster parents, and mostly via maternal, not paternal, effects. Paternal effects were only significant during the chick-rearing period and depended on the mother's early life BSO treatment (figure 3). Mothers have more mechanisms available to influence offspring quality as they can manipulate egg composition (below). Similarly, in another zebra finch population, Alonso-Alvarez and colleagues (2007a) reported that effects derived from being reared in an enlarged brood in terms of reduced size-corrected body mass were only matrilineally transferred to descendants.

The (mostly prenatal) effects transmitted by biological parents were exclusively exerted by mothers and depended on offspring sex in an intriguing pattern. Literature usually reports higher body mass among birds raised in reduced broods (e.g. Alonso-Alvarez et al. 2006; Griffith and Buchanan 2010). This was indeed met by female nestlings, but not from those females whose biological mother endured early-life OS (BSO-treated; figure 2a). The latter supports the idea of a transgenerational constraining effect due to early-life OS, at least via females. However, surprisingly, the males raised in reduced broods did not gain more mass when their biological mothers were controls, but when they were early-treated with BSO (see also body mass results in Appendix A, table A1).

This male offspring result is particularly puzzling. It contradicts the "environmental matching" hypothesis (Monaghan 2008) since the quality of the early conditions of both mother

and offspring did not match in the heavier male nestlings (table A1). Male nestlings were heavier when mothers and offspring experienced adverse and benign early environments, respectively. Furthermore, males were heavier than females only when their biological mothers were exposed to early OS (i.e. BSO-treated mothers; figure S1). All these results may, nonetheless, suggest some glutathione-based programming mechanism acting on the male offspring phenotype. Nevertheless, to accept that this pattern could be adaptive the mother transmitting the effect should obtain higher fitness returns.

Here we should first consider that early body mass often positively predicts sexual maturity (see e.g. in different taxa: Jorgenson et al. 1993; Kuzawa et al. 2010). Thus, attaining a high body mass early in life could favor early reproduction, which is an optimal strategy when survival expectancies are low (Stearns 1992). This could be true for the offspring from biological mothers that experienced early OS. However, why this body mass improvement only appeared among nestlings raised in reduced broods and exclusively among male offspring? In the first case, we may argue that the mechanism in question may probably depend on resource availability (i.e. higher in reduced broods). In the second case, from an evolutionary perspective, the fitness returns of investing in males should be higher than those obtained when investing in females (Lessells 2002). This theoretically requires males showing higher variability in reproductive value than females (Trivers 1972). Unfortunately, the latter is difficult to establish from current data and precedent studies.

No sexual dimorphism (body mass, tarsus length) was found among F1 birds (Romero-Haro and Alonso-Alvarez 2015; also Rozman et al. 2003 for wild zebra finches), although female-biased body mass has often been reported (Boag 1987; Zann 1996). However, F2 males were here

significantly heavier than F2 females (P = 0.017; least-square means ± SE: 10.75 ± 0.12 g, 10.48 ± 0.13 g, respectively). Moreover, males raised by foster early-BSO-treated mothers were lighter than those males raised by control mothers (figure 2b), suggesting that males could be more expensive to rear. Alternatively, early-BSO-treated mothers could have avoided feeding males, prioritizing female nestlings, but no effect was found among females (figure 2b). In any event, Martins (2004) showed that hand-reared female nestlings, but not males, gained less body mass when food intake was reduced. Contrarily, however, zebra finch mothers seem to preferentially provision sons over daughters (see Mainwaring et al. 2011), and only males, not females, reported a positive phenotypic correlation between 8-day old body mass and fecundity (number of eggs sired; Bolund et al. 2010). Accordingly, a programming mechanism improving male, but not female, body mass gain could increase maternal fitness.

Another puzzling result from the three-way interaction is that those males from control biological mothers did not gain more body mass when raised in reduced broods than males in enlarged broods. These males could, perhaps, have invested the resource surplus in developing other non-assessed traits, such as colored sexual signals, as males exhibit more conspicuous colorations than females. However, this sexual dichromatism emerges several weeks later (Zann 1996) and pigment production mostly depends on micronutrients whose role in body mass gain could be minor (e.g. Marri and Richner 2015). Alternatively, mothers could have produced males that restrained mass gain under benign conditions to avoid costs derived from growing faster (e.g. Lee et al. 2016 and references therein). Costs of accelerated growth have been found in many parameters, including OS in rats (Tarry-Adkins et al. 2013) and zebra finches (Alonso-Alvarez et al. 2007b). However, the cost of higher growth rates should be higher for males as female

nestlings were able to gain more mass when raised in reduced broods. We have not found any avian study reporting such sex-biased cost of accelerated growth. This result, hence, remains unexplained.

Regarding the most proximate mechanisms, biological mothers could have influenced their offspring before egg development by transmitting non-genetic information via the ovum. Environmental cues can be translated to epigenetic changes in germ cells and, in turn, transferred to descendants affecting their phenotypes (e.g. Nilsson et al. 2018; Norouzitallab et al. 2019). Bird mothers can modulate the allocation of macronutrients to eggs in a sex-biased way (Anderson et al. 1997; Chin et al. 2012). Sex-biased allocation of non-energetic micronutrients such as hormones (e.g. Petrie et al. 2001; Müller et al. 2002), antibodies (e.g. Saino et al. 2003; Martyka et al. 2011) or antioxidants such as carotenoids (Badyaev et al. 2006; but see Romano et al. 2008) have also been reported. Sex-biased investment in egg composition may, in turn, affect nestling body mass (e.g. Williams 1994; Marri and Richner 2014; Boncoraglio et al. 2011). Biological mothers could also have altered their incubatory behavior. In zebra finches, the egg temperature can thus affect female, but not male, hatching body mass (Gurley et al. 2018). Lastly, biological mothers could have altered feeding/caring hatchlings until the cross-fostering event. Nonetheless, the fact that hatchling energetics largely relies on egg yolk reserves during the first 24-48 hours (Zann 1996) probably makes this mechanism less relevant.

Contrarily to results from biological mothers, the findings in foster parents clearly support the constraining transgenerational effect of early OS exposure (figures 2b and 3). In the case of tarsus, results also suggest that one parent was able to compensate for the reduced contribution of its partner. Females are traditionally considered more disposed to compensate for a lower

partner contribution because they initially invest more (larger gametes) and also have higher paternity certainty (Trivers 1972; Kokko and Jennions 2008). However, the effect did not depend on parental sex. The chick-rearing behavior impairment would be modest enough to allow compensation by any sex, but compensation would not be possible when both were affected. Nonetheless, we cannot discard that nestlings prioritized resource allocation to tarsus compared to other body traits when resource availability was not dramatically low (i.e. only one BSO parent).

Results from foster mothers also indicate that early glutathione levels were able to influence the offspring phenotype by postnatal mechanisms. The cross-fostering manipulation would discard that the effect was due to the immediate impact of the BSO exposure on F1 germline (above). Thus, low early glutathione levels would have induced transgenerational effects across the soma, that is, by changes in postnatal parental behavior. For instance, in female burying beetles (*Nicrophorus vespilloides*), insects with elaborate parental care, those individuals that suffered low food availability during the larval period provided low-quality parental care to foster broods, impairing offspring survival (McLean et al. 2014). The effect of early adverse conditions could have directly affected the central nervous system development of the F1 parents. For example, in humans and rodents, the neural system that regulates parental care behavior is sensitive to early life social stress, leading to poor parental behavior provided in adulthood (Bester-Meredith and Marler 2003; Kundakovic and Champagne 2015). In contrast, captive male zebra finches exposed to high corticosterone levels during growth invested more in chick-rearing behaviors in adulthood (Crino et al. 2014).

In summary, although we have previously shown that low early glutathione levels favored early-breeding traits and allowed adult zebra finches to cope with OS linked to reproduction (i.e.

Romero-Haro and Alonso-Alvarez 2015; Romero-Haro et al. 2016), these levels also constrained the parental capacity to allocate resources to the offspring. Nonetheless, low early glutathione levels seem also to trigger some prenatal maternal mechanism (figure 2a) that favors male nestling development over females under benign nest conditions. If such a mechanism is adaptive or not can currently not be answered without additional information on F2 life-history. In any event, all these results constitute, as far as we know, the first experimental demonstration that OS endured during early development can determine the parental investment later in life and offspring phenotype. The work exemplifies the importance of longitudinal and transgenerational approaches in studying the role of the OS in modulating life histories because all these effects can only be perceptible in the subsequent generations.

