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RESEARCH ARTICLE

Immediate and delayed effects of growth conditions on ageing parameters in nestling zebra finches

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ABSTRACT

Conditions experienced during development and growth are of crucial importance as they can have a significant influence on the optimisation of life histories. Indeed, the ability of an organism to grow fast and achieve a large body size often confers short- and long-term fitness benefits. However, there is good evidence that organisms do not grow at their maximal rates as growth rates seem to have potential costs on subsequent lifespan. There are several potential proximate causes of such a reduced lifespan. Among them, one emerging hypothesis is that growth impacts adult survival and/or longevity through a shared, end point, ageing mechanism: telomere erosion. In this study, we manipulated brood size in order to investigate whether rapid growth (chicks in reduced broods) is effectively done at the cost of a short- (end of growth) and long-term (at adulthood) increase of oxidative damage and telomere loss. Contrary to what we expected, chicks from the enlarged broods displayed more oxidative damage and had shorter telomeres at the end of the growth period and at adulthood. Our study extends the understanding of the proximate mechanisms involved in the trade-off between growth and ageing. It highlights that adverse environmental conditions during growth can come at a cost via transient increased oxidative stress and pervasive eroded telomeres. Indeed, it suggests that telomeres are not only controlled by intrinsic growth rates per se but also may be under the control of some extrinsic environmental factors, which could complicate our understanding of the growth–ageing interaction.

KEY WORDS: Birds, Flight performance, Growth conditions, Oxidative stress, Telomeres

INTRODUCTION

Conditions experienced during growth are of crucial importance as they can have a significant influence on the optimisation of life histories (Ricklefs, 1979; Lindström, 1999). Indeed, early life conditions are known to directly impact growth patterns (De Kogel, 1997), metabolism (Desai and Hales, 1997), immune function (Saino et al., 1997) and sexual attractiveness (Gustafsson et al., 1995). Moreover, the ability of an organism to grow fast and achieve a large body size often correlates with short- and long-term fitness benefits (Richner, 1992; Reeve, 2000; Bonduriansky, 2001). Growth is a vulnerable stage for individuals, and organisms would therefore be expected to grow as quickly as they could in order to escape this risky stage of life (Arendt, 1997). Because growing fast is often associated with a large 'final' body size, especially in species with determinate growth, fast growth is also thought to greatly influence fitness at adulthood. Indeed, adult body size is frequently under strong natural and sexual selection, and larger individuals are often better 'equipped' to defend their food resources, territories or mates against conspecifics. In addition, larger individuals are often the favourites when it comes to choosing a mate (Shine, 1988; Arendt, 1997). However, the accumulation of counterintuitive data underlines that individuals presenting a more rapid juvenile growth rate tend to have a reduced adult lifespan (Metcalfe and Monaghan, 2001; Olsson and Shine, 2002; Rollo, 2002; Dmitriew, 2011; Lee et al., 2012). Consequently, one can conclude that, even if fast growth rates should be the norm and individual fitness decreases as a function of developmental time (Roff, 1980; Ricklefs, 2010), growth remains a flexible life history trait that can change quickly both up and down in response to environmental variations (such as resource availability and foraging risk), with potential costs (Dmitriew, 2011).

Environmental conditions in which organisms grow up are crucial as they determine access to food. Indeed, when resources decline in quantity and/or quality, individuals grow slower and achieve a smaller size at maturity (Morey and Reznick, 2000; Day and Rowe, 2002). However, even if animals have unlimited access to resources, their growth rates are lower than what is physiologically possible (Metcalfe and Monaghan, 2001). This suggests that intrinsic factors also constrain growth rate, and that rapid growth carries inevitable physiological and ultimately life history costs (Metcalfe and Monaghan, 2001; Metcalfe and Monaghan, 2003). Ultimately, the realised growth rate will be the result of a compromise between the advantages that rapid growth procures and the short- and long-term costs it entails.

In order to identify the nature of the effects of growth conditions and the costs associated with growth rate plasticity, previous studies have been conducted using experimental approaches, usually through the modification of early developmental conditions. These experimental designs manipulate the quantity and/or quality of nutrition, or the environmental growth conditions through temperature (Lee et al., 2013) or brood/pup size (Alonso-Alvarez et al., 2007). These costs have particularly been highlighted by the study of growth compensation (Metcalfe and Monaghan, 2001; Langley-Evans, 2006) showing that a faster than normal growth rate following an initial growth stint allows the young organism to catch up with standard adult body size (Bize et al., 2006), but at the expense of a wide array of metabolic dysfunctions. For instance, glucose regulation (Ozanne and Hales, 2002; Bateson et al., 2004), locomotive performances (Álvarez and Metcalfe, 2005; Criscuolo et al., 2011) or decreased resistance to fasting (Gotthard et al., 1994) have been found to be impaired by high growth rates in early life (Metcalfe and Monaghan, 2003; Dmitriew, 2011). These short- and long-term costs are the main reason invoked for the ubiquitous observation that growth rate is

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naturally constrained to sub-maximal levels in most species despite evident benefits of reaching adult size as fast as possible.

However, any of these manipulations of growth conditions might have had a direct impact on the above physiological and metabolic pathways regardless of a putative effect on growth rates. As such, one crucial task that remains is to disentangle the effects of growth per se versus the effects of growth conditions on these physiological and metabolic pathways.

In the light of this issue, the accumulated evidence of the link between early life conditions and senescence (Nussey et al., 2009; Lee et al., 2013) re-launches our interest in the mechanisms by which growth rate and/or conditions may alter longevity. Indeed, besides the ultimate consequences of fast growth presented above, several proximate causes of such a reduced lifespan might be involved (Metcalfe and Monaghan, 2003). Among them, one emerging hypothesis is that growth may impact adult survival and/or longevity through a shared end point, ageing mechanism: telomere erosion (Allsopp et al., 1992; Jennings et al., 1999). Telomeres are highly conserved non-coding repetitive DNA sequences located at the end of linear chromosomes, which prevent the chromosome ends from being recognised as double-stranded breaks (Blackburn, 1991). Telomeric DNA is progressively lost because of the inability of telomeres to be completely replicated by DNA polymerase; progressive telomere shortening occurs over cell divisions. Once a lower critical size is reached, the telomere signalling pathway induces cell division arrest and/or cell senescence (Harley et al., 1990; Blackburn, 1991). Telomere loss is particularly important during development (Jennings et al., 1999; Hall et al., 2004), a phenomenon likely to be due to the convergence of the high rate of cell division and of a potentially high energy metabolism. In fact, telomere dynamics can be viewed as a balance of pro- and anti-erosion factors, and telomere loss is further exacerbated by oxidative stress (Von Zglinicki, 2000), which comes from the imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity (Finkel and Holbrook, 2000). However, there are anti-erosion factors, such as telomerase activity (mainly active in germinal and stem cells) and shelterin protein complex stability that favour telomere maintenance (Blackburn, 2000; de Lange, 2009) and may partly explain the high variability in telomere length among age-matched individuals (Hall et al., 2004).

So far, a few experimental studies have yielded data indicating that growth conditions could have a negative influence on telomere length. Manipulation of maternal nutrition in rats showed that accelerated postnatal growth of pups was associated with shorter telomeres at adulthood (Jennings et al., 1999; Tarry-Adkins et al., 2008; Tarry-Adkins et al., 2009; Tarry-Adkins et al., 2013). In wild king penguin chicks (*Aptenodytes patagonicus*) (Geiger et al., 2012), telomere shortening was related to growth trajectories, with small chicks that grew faster displaying accelerated telomere loss. In this last study, such telomere loss seemed to be related to a higher oxidative challenge. At the individual level, increased susceptibility to oxidative stress was also revealed to be a potential cost of accelerated somatic growth in zebra finches (*Taeniopygia guttata*) (Alonso-Alvarez et al., 2007), and accelerated growth in response to high altitude was associated with a high level of oxidative damage to DNA in coal tits (*Periparus ater*) (Stier et al., 2014). Moreover, oxidative stress that derives from stressful events in early life was found to accelerate telomere loss (Haussmann et al., 2012; Blackburn and Epel, 2012). This last report actually raises the point that some of the deleterious effects of growth on the ageing rate of the adult organism may be due to early stress (i.e. environmental conditions of growth) rather than being a consequence of growth rate per se. Indeed, most studies have manipulated growth in several ways (food restriction, modification of temperature), but these manipulations may influence proximate mechanisms independently of the effects on growth rate. As such, the central task that remains is to determine whether the effects on telomere dynamics are due to growth rate (i.e. intrinsic factor) or to environmental conditions of growth (i.e. extrinsic factors).

Although the trade-off between growth and longevity has been well documented, links between growth and telomere loss have mainly been studied in the case of growth compensation. We still need to test experimentally whether rapid growth rate actually modifies telomere loss during the growth period, and whether such a modification is likely to be persistent with age. In fact, the effects of a bad start and of subsequent growth acceleration have rarely been disentangled (Metcalfe and Monaghan, 2001). To clarify this point, we experimentally modified growth conditions by manipulating brood size in zebra finches (*T. guttata* Reichenbach 1862), thereby creating control broods (non-manipulated number of nestlings), enlarged broods by adding two nestlings (stressful growth conditions as a result of altered individual nutrition and/or a stressful social environment) and reduced broods by removing two nestlings (overall improved growth conditions).