ETHICS

This research project was approved by the animal experimentation committee of the University of Castilla-La Mancha under license number CEEA: 1201_08.

ACKNOWLEDGMENTS

We thank Barbara Tschirren, Neeltje J. Boogert and Gabriele Sorci for reviewing early versions of the manuscript. We also thank E. Ferrero, L. Ramirez, E. García and L. Perez-Rodriguez for their help with the blood sampling and laboratory work and Ana Piriz, Sol Rodríguez-Martínez and Martina Carrete for their help during paternity analyses at the Laboratorio de Ecología Molecular,

Estación Biológica de Doñana, CSIC (LEM-EBD). We also thank Editor-in-Chief Daniel I. Bolnick, Associate Editor Greg Demas and two anonymous reviewers for constructive comments on the manuscript. Financial support was obtained from the projects CGL2009-10883-C02-02, CGL2015-69338-C2-2-P and PID2019-109303GB-I00 from Ministerio de Ciencia e Innovación (MICIN, Spain). AARH was funded by a Formación de Personal Investigador (FPI) grant also from MICIN and by the Marie Skłodowska-Curie grant agreement No 842085 from the European Union's Horizon 2020 research and innovation programme.

Authors' contributions

AARH and CAA designed and performed the research, made the laboratory analyses, analysed the data, discussed the results and wrote the manuscript.

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TABLE 1. TESTING THE POTENTIAL INFLUENCE OF EARLY-OXIDATIVE STRESS TREATMENTS OF PARENTS AND BROOD SIZE ON THE NESTLING PHENOTYPE.

Dependent variable	Slope	SE	*F*	*df*	*P*
RCB Total Glutathione					
Brood size manipulation	0.227	0.084	7.38	1, 146	0.007
Sampling age	-0.099	0.03	11.13	1, 362	0.001
Body Mass					
Sex	0.385	0.271	5.71	1, 344	0.017
Biological mother early treatment	0.473	0.318	0.00	1, 76.5	0.950
Sex x biological mother early treatment	0.985	0.375	4.09	1, 343	0.044
Brood size manipulation	0.258	0.246	7.69	1, 131	0.006
Brood size manipulation x Biological mother early treatment	0.46	0.334	0.08	1, 339	0.778
Sex x brood size manipulation	0.294	0.31	0.96	1, 355	0.329
Sex x brood size manipulation x Biological mother early treatment	1.048	0.462	5.14	1, 344	0.024
Foster mother early treatment	0.074	0.18	1.88	1, 134	0.172
Sex x Foster mother early treatment	0.53	0.218	5.92	1, 336	0.016
Tarsus Length					
Early treatment foster mother	0.613	0.250	3.46	1, 387	0.064
Early treatment foster father	0.521	0.203	1.83	1, 364	0.177
Early treatment foster mother x Early treatment foster father	0.620	0.313	3.92	1, 378	0.048
Sampling age	+0.257	0.061	17.29	1, 391	<0.001

Note.- Mixed models testing the differences in erythrocyte glutathione levels, tarsus length and body mass of 12 days old zebra finches depending on the brood size manipulation treatment (enlarged vs reduced) and the early development treatment of each biological and foster parent (control vs early BSO exposed).

Figure 1: Effect of brood size manipulation on erythrocyte glutathione levels of nestlings. Least-squared means ± 95% CI from mixed models. Sample sizes under the bars.

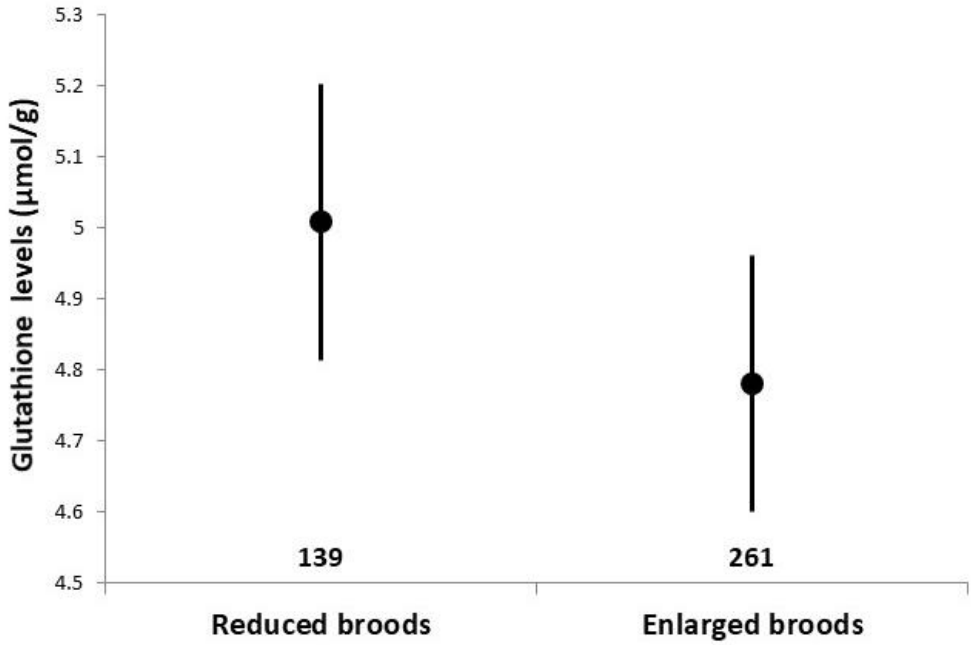


Figure 2: Effects of early treatments of mothers on offspring body mass at 12-d old. (a.) Effect of the early-life treatment of the biological mother (prenatal effects) and offspring nest environment (reduced or enlarged brood) on male and female nestling body mass. (b.) Impact of the early-life treatment of the foster mother (postnatal effects) on the nestling body mass depending on its sex. Least-squared means ± 95% CI from mixed models, and sample sizes under the bars.