Using telomere dynamic comparisons between broods, we investigated whether rapid growth (chicks in reduced broods) is effectively terminated at the cost of a short-term (end of growth) and long-term increase (at adulthood) of oxidative damage and telomere loss. In parallel, we investigated how the body maintenance capacity of chicks (i.e. antioxidant capacity and flying performance) is affected by the manipulation, and we also tested at different time scales whether initial stressful growth conditions (enlarged broods) trigger an enhanced telomere shortening independently of growth rate, and whether this is associated with body maintenance status.

RESULTS

Effects on nestling development, fledging age and flight performance

Body masses at cross-fostering were not different between experimental groups or sex (P=0.203, F=1.69, d.f.=2–25.9 and P=0.279, F=1.19, d.f.=1–62.9, respectively).

The interaction between time period and brood size manipulation significantly affected body mass gain for both males and females (Fig. 1, Table 1). During the first period of growth (0–20 days), nestlings in enlarged broods grew more slowly than those in the

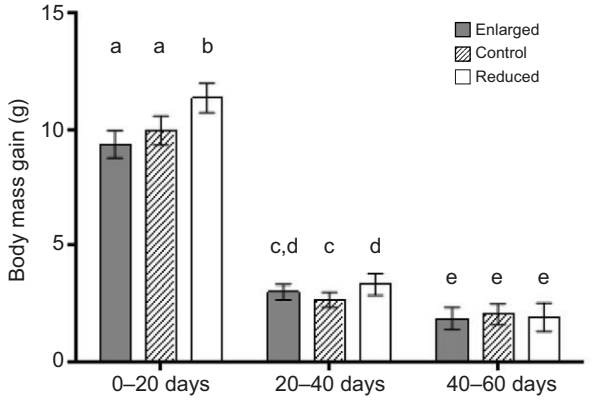

Fig. 1. Body mass gain for both sexes in the three experimental groups (enlarged, reduced and control) during different time intervals. Sample size up to day 60: enlarged, N=30; reduced N=15; and control, N=25. Bars represent ±s.e. Different letters indicate significant differences (P<0.05).

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Table 1. Results of general linear mixed models on body mass gain, body mass, fledging day, flight performance, antioxidant levels, DNA oxidative damage and telomere length

Variable	d.f.	*F*	*P*
Body mass gain			
Time (0–20 versus 20–40 versus 40–60 days)	2, 154	29.4	<0.001
Sex	1, 151	0.47	0.493
Brood manipulation	2, 16	4.89	0.022
Time × brood manipulation	4, 148	3.15	0.016
Initial body mass	1, 166	11.17	0.001
Body mass			
Time (day 10, 20, 30, 40, 60, 360)	5, 328	133.99	<0.001
Sex	1, 341	0.53	0.467
Brood manipulation	2, 39	10.76	<0.001
Time × brood manipulation	10, 328	0.86	0.574
Growth rate (*K*)			
Sex	1, 57	1.44	0.233
Brood manipulation	2, 30	8.39	0.001
Sex × brood manipulation	2, 52	0.46	0.628
Fledging day			
Sex	1, 66	1.32	0.255
Brood manipulation	2, 66	18.27	<0.001
Flight performance			
Time (45 versus 360 days)	1, 52	0.77	0.383
Sex	1, 48	2.23	0.142
Brood manipulation	2, 25	2.35	0.116
Time × brood manipulation	2, 51	5.17	0.009
Body mass	1, 51	14.26	<0.001
Antioxidant levels			
Time (10 versus 30 versus 360 days)	2, 154	6.69	<0.001
Sex	1, 154	3.34	0.069
Brood manipulation	2, 154	11.57	0.001
Time x brood manipulation	4, 154	1.83	0.12
Body mass	1, 154	0.11	0.74
DNA oxidative damage			
Time (10 versus 30 versus 360 days)	2, 101	22.02	<0.001
Sex	1, 111	0.37	0.54
Brood manipulation	2, 30	1.88	0.169
Time × brood manipulation	4, 97	5.74	<0.001
Antioxidant levels	1, 98	9.78	0.002
Body mass	1, 108	0.19	0.655
Telomere length			
Time (10 versus 30 versus 360 days)	2, 120	3.12	0.04
Sex	1, 108	0.08	0.778
Brood manipulation	2, 23	6.19	0.006
Time x brood manipulation	4, 112	3.43	0.01
DNA oxidative damage	1, 92	8.67	0.004
Body mass	1, 19	0.27	0.602

To control for pseudo-replication, nest identity was included as a random factor in each model.

reduced group (P<0.001), but were not significantly different in terms of growth rates from the nestlings in the control group (P=0.289). During days 20–40, nestlings from the enlarged group did not differ in their rate of growth from those from the reduced and the control groups. During the 40–60 day time period, growth rates were not significantly different for all experimental groups.