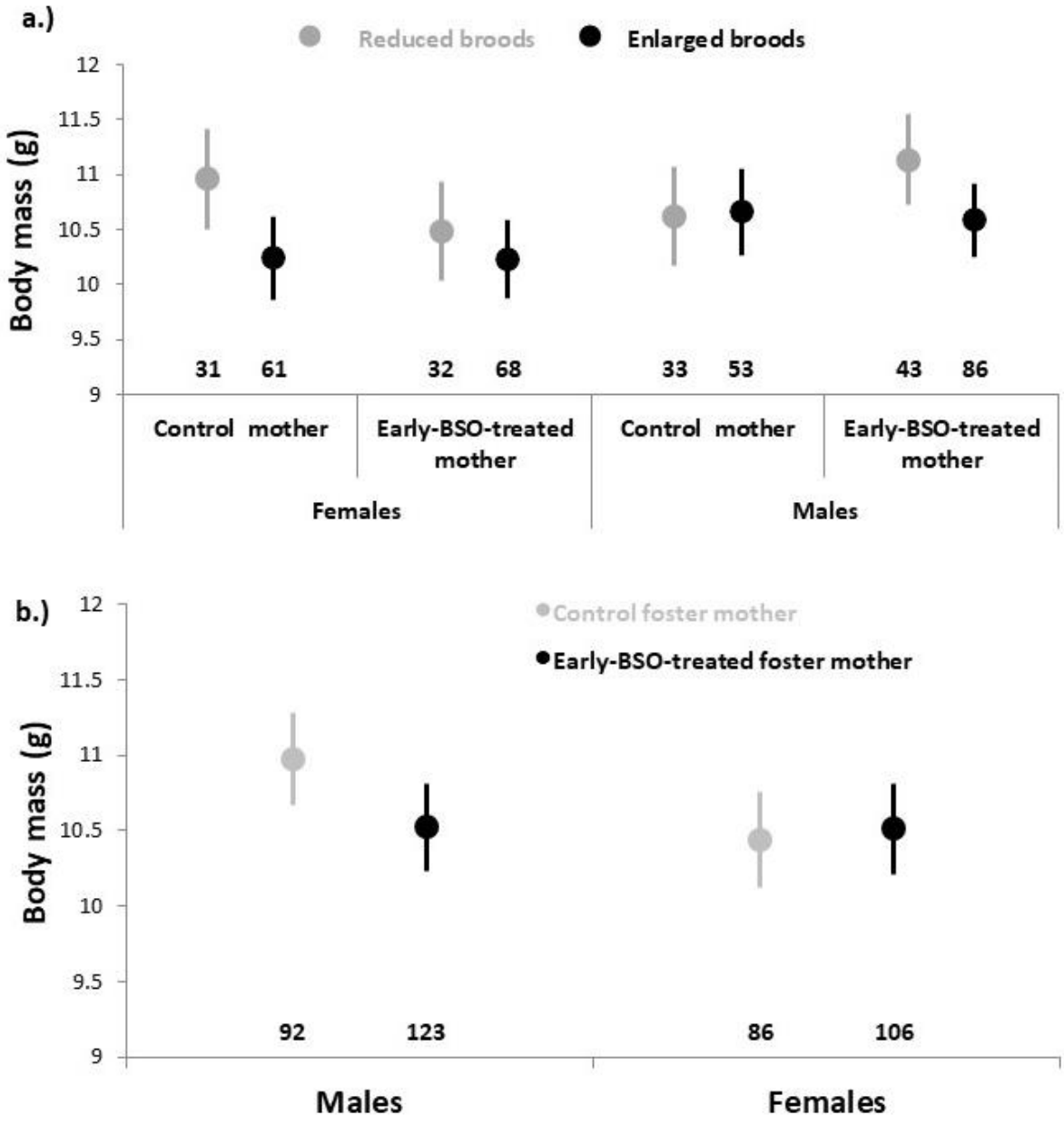
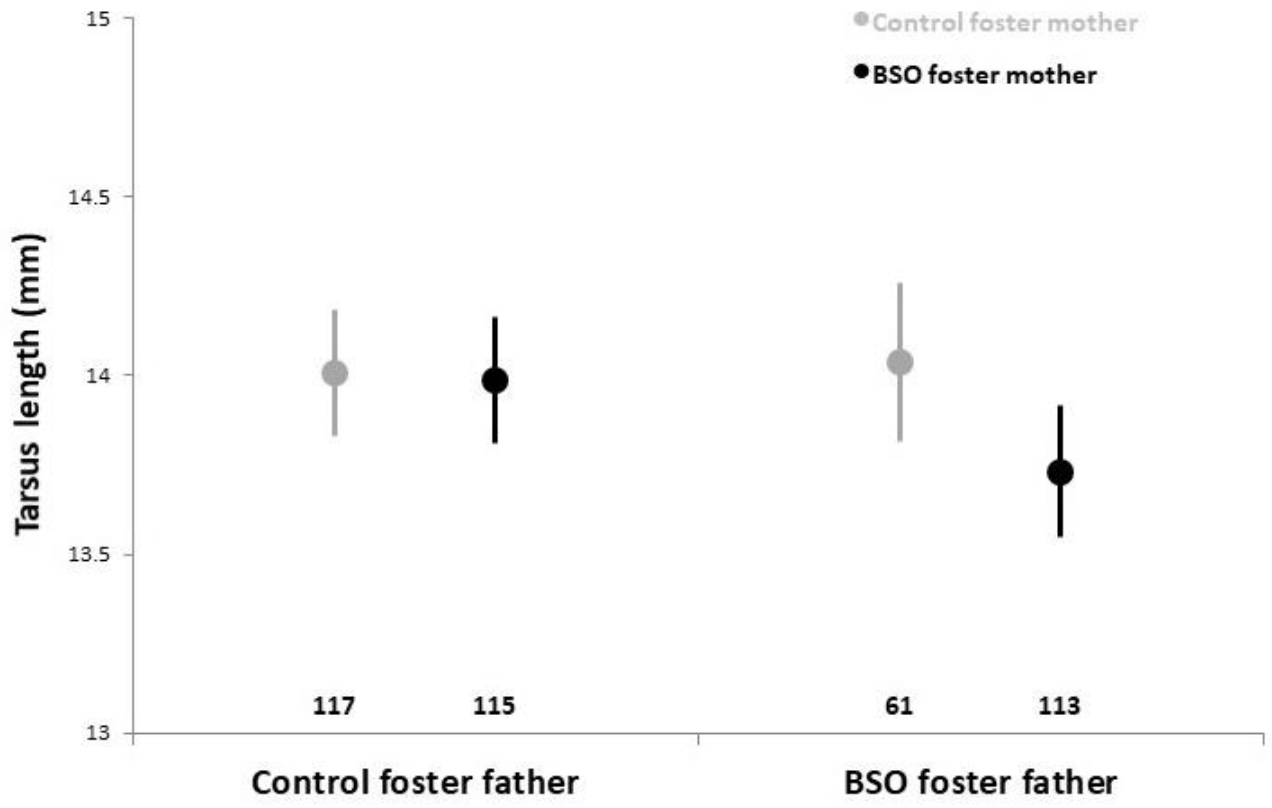


Figure 3: Effect of the early life BSO treatment of the foster parents on nestling tarsus length. Least-squared means ± 95% CI from mixed models, and sample sizes under the bars. Tarsus length is here untransformed (see Figure S6 in supplementary material for transformed data).



APPENDIX A: Oxidative stress experienced during early development influences the offspring phenotype. Predictions and extended methods.

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Table A1. Predictions. Parental past and present conditions induced by a challenging environment impacts on nestling phenotypic trait values. HYPOTHESIS 1: Adverse conditions constrain pre- or postnatal parental investment. HYPOTHESIS 2: Early adverse conditions trigger some parental anticipatory programming mechanism on descendants. This allows offspring to avoid the costs derived from developing under an adverse early environment. When parental and offspring conditions do not match, the impact on the nestling phenotype would be negative. Prenatal effects could exert a stronger impact than postnatal ones as the former would include more pathways to influence descendants (via germline, egg composition or incubation vs nestling care). Similarly, maternal effects could be stronger than paternal effects due to the mother's capacity to manipulate egg quality/composition but also because females usually invest more due to higher certainty of paternity and sexual asymmetry in reproductive investment (see Discussion). In the case of the offspring sex, parents should invest more in the sex with higher fitness returns (reproductive value). This is still unclear in zebra finches (see Discussion). **Body mass Results** summarized for each sex separately**.** Only the body mass reported an early parental x offspring effects interaction. Grey cells indicate results that did not meet any prediction.

		H1 Constraint	**H2 Programming**		**Body mass Results**	
Parental early environment	**Offspring early environment**	**Constrained parental investment**	**"Phenotypic matching" parental investment**		**Female offspring**	**Male offspring**
Non-adverse (serum injected)	Benign (Reduced brood)	(+)	(+)		(+)	(–)
Non-adverse (serum injected)	Adverse (Enlarged brood)	(–)	(–)		(–)	(–)
Adverse (BSO-treated, low GSH values)	Benign (Reduced brood)	(–)	(–)		(–)	(+)
Adverse (BSO-treated, low GSH values)	Adverse (Enlarged brood)	(–)	(+)		(–)	(–)

METHODS

The present experiment was carried out in the experimental facilities located at Finca Dehesa Galiana (Ciudad Real, Spain). The focal individuals were the F2 generation of a transgenerational study about the role of total glutathione (tGSH) levels in the resolution of trade-offs across the entire lifetime. The tGSH levels were manipulated in the parents of these birds during their development (F1 generation). In addition, a cross-fostering experiment producing enlarged or reduced broods was performed for manipulating both the reproductive investment of F1 adults and early development conditions of F2 nestlings (Figure A1).

EARLY DEVELOPMENT IN F1 BIRDS: GSH LEVELS MANIPULATION

Eighty randomly formed zebra finch pairs (F0; wild-type phenotype) obtained from five commercial breeders across Spain were placed in breeding cages and bred over five months producing 409 nestlings (F1). The origin of the bird was randomly mixed among pairs. The early environment of F1 birds was manipulated when nestlings reached a minimum body mass of 3 g (mean + s.e.: 4.82 g + 0.03). Half of the chicks in each brood were randomly assigned to a treatment receiving DL-buthionine-S,R-sulfoximine (BSO; Sigma, ref. B2640) diluted in sterilized physiological serum (50 mg/ml; n = 206) and the other half (n = 203) received sterilized physiological serum only (controls), all of them by means of four subcutaneous injections of 0.06 ml each one. Thus, BSO chicks received a total of 12 mg BSO. We randomly allocated a treatment to the heaviest chick in a brood and then successively alternated the treatment category among its siblings while decreasing body mass (e.g., control, BSO, control, BSO). BSO is a specific inhibitor of the first enzyme in the glutathione biosynthesis pathway and, of course, the BSO birds showed lower levels of GSH during development than control ones (see Romero-Haro and Alonso-Alvarez 2015). No effect on F1 mortality was detected (also Romero-Haro and Alonso-Alvarez 2015).

BROOD SIZE MANIPULATION

F1 birds produced by our F0 population were released in the outdoor aviary until it reached about one bird per m^3, which would be a value low enough to avoid social stress in this species at least under captivity (Poot et al. 2012). The F1 birds released in the outdoor aviary received food (a

commercial mix of seeds; KIKI, Callosa del Segura, Spain), a commercial supplement favoring reproduction (crumbled bread mixed with eggs, vitamin A, C, D3 and E; Briss, Italia), water and coconut fiber for nest construction all *ad libitum* throughout the study. In the subset of birds released into the aviary, the BSO-treated animals showed lower levels of tGSH throughout development than control birds (see Romero-Haro et al. 2016).

Free-mating and breeding were allowed for 7 months, but the cross-fostering manipulation was only performed during 120 consecutive days due to logistic limitations. This cross-fostering aimed to (i) increase/decrease the original breeding effort of the F1 parents, (ii) maintain the same first reproductive treatment (brood size enlargement or reduction) for F1 parents with several manipulations, (iii) distribute siblings (F2, focal birds of this work) in at least two broods of different brood size treatments, and (iv) avoid leaving any F2 chick in their own nest.

Although the control mother and BSO father combination was less numerous than the others, the pair composition in terms of early-OS treatments did not significantly differ among reproductive events (χ^2 =1.64, P = 0.200; table A2). Note that individuals in a pair acted as both foster and biological parents. The number of broods produced by each combination (range 1-3) neither differ among early-treatment combinations (father early treatment: χ^2 = 0.41, P = 0.521; mother early treatment: χ^2 = 1.11, P = 0.291; interaction: χ^2 = 1.39, P = 0.239; Generalized Linear Model, PROC GENMOD in SAS, multinomial error with clogit link function father early treatment).

Table A2: Sample size of the different pair compositions (F1 birds) depending on the early-treatment of each parent.

		Mother early treatment		
		Control	**BSO**	Total
Father early treatment	**Control**	28	29	57
	BSO	16	28	44
Total		44	57	101

Additionaly, we analyzed whether the brood size treatments were equally assigned to broods produced by each pair combination (table A3). We tested the brood size group as a binary

dependent variable, testing the interaction of the early treatment of the foster father and mother (table A3; GLIMMIX mixed model with binomial distribution and log link; pair identity as a random term). As mentioned, some pairs were engaged in more than one event (range: 1-3). The unavailability of synchronous broods did not allow the same brood size manipulation (enlargement or reduction) to be consistently maintained in all the broods of 18 F1 birds (nine pairs). Moreover, one-nestling broods of another nine pairs were erroneously enlarged considering that these broods could not, alternatively, be reduced. All these birds were excluded from the statistical analyses of reproductive costs in Romero-Haro et al. (2016). However, we here focused on F2 individuals, and hence, this source of variability is unrelevant, i.e. only the parental identity with early treatment groups and the brood size treatment endured by the nestling are relevant factors influencing the F2 phenotype. Moreover, no significant biases in original brood sizes among treatments were detected (below). Therefore, all breeding events were studied here. No early mother or father treatment or its interaction reported a significant effect, thus revealing no bias on the brood size group assignment (mother early treatment: $F_{1,158} = 0.48$, $P = 0.488$; father early treatment: $F_{1,158} = 0.29$, $P = 0.589$; interaction: $F_{1,158} = 2.40$, $P = 0.123$; table A3). When the interaction was removed the early treatment groups remained non-significant (both $P > 0.70$). Alternatively, if the early treatment of the parents is tested as the dependent binary variable, the brood size group and other-parent early treatment factors being tested, all the tests including the interaction reported $P > 0.11$.

Table A3. Number of breeding events produced by early treatment pair combinations of the foster (rearing) parents in reduced or enlarged brood size treatments.

		Mother early treatment		
Reduced broods		**Control**	**BSO**	Total
Father early treatment	**Control**	22	26	48
	BSO	18	19	37
	Total	40	45	85

		Mother early treatment		
Enlarged broods		**Control**	**BSO**	Total
Father early treatment	**Control**	24	21	45
	BSO	10	22	32
	Total	34	43	77

The models testing original clutch or brood sizes of each breeding event did not detect significant differences with the early treatment of biological parents (clutch, father: $F_{1,158} = 0.01$, $P = 0.753$; brood, father: $F_{1,158} = 0.35$, $P = 0.553$; clutch, mother: $F_{1,158} = 0.01$, $P = 0.995$; brood, mother: $F_{1,158} = 0.07$, $P = 0.787$) or its interaction (clutch: $F_{1,158} = 0.05$, $P = 0.819$, brood: $F_{1,158} = 0.25$, $P = 0.620$; PROC GLIMMIX in SAS, Poisson error, log link, brood identity as random term). The number of removed or added hatchlings to the foster brood did not significantly differ with the foster father or mother early treatments ($F_{1,108.8} = 0.59$, $P = 0.442$ and $F_{1,105.5} = 0.02$, $P = 0.896$, respectively) or its interaction ($F_{1,108.8} = 1.18$, $P = 0.280$, PROC GLIMMIX in SAS, Poisson error, log link, brood identity as random term). For the sake of simplicity, the different manipulations were clustered in two categories: enlarged or reduced broods.

A total of 522 chicks were cross-fostered in 41 manipulations when they were two days old. Each cross-fostering manipulation involved a different number of broods (mean: 4.24 + 1.88, range: 2–9; $N = 173$ broods, 80 enlarged, 87 reduced and 6 without a change in brood size).

As a result, 174 chicks were reared in reduced broods and 342 chicks were reared in enlarged broods. Six chicks were cross-fostered, but its brood size was not modified, so these chicks were removed from the statistical analyses. Among chicks allocated to enlarged or reduced broods, its original brood size did not differ between brood size manipulation groups ($F_{1,439} = 0.05$,

P = 0.818), biological father ($F_{1,262.9}$= 1.58, P = 0.210) or mother ($F_{1,198.4}$= 0.84, P = 0.361) early treatments, offspring sex ($F_{1,439}$= 0.02, P = 0.899) or interactions (all P-values > 0.20; PROC GLIMMIX in SAS with Poisson error and log link and original brood size identity as a random factor). The number of days elapsed from the hatching date to the date of cross-fostering (nestling age) for each chick (mean ± SD: 1.6 ± 1.06, range: 0-5) did not differ between nestling sexes ($F_{1,439}$= 1.21, P = 0.271), brood size manipulation treatments ($F_{1, 439}$= 0.30, P = 0.586), the treatments of biological (father: $F_{1,466}$= 1.29, P = 0.257; mother: $F_{1,439}$= 1.86, P = 0.174) or foster parents' treatments (father: $F_{1,466}$= 0.18, P = 0.669; mother: $F_{1,439}$= 0.01, P = 0.939) and interactions (all P-values > 0.12; PROC GLIMMIX in SAS with Poisson distribution and log link, original brood identity as a random factor). Moreover, the models reported in table 1 (main text) did not show any change (even in *posthoc* pairwise comparisons) when this variable was added as a covariate.

Forty-five chicks remained in its same nest after the cross-fostering due to unavailability of synchronous broods. Nevertheless, this variable did not differ between sexes ($F_{1,439}$= 2.10, P = 0.148), the early treatment of biological (father: $F_{1,330}$= 0.02, P = 0.886; mother: $F_{1,280.2}$= 1.16, P = 0.282) or foster (father: $F_{1,439}$= 0.23, P = 0.633; mother: $F_{1,439}$= 1.41, P = 0.236) parents and the brood size manipulation treatments ($F_{1,439}$= 0.10, P = 0.758) or interactions (all P > 0.1; PROC GLIMMIX in SAS with binomial error and logit link and brood identity as a random term).