During the growth period (days 10, 20 and 30) and afterwards (days 40, 60 and 360), brood size manipulation significantly affected body mass for both males and females (Table 1, Fig. 2). Nestlings in the enlarged group and control group were not significantly different in terms of body mass (P=0.101) but were lighter than those reared in the reduced group (both P<0.001). A similar pattern was found for the growth rate K (calculated from the logistic equation); brood size manipulation significantly affected growth rate for both males and females (Table 1). Nestlings in the enlarged group and control group were not significantly different in terms of growth rate (P=0.298) but grew more slowly than those reared in the reduced group (both P=0.031).

Brood size manipulation had significant effects on chick fledging dates (mean age at fledging: enlarged: 21 days; control: 19.04 days; reduced: 16.8 days) (Table 1). Chicks from the enlarged group fledged later than those from the control and the reduced groups (P=0.002 and P<0.001). Similarly, chicks from the control group fledged later than those from the reduced group (P=0.003).

Significant variation in the flight performance of males and females was explained by the interaction between brood size manipulation and time (Fig. 3, Table 1). Just after the fledging time (45 days), birds from the enlarged group exhibited lower flight performances than those from the reduced group (P<0.001; no significant differences were found with the control group, P=0.501). When birds were 1 year old, this difference disappeared, as all groups exhibited the same flight performance (i.e. LSD pairwise comparisons, all P>0.1).

Effects on oxidative stress, telomere length and survival

Brood size manipulation as well as time (10 versus 30 versus 360 days) had significant effects on antioxidant levels for both males

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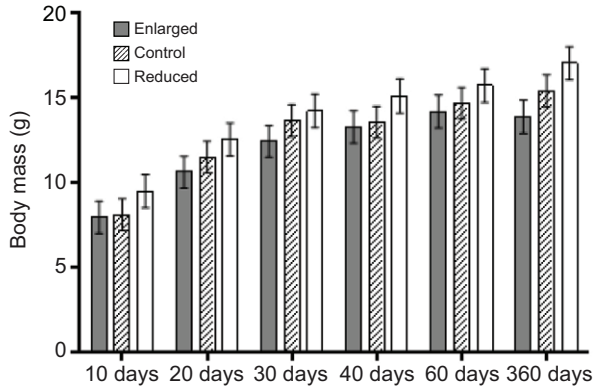

Fig. 2. Body mass for both sexes in the three experimental groups at different time points. Sample size up to day 60: enlarged, *N*=30; reduced *N*=15; and control, *N*=25; sample size at day 360: enlarged, *N*=14; reduced, *N*=13; and control, *N*=20. Bars represent ±s.e.

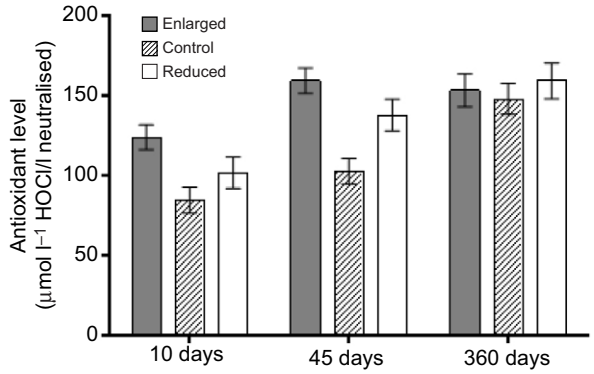

Fig. 4. Antioxidant levels in the three experimental groups at different time points. Antioxidant levels were measured as µmol l^{-1} hypochlorous acid (HOCl) per ml neutralised. Sample size at day 10 and 30: enlarged, *N*=30; reduced *N*=15; and control, *N*=25; sample size at day 360: enlarged, *N*=14; reduced, *N*=13; and control, *N*=20. Bars represent ±s.e.

and females (Fig. 4, Table 1). All experimental groups exhibited an increase in antioxidant defences over the growth period. Independently of the time period, chicks raised in enlarged broods displayed higher levels of antioxidant levels compared with the controls ($P<0.001$) but levels were not significantly different when compared with those of the reduced group (Fig. 4, $P=0.112$).