When the chicks were 12 days old (mean 12.25 ± 1.30 d, range: 9-17 d), a blood sample was taken from the brachial vein and the body mass and tarsus length were recorded. We should note that F1 birds were sampled at 14 days old for analyzing its phenotype, including glutathione levels (Romero-Haro and Alonso-Alvarez 2015). F1 birds were raised in an indoor aviary with breeding cages (one per breeding pair). Here we sampled the nestlings (F2) two days earlier to avoid that the birds left the nestbox before being identified with a metal numbered ring. Note that the same sampling protocol has successfully been used in the wild to avoid the cited problem (Andrew et al. 2017, see Methods section, first parag.). Unfortunately, 99 chicks died before this age (19,2 %), but the 12 days-old mortality did not differ with the offspring sex ($F_{1,439}$= 1.13, P = 0.288), the brood size manipulation treatment ($F_{1,196.7}$= 0.88, P = 0.349) or the early treatment of the foster parents (father: $F_{1,157.6}$= 0.38, P = 0.538; mother: $F_{1,506}$= 0.58, P = 0.448; GLIMMIX in SAS with binomial error and logit link, foster brood identity as random terms). Of them, 39 cadavers

could be recovered for molecular sexing and genetic parenthood analyses. The rest of the corpses could not be analyzed as they were very rotten and contaminated or because they have disappeared. We should note that parents usually throw corpses out of the nestbox. The aviary was monitored every other day. Thus, it is very feasible that ants (abundant into the aviary in summer) quickly made them disappear. In fact, we found ants carrying out corpse fractions.

Glutathione quantification

Glutathione was quantified following Griffith's method (1980) with modifications. Briefly, the blood pellet in the tube was thawed and immediately diluted (1:10 w/v) and homogenized in a stock buffer (0.01M PBS and 0.02M EDTA), working on ice to avoid oxidation. Three working solutions were created in the same stock buffer as follows: 0.3 mM NADPH (solution I), 6 mM DTNB (solution II), and 50 units of glutathione reductase mL-1 (solution III). An aliquot (250 µL) of a homogenate of blood cells was vortexed with 250 µL phosphate buffer and 0.5 mL of diluted trichloroacetic acid (10% in H_2O) three times, for 5 s each time, within a 15-min period. In the meantime, samples were protected from light and refrigerated to prevent oxidation. The mixture was then centrifuged (1,125 g for 15 min at 6ºC), and the supernatant removed. Subsequent steps were performed in an automated spectrophotometer (A25-Autoanalyzer, Biosystems, Barcelona, Spain). Solutions I and II were mixed at a ratio of 7:1 v/v, respectively. One-hundred sixty µL of this new mixture was automatically added to 40 µL of sample (i.e., supernatant) in a cuvette. Then, 20 µL of solution III was added after 15 s, and the absorbance at 405 nm was monitored after 30 and 60 s. The change in absorbance was used to determine total GSH levels by comparing the output with the results from a standard curve generated by serial dilution of glutathione from 1 mM to 0.031 mM. Results are given in mM per gram of pellet. Another bird sample (n = 28) from the same population whose blood was assessed twice reported a high repeatability (r = 0.95, P < 0.001).

Molecular sexing

Nestlings that were not sexed by their plumage dimorphic traits were molecularly sexed from a subsample of the RBC fraction when there was a blood sample available or instead from the muscle tissue when they died. DNA from sex chromosomes was amplified with polymerase chain reaction using the primers 002R, 0057F (Round et al. 2007).

Paternity genetic analyses

All the F1 parents released in the aviary were genotyped. Five chicks of the 417 F2 that reached 12 days old were not bled because were very light at this age. So, 412 F2 birds from a blood sample and 39 from cadavers were genotyped.

DNA was extracted from blood samples or bodies using magnetic beads (SpeedBeads™ magnetic carboxylate modified particles, GE Healthcare). We used twelve fluorescently labeled forward microsatellite loci: Tgu1, Tgu3, Tgu4, Tgu5, Tgu6, Tgu7, Tgu8, Tgu9, Tgu10, Tgu11, Tgu12 and Tgu13 (Forstmeier et al. 2007). All loci were PCR amplified in three independent multiplex reactions. Primer mix 1 contained loci Tgu1, 5, 7 and 12, mix 2 loci Tgu4, 6, 8, 9, 10 and 11 and mix 3 loci Tgu3 and 13. Each 25 µL PCR sample contained 12.5 µL QIAGEN Multiplex PCR master mix, 0.1 µL bovine serum albumin (BSA), 1 µL template DNA and ddH_2O (6.4, 4.4 and 8.4 µL in the mixes 1, 2, and 3 respectively). One microliter (0.5 forward and 0.5 reverse) of 10 µM primers was used of Tgu1, Tgu6, Tgu8, Tgu9, Tgu11 and Tgu12 and 1.5 µL (0.75 forward and 0.75 reverse) of Tgu3, Tgu4, Tgu5, Tgu7, Tgu10 and Tgu13. The cycling conditions consisted of a 5-minute denaturation step at 95ºC, 35 cycles of 30 seconds at 95ºC, 90 seconds at 55ºC for mix 1 and 3 and 53ºC for mix 2 and 30 seconds at 72ºC, and a final extension step of 30 minutes at 60ºC. PCR products were run on 1.5% agarose gels to check for amplification. DNA fragment separation and detection were conducted in a 16 capillary sequencer (ABI PRISM 3130XL, Applied Biosystems) based on fluorescence. Allele sizes were assigned both automatically using GeneMapper 5.0 (Applied Biosystems, Foster City, CA) and manually. Tgu13 was excluded from analyses because of the high presence of null alleles as it had already been described (Forstmeier et al. 2007).

Parentage analyses were performed in CERVUS 3.0 using a maximum likelihood method. Both parents and the trio had a high LOD score for a given nestling. The assignments were also checked and confirmed manually and parents had none or only one mismatch (due to null alleles). We were not able to resolve parent-offspring matching with a strong level of confidence only for 4 chicks of 451 (0.89%) and in three chicks (0.67%) only the mother was assigned. Anyway, these last 7 chicks were not included in main analyses because they did not reach the recording age: the paternity analyses were done from bodies and morphological measurements and blood samples for glutathione lab analyses were not taken, besides the parent early treatments were unknown.

Tarsus length normalization

Tarsus length and its residuals from the mixed model were not normally distributed. They values were negatively skewed (asymmetry: -0.70). We tried different transformations and only Box-Cox provided an effective normalization. This procedure has been used for tarsus length in birds before (e.g. Polo et al. 2015; see also Rigby and Stasinopoulos 2004).