The interaction between brood size manipulation and time significantly affected oxidative damage on DNA (Fig. 5, Table 1). At 10 days, all experimental groups exhibited the same levels of oxidative damage. However, at the end of the growth period (30 days), birds from the enlarged brood group displayed higher levels of oxidative damage compared with those from the control and reduced groups (Fig. 5, $P=0.09$ and $P=0.011$, respectively). A year after the experiment, all groups displayed the same levels of oxidative damage on DNA.

Brood size manipulation, time (i.e. 10 days versus 30 days versus 1 year after the experiment) and the interaction between brood size manipulation and time had significant effects on telomere length (Table 1). All birds presented a progressive decrease in their telomere length over 1 year (Fig. 6). However, telomere loss was not observed in all experimental groups over the growth period (during the first 30 days): only chicks from the enlarged group exhibited a significant decrease in telomere length during this period. Compared with chicks in the control and reduced groups, chicks raised in enlarged broods displayed the most extensive telomere loss at the end of the growth period (30 days) ($P<0.001$ and $P=0.005$, control and reduced, respectively).

The apparent deleterious effects of brood enlargement on telomere length persisted 1 year after the experiment, and the enlarged group displayed the shortest telomeres (LSD *post hoc* comparisons, $P<0.001$ for both control and reduced groups, Fig. 6). Conversely, at day 360, birds from the control and reduced groups displayed no significant differences in their telomere lengths (LSD *post hoc* comparisons, $P=0.955$; Fig. 6).

Body mass did not significantly co-vary with telomere length, DNA oxidative damage or antioxidant levels (Table 1, $P=0.602$, $P=0.655$, $P=0.740$, respectively). To further explore the effects of growth rates on these variables, we also ran similar analyses by replacing body mass by *K* (logistic growth rate constant) as a covariate. As for body mass, *K* did not significantly co-vary with telomere length, DNA oxidative damage or antioxidant levels (results not presented, $P=0.618$, $P=0.990$, $P=0.453$, respectively).

Offspring survival over the first year after manipulation did not significantly differ between experimental groups (sex effect $P=0.910$, brood size manipulation effect $P=0.985$). Telomere length, DNA oxidative damage and antioxidant levels at 30 days were not significantly linked to survival either ($P=0.462$, $P=0.275$ and $P=0.781$, respectively).

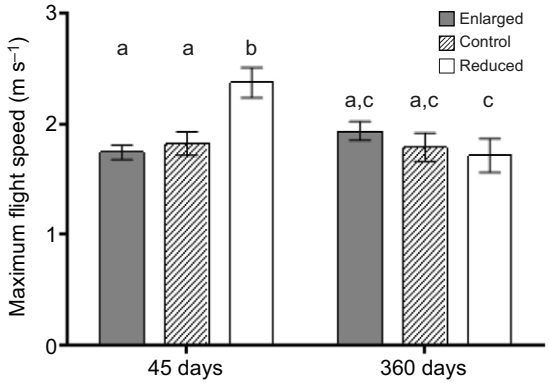

Fig. 3. Flight performance expressed as maximum flight speed in the three experimental groups at different time points. Sample size at day 45: enlarged, *N*=30; reduced *N*=15; and control, *N*=25; sample size at day 360: enlarged, *N*=14; reduced, *N*=13; and control, *N*=20. Bars represent ±s.e. Different letters indicate significant differences ($P<0.05$).

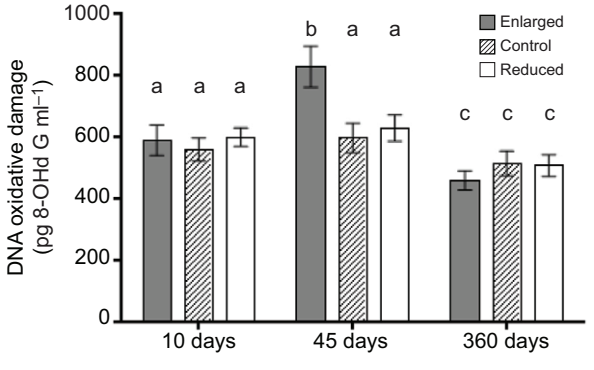

Fig. 5. Oxidative damage on DNA in the three experimental groups at different time points. Oxidative damage was measured in terms of 8-hydroxy-2-deoxyguanosine (8-OHdG) levels. Sample size at day 10 and 30: enlarged, *N*=30; reduced *N*=15; and control, *N*=25; sample size at day 360: enlarged, *N*=14; reduced, *N*=13; and control, *N*=20. Bars represent ±s.e. Different letters indicate significant differences ($P<0.05$).

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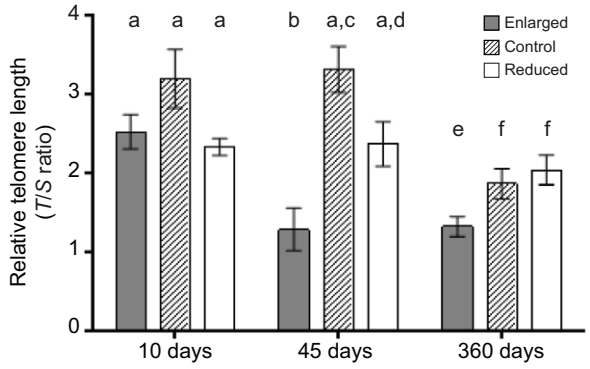

Fig. 6. Relative telomere length in the three experimental groups at different time points. Relative telomere length was measured as the ratio of telomere repeat copy number (*T*) to a control single gene (GAPDH) copy number (*S*). Sample size at day 10 and 30: enlarged, *N*=30; reduced *N*=15; and control, *N*=25; sample size at day 360: enlarged, *N*=14; reduced, *N*=13; and control, *N*=20. Bars represent ±s.e. Different letters indicate significant differences ($P<0.05$).

DISCUSSION

Our experimental manipulation of brood size at hatching in a captive population of zebra finches shows that chicks from reduced broods were growing at a faster rate and attained a heavier body mass, but without any apparent deleterious impact on blood oxidative markers and telomere length, compared with chicks from control broods. In contrast, although chicks from enlarged broods were growing at the same rate and exhibited similar body masses to control chicks, they suffered from greater oxidative damage and telomere loss during growth than control broods, this latter effect persisting at adulthood.

Brood size manipulation, body mass growth and flight performance

The brood size manipulation was successful in creating differences in growth trajectories. Chicks raised in reduced broods showed faster growth rates (until 20 days) and were heavier at all time points compared with control and enlarged groups. These results are in accordance with previous literature relating the effects of brood size manipulation on growth patterns (Dijkstra et al., 1990; De Kogel, 1997; Pettifor et al., 2001) but differ from some others where reduced and control chicks presented no differences in their body masses (Dijkstra et al., 1990). The absence of an un-manipulated group in some studies (e.g. Alonso-Alvarez et al., 2007) means it is difficult to make any generalisation of our control–reduced–enlarged two-by-two group comparisons of body mass data. For instance, chicks reared in enlarged broods did not significantly differ from those reared in control broods in terms of body mass or growth rate, rather suggesting that our treatment had no effect. However, we showed here that chicks reared in enlarged broods suffered from a mass-independent deleterious impact like delayed fledging date or reduced flight performance, underlining that costs that remained undetected using body mass growth measurements probably exist.

A neonatal energy stress is known to disturb the growth–body maintenance trade-off (Metcalfe and Monaghan, 2001). However, it may also alter the fine-tuned processes that regulate the progressive development and maturation of the different organs specific to each species (Ricklefs, 1979; Bize et al., 2006). In accordance, the fledging date of chicks from the enlarged group was delayed compared with that of chicks from the control and reduced groups. Therefore, brood enlargement is likely to have delayed their age of maturity, leading to an altered functioning of key tissues, such as muscle, thereby partially explaining the reduced flight ability of enlarged brood chicks when challenged at 45 days (Criscuolo et al., 2011). However, as birds from enlarged broods were doing as well as control chicks, it is likely that our differences in flight performance arise from a better ability of flight escape in birds reared in reduced broods, maybe due to an earlier functioning maturity of flight muscles at 45 days, a difference that disappeared at adulthood (when birds were 360 days), while growth ended and tissues were mature.