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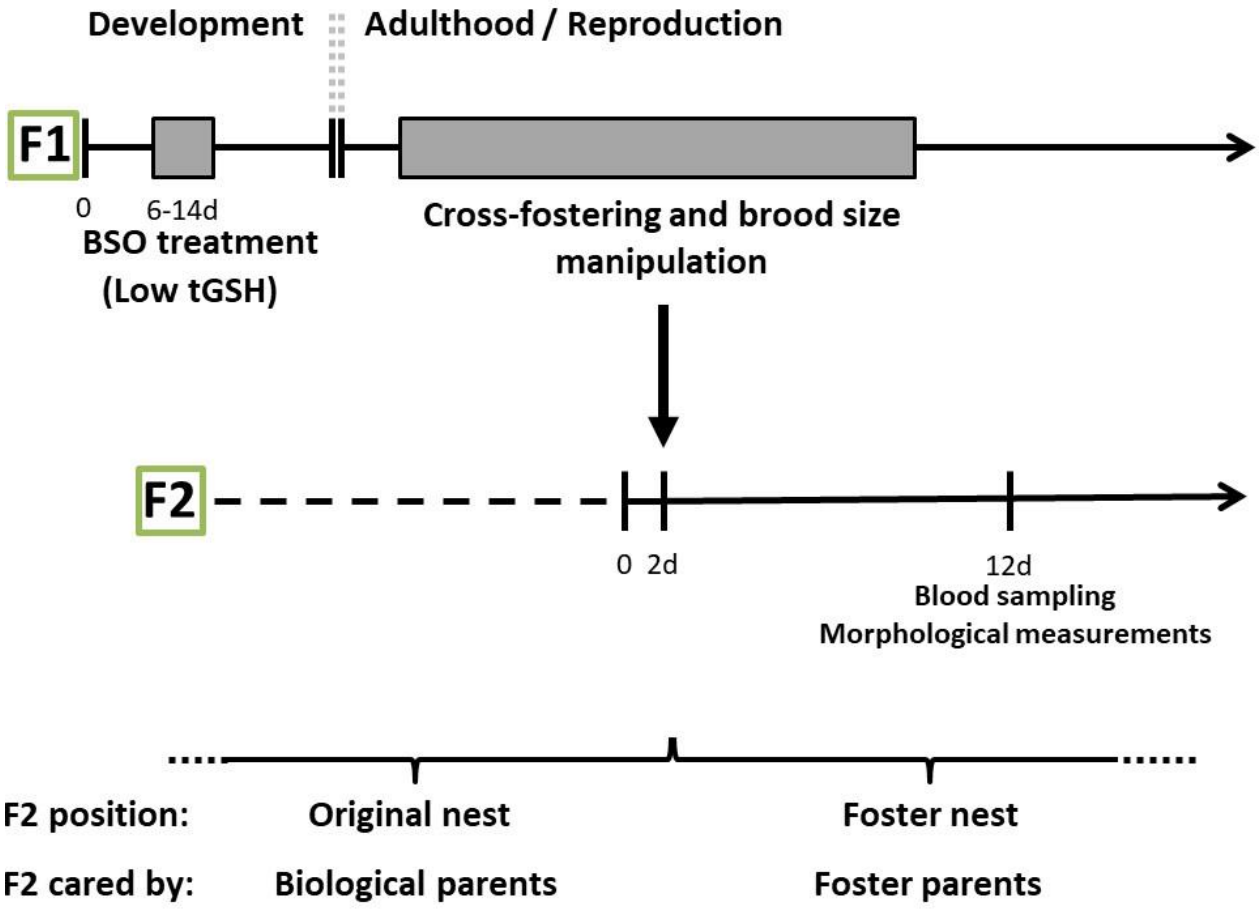
Figure A1: Chronogram showing the experimental phases. F0 pairs produced F1 offspring that were early treated with every two days BSO injections inducing a reduction in total glutathione (tGSH) synthesis at 14d old. A random subsample of F1 adult birds was allowed to freely mate and breed in a large outdoor aviary. Hatchlings (F2) were cross-fostered during the first days of their life (mean 2-d old) to increase or reduce the original brood size to test the effect of the early development treatment of their parents under two differentiated environments (adverse or benign, respectively). Parental effects of the biological parents would take place across germline (mother and father), egg quality (mother), incubation (both parents) or nest caring and hatchling feeding in the two first days of life, whereas foster parents would contribute by nestling care (food, temperature, protection; full description below).



Supplementary material: Oxidative stress experienced during early development influences the offspring phenotype

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ADDITIONAL ANALYSES AND FIGURES

COMPARING EFFECT ON GSH VALUES OF F1 AND F2 MANIPULATIONS

Least square means ± SE for F1 control and BSO birds were 4.69 ± 3.85 and 4.07 ± 3.92 µmol/g, respectively, whereas F2 birds reported 5.01 ± 0.01 and 4.78 ± 0.09 µmol/g, for nestlings in reduced and enlarged broods, respectively. Accordingly, the BSO manipulation led to a mean 13.2% decrease in erythrocyte GSH values, whereas the brood size groups showed a mean 4.4% difference. Even when considering the larger impact of the BSO manipulation, the GSH values broadly overlapped between the two groups (see raw data in Fig 5 of Romero-Haro & Alonso-Alvarez 2015 Am Nat 185, 390-405), which suggests that the manipulation generated a phenotype quite similar to one found in a natural scenario. We should also mention other differences in experimental conditions that could explain the differences in values. F1 birds were bleed at 14d old in an indoor aviary and housed in individual small cages, whereas birds in the brood size manipulation were sampled at 12d old in a large outdoor with many nest-boxes. The date of bleeding was also used for metal ringing. We advanced two days this date to avoid nestlings leaving the nestbox and losing its identity. We could additionally consider differences in temperature and light cycles between both environments (outdoor subject to larger ranges).

Lastly, we should consider that the decrease of glutathione levels, and not its magnitude, could be enough to trigger an anticipatory mechanism.

BIOLOGICAL MOTHER EARLY-TREATMENT X OFFSPRING SEX INTERACTION

The body mass model in table 1 (main text) reported a significant interaction ($P = 0.044$) between sex and biological mother treatment. This interaction was driven by a significant difference between male and female body mass among nestlings whose biological mothers were early treated with BSO ($P = 0.001$; figure S1). Other pairwise comparisons provided P-values > 0.20. This is an interaction that should greatly depend on the three-order one reported in table 1 and figure 2a. It shares the two factors with that interaction.

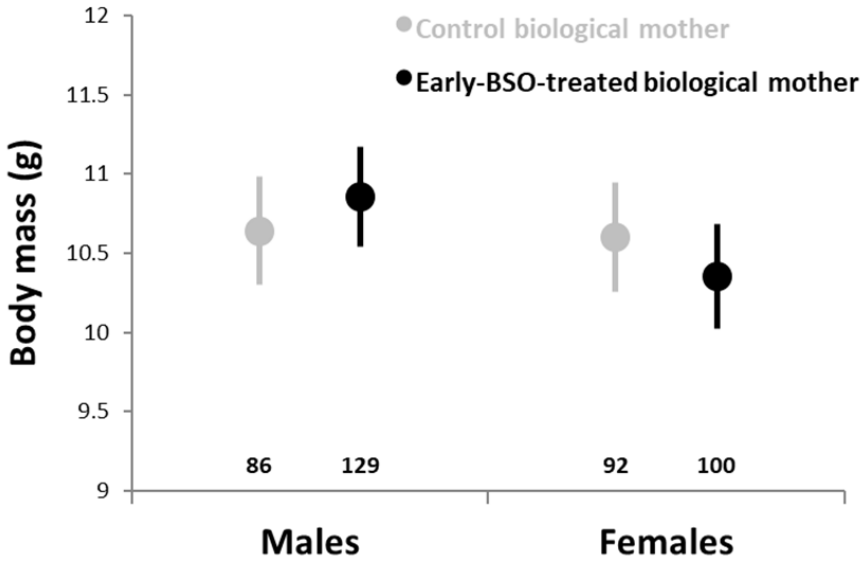

Figure S1: Effect of the biological mother early treatment on male and female nestling body mass. Least-squared means ± 95% CI from the mixed models.

SIZE-CORRECTED BODY MASS (BODY CONDITION)

In the model testing body mass but controlling for size (tarsus length), the interaction between the early treatment of the foster mother and nestling sex was close to the borderline of significance (P = 0.054; table S1). Males showed higher body condition than females but only when the foster mother was a control (P = 0.003, d = 0.45; Figure S2). When both male and female nestlings were reared by a BSO mother, this was not significant (P = 0.608). Other interactions were removed at P > 0.08. Finally, as expected, the nestlings reared in reduced broods showed higher size-corrected body mass than those from enlarged ones (P = 0.016, d = 0.26; mean ± SE: 10.76 ± 0.101 g and 10.49 ± 0.092 g).

Table S1. Mixed models testing the variability in body mass controlled for tarsus length (body condition) of 12 days old zebra finches depending on the brood size manipulation treatment (enlarged vs reduced) and the early development treatment of each foster and genetic parent (control vs BSO exposed).

	Slope	SE	*F*	*df*	*P*
Brood size manipulation	-0.27	0.110	6.01	1, 131	0.016
Sex	0.056	0.110	6.78	1, 332	0.010
Foster mother early treatment	-0.127	0.143	0.08	1, 137	0.774
Sex x foster mother early treatment	0.32	0.166	3.73	1, 324	0.054
Sampling age	-0.172	0.037	22.26	1, 392	<0.0001
Tarsus length	0.918	0.054	292.08	1, 363	<0.0001

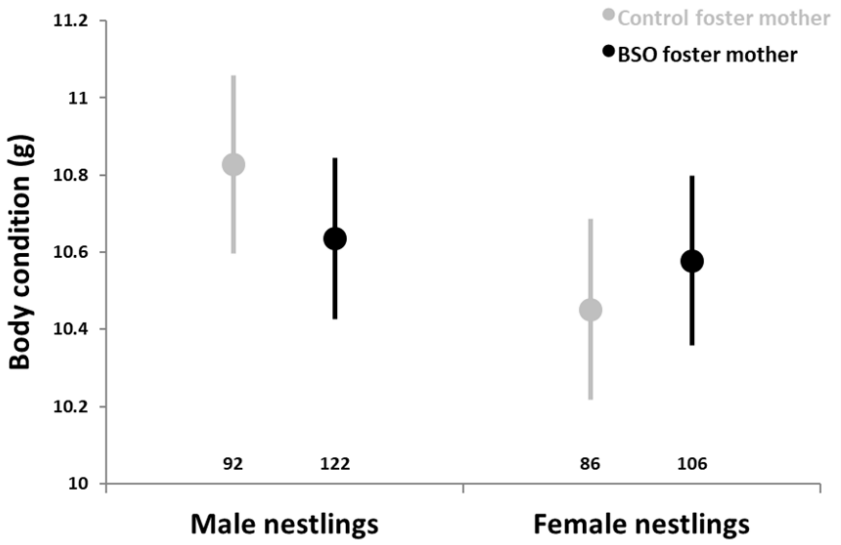

Figure S2: Effect of the foster mother early treatment on male and female nestling body condition (i.e. body mass controlled for tarsus length). Least-squared means ± 95% CI from the mixed models.