Brood size enlargement and deleterious effects on ageing

An alteration of growth conditions, such as brood size manipulation, can impact several traits beyond and independently of growth rate. Consequently, the observed detrimental effects of growth on ageing might come from several factors other than growth rate variability, like a lack of specific nutrients (Arnold et al., 2007), a restructuring of the growth hormone–insulin-like growth hormone axis (Carter et al., 2002), or an elevation in corticosterone levels. Chronic exposure to high levels of stress is known to increase oxidative damage (Costantini et al., 2008a; Zafir and Banu, 2009). Chicks raised in enlarged brood were certainly facing a food shortage and increased intra-brood competition. As a consequence, they may have responded by increasing their begging activity, a process that has previously been related to enhanced corticosterone levels (Kitaysky et al., 2003). This may in return have triggered oxidative stress and telomere loss, which have recently been shown to be increased following corticosterone exposure in early life (Haussmann et al., 2012), even if the ways through which corticosterone alters the oxidative balance remain elusive (Haussmann and Marchetto, 2010). When investigating effects on oxidative stress, it is best to consider both aspects of the imbalance, antioxidant levels as well as oxidative damage (which are caused by ROS) (Balaban et al., 2005), as measurements of only one of these parameters can give a misleading picture (Costantini and Verhulst, 2009; Monaghan et al., 2009). Oxidative stress is due to the imbalance between ROS production (mainly by mitochondria) and the antioxidant networks (mitochondrial, cellular and systemic) (Halliwell and Gutteridge, 1999). Interestingly, in our study, despite the fact that chicks from enlarged broods exhibited higher antioxidant levels than control broods (but no different from reduced broods), they still suffered from higher DNA oxidative damage levels at the end of the growth period than control and reduced broods. These results are in concordance with previous findings (Alonso-Alvarez et al., 2006; Alonso-Alvarez et al., 2007). Therefore, chicks from the enlarged broods were not able to compensate for an increased ROS production.

Brood size enlargement not only had an impact on oxidative stress but also affected short- and long-term telomere dynamics. Compared with chicks of the control and reduced groups, those raised in enlarged broods displayed a more severe telomere erosion during the growth period. Moreover, enlarged brood chicks presented the shortest telomeres 1 year after the experiment. Our findings are in accordance with those of a recent study (Boonekamp et al., 2014) but differ from the findings of an earlier study (Voillemot et al., 2012) in which there was no effect of brood size manipulation on telomere length. This discrepancy might be due to the fact that Voillemot et al. (Voillemot et al., 2012) measured telomere length only once and early during growth (at day 12). However, our results may also be interpreted as a support for Voillemot and colleagues' conclusion, i.e. that telomeres are not only controlled by intrinsic growth rates per se but may also be under the control of some extrinsic environmental factors. In our case, enlarged and control

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broods displayed the same pace of growth, but did not exhibit the same levels of oxidative stress or the same pattern of telomere erosion. Thus, modifications of growth conditions, such as brood size manipulation, could impact individual physiological and molecular pathways impinging on ageing independently of growth rates. As such, the potential stressful conditions (increased competition and crowding) experienced by chicks from enlarged broods might be the cause of the effects we observe on ageing parameters. A very recent study showing that a competitive disadvantage during growth triggered an acceleration of telomere erosion in European starling (*Sturnus vulgaris*) chicks (Nettle et al., 2015) supports this hypothesis. This suggests that the environmental (and non-energy related) conditions in which an individual grows up might have predominant effects on ageing markers compared with growth rates per se. This hypothesis is further supported by the absence of a link between body mass or growth rate (*K*) on telomere length, oxidative damage or antioxidant levels.

Long-term DNA oxidative damage being similar for all groups, the shorter telomeres of enlarged brood chicks at adulthood might be the consequence of a reduced capacity to repair damaged telomeres. Effects on telomerase function are likely to be an important issue. Chronic stress has been shown to increase telomere shortening (Epel et al., 2004; Kotrschal et al., 2007; Hatakeyama et al., 2008) and to lower telomerase activity (Epel et al., 2010). In addition, early exposure to corticosterone is associated with a long-term increase in stress reaction of the whole adreno-cortical system (Spencer et al., 2009). This may account for the shorter telomeres at adulthood of enlarged brood chicks (Haussmann et al., 2012).

Brood size reduction, faster growth and ageing

Our results indicate that individuals raised in reduced broods did better in terms of growth and of short-term flight performance, suggesting that they were raised under super-optimal energy conditions compared with individuals that grew up in control and enlarged broods. However, they also exhibited no significant difference in telomere length compared with control chicks, suggesting that their growing conditions either spared them from a high environmental stress or allowed them a different trade-off between growth and ageing. Indeed, environmental stress may be of tremendous importance in our case (i.e. chick competition, parent–offspring conflict), given the demonstrated deleterious impact on telomere length in humans (Blackburn and Epel, 2012).

Alternatively, in reduced broods, parents took care of fewer chicks than initially expected, thus investing more per chick and enhancing chick quality without immediate ageing costs. This idea is supported by the fact that reduced brood chicks presented an increase of their plasma antioxidant capacity at a similar level to that recorded in chicks from the enlarged group. This means that rapid growth may have enhanced ROS production, but that good rearing conditions may have provided chicks from the reduced broods with adequate antioxidant resources (Beaulieu and Schaefer, 2013). Future experiments should focus on the enzymatic and non-enzymatic antioxidant buffering activities in a similar experimental design.