ALTERNATIVE VIOLIN FIGURES

Here we represent the main results by means of violin plots created from the residuals of the models shown in Table 1 (i.e. excluding the factors and interactions).

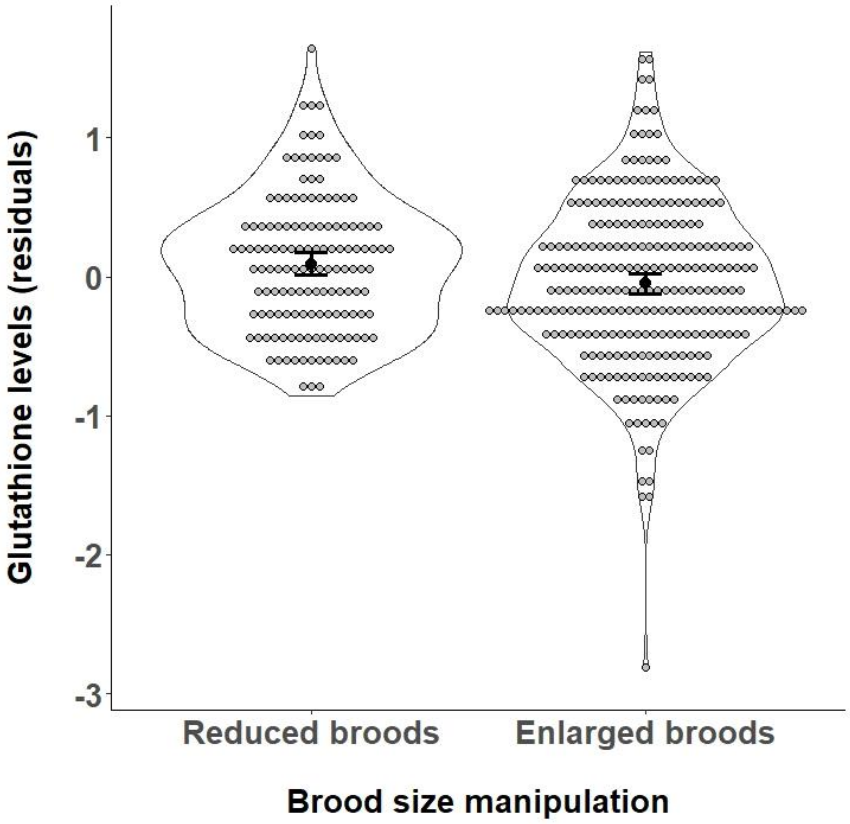

Figure S3. Erythrocyte glutathione levels depending on brood size manipulation treatment. Violin plots showing frequency distributions, individual data (residuals from the mixed model) and their means ± 95% CIs. The elimination of the lowest value did not modify the highly significant effect of the factor (P = 0.007).

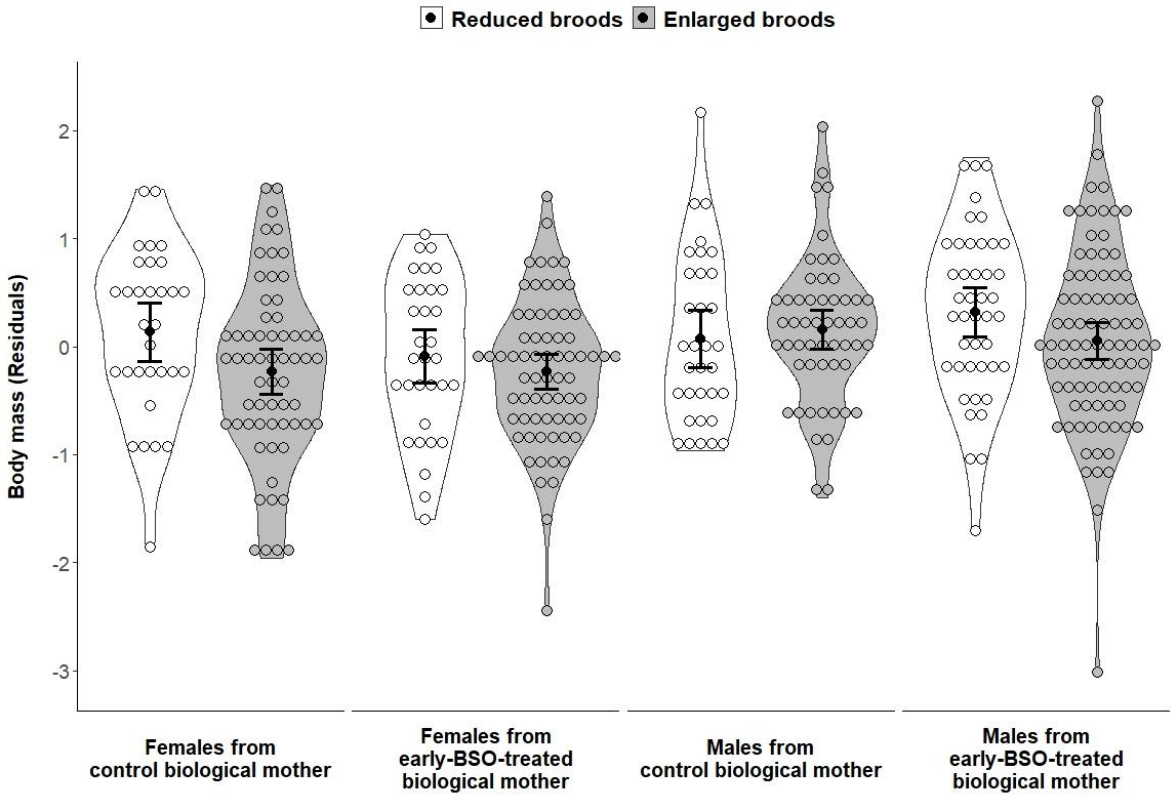

Figure S4: Effect of the early life BSO treatment of biological mothers and offspring nest environment (brood size) on female (left) and male (right) nestling body mass. Violin plots showing frequency distributions, individual data (residuals from the mixed model) and their means ± 95% CIs. The elimination of the lowest value did not alter the significance of the interaction (P = 0.024).

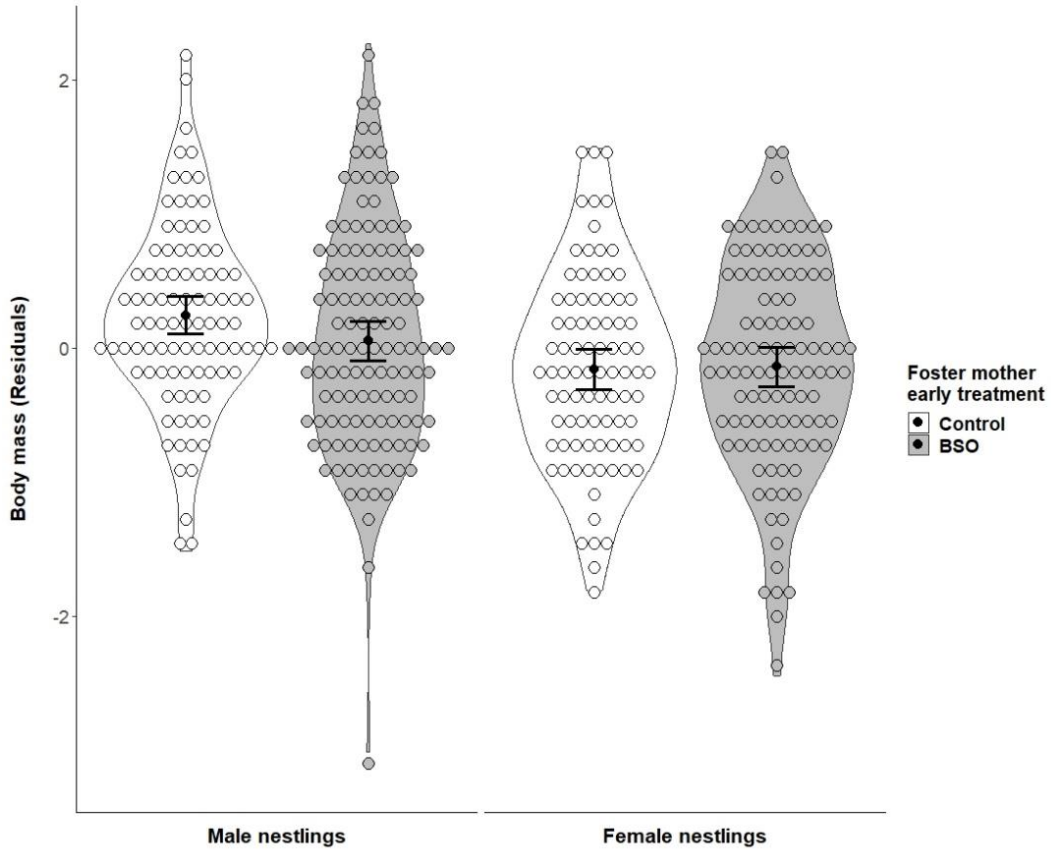

Figure S5. Effect of the foster mother early treatment on male and female nestling body mass. Violin plots showing frequency distributions, individual data (residuals from the mixed model) and their means ± 95% CIs. The elimination of the lowest value did not alter the significance of the interaction (P = 0.016).

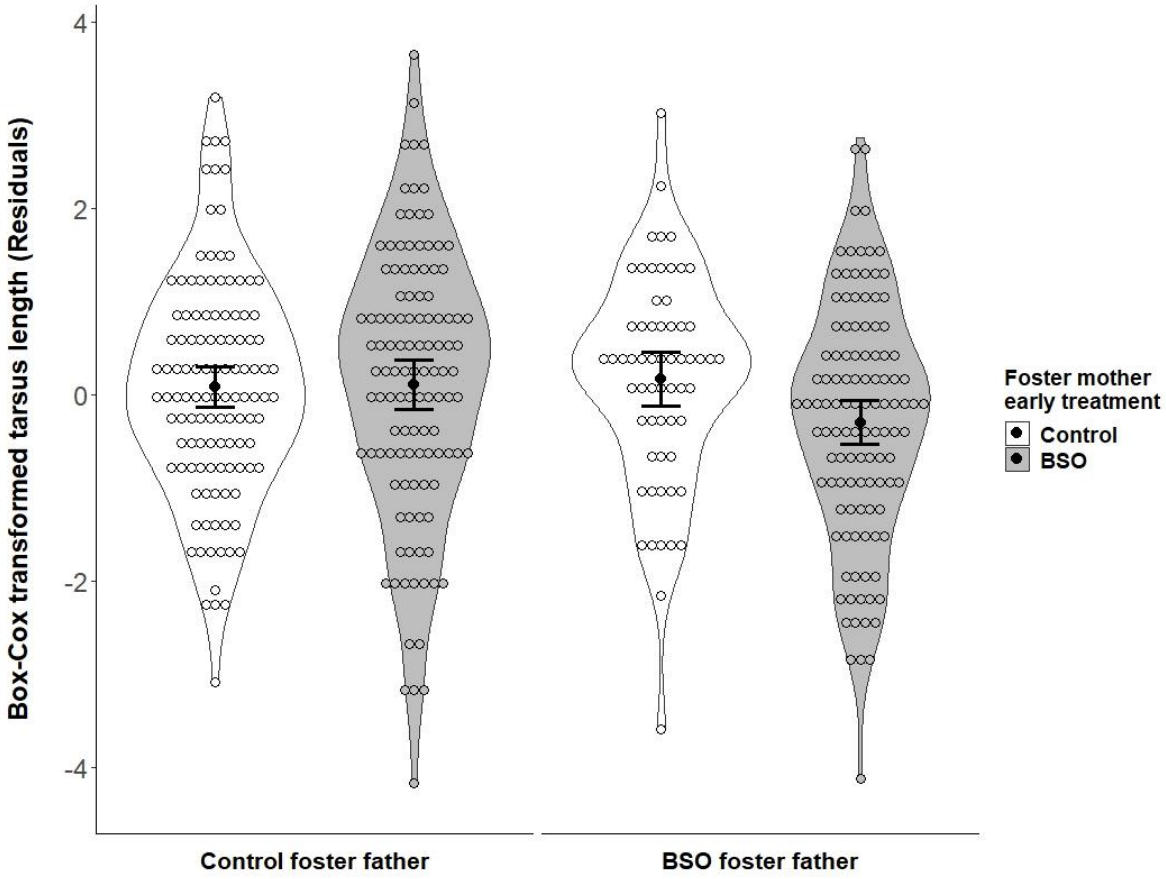

Figure S6: Effect of the early life BSO treatment of the foster parents on Box-Cox-transformed tarsus length of the offspring. Violin plots showing frequency distributions, individual data (residuals from the mixed model) and their means ± 95% CIs.

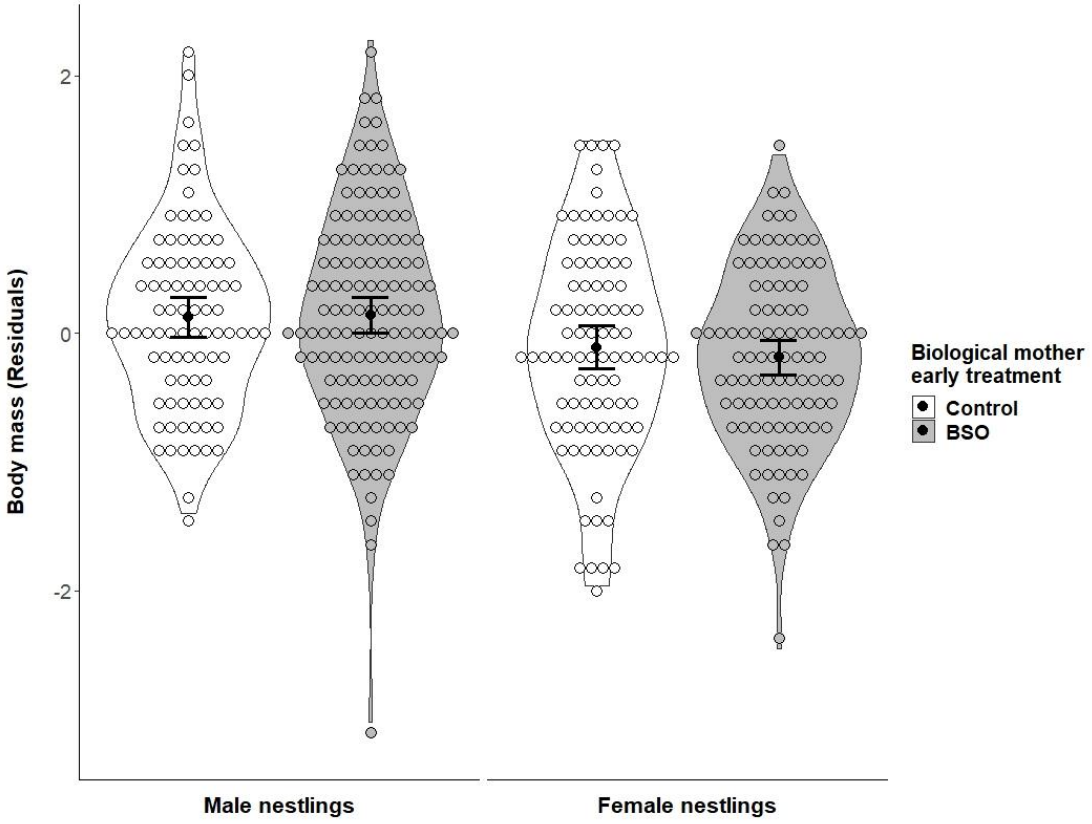

Figure S7. Effect of the biological mother early treatment on male and female nestling body mass. Violin plots showing frequency distributions, individual data (residuals from the mixed model) and their means ± 95% CIs. The elimination of the lowest value did not alter the significance of the interaction (P = 0.040).