Brood size manipulation, ageing and survival

Our study highlighted that adverse environmental conditions during growth can come at a cost via increased oxidative stress and long-term erosion of telomeres. Telomere length measured early in life or at adulthood has been found to predict subsequent survival and life expectancy (Cawthon et al., 2003; Haussmann et al., 2003; Bize et al., 2009; Heidinger et al., 2012). Similarly, a more recent study in free-living jackdaws (*Corvus monedula*) (Boonekamp et al., 2014) showed that nestlings in enlarged broods lost more telomere repeats and that this loss was linked to post-fledging survival. However, despite their effects on oxidative stress, telomere dynamics and telomere length, our rearing conditions did not modify survival rates 1 year after the experiment. Following the survival rate of adult zebra finches over a year is probably not a sufficient time to highlight the impact of growth retardation on subsequent individual lifespan and fitness. It underlines that we still need to acquire more data on the accurate nature of the determinants of telomere length in early life (i.e. environmental stress) (Monaghan, 2014), on the way potentially important maintenance processes, such as telomerase activity, are affected in the long term, and how this links with survival and reproduction over adulthood. Merging cell and organismal levels of comprehension of ageing causes and consequences will certainly enable us to test whether telomere dynamics are a reliable proxy of individual fitness, as is more and more often hinted (Monaghan and Haussmann, 2006; Haussmann and Marchetto, 2010; Monaghan, 2010).

MATERIALS AND METHODS

Experimental design

The present study was conducted on a captive population of zebra finches. Twenty-five pairs (randomly formed) were placed in breeding cages (0.57×0.31×0.39 m; randomly placed in the room) with food (a commercial mix of seeds for exotic birds enriched with vitamins and egg) and water *ad libitum*. Nest boxes and straw were provided for breeding. The cages were put in a room with a constant temperature of 24±1°C and light conditions were 13 h light:11 h dark. The brood size was manipulated in order to create three groups: nine enlarged broods (30 chicks), nine reduced broods (15 chicks) and seven control broods (25 chicks). Chicks, from 1 to 3 days old, were randomly cross-fostered to form enlarged (two chicks added, mean brood size 4.8±0.75), reduced (two chicks removed, 1.8±0.64) and control broods (number of chicks unchanged, 2.8±0.64). Pre-manipulated brood size did not differ between groups (P=0.078). Brood size manipulation was successful as post-manipulation brood size significantly differed between groups (P<0.001). Birds were separated from their parents when the younger chick from the brood was 35 days old. Afterwards, chicks were released into a large aviary, and they were followed individually up to 1 year after treatment to evaluate the long-term effects of manipulated growth conditions on chick health, flight performance and survival.

Blood samples were collected from the brachial vein of the chicks during the growth period (i.e. 30 µl at 10 and 30 days), the same individuals being re-sampled (if still alive) 1 year after treatment (i.e. 50 µl at 360 days; 14 individuals in the enlarged group, 20 individuals in the control group, 13 individuals in the reduced group). Roughly, one-third of the birds died within a year, which is a mortality rate comparable to what was previously observed in the same species (Heidinger et al., 2012).

Body mass growth trajectory was assessed by weighing the chicks every day from hatching until they reached 30 days of age (end of the growth period). Body mass growth curves were fitted with the following logistic equation: $y(x)=A[1+\exp(-K\times x-B)]$, which was the best-fitting model based on R^2. $y(x)$ represents the mass (in g) of a chick at age x, A is the asymptotic mass (i.e. mass at fledging), K is the growth rate constant (an increase in K value implies an increase in the rate at which mass increases from the initial value to the asymptotic value) and B is a constant determining the initial mass. Birds were also weighed at the time of the flight performance measurements (at 45 and 360 days). The study complied with the 'Principles of Animal Care' publication no. 86-23, revised 1985, of the National Institutes of Health, and with current legislation (L87-848) on animal experimentation in France.

Flight performance measurements

Flight performance, which is directly linked to an individual's capacity to forage and escape predators, is used as a proxy for individual maintenance (Lindhe Norberg, 2002). Flight performance was first measured when birds were 45 and 360 days old, using vertical take-off when alarmed. To test flight

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ability, we used a similar set up to that used in two previous studies (Kullberg et al., 2002; Criscuolo et al., 2011). Measurements were made in a vertical plastic tube. The week before the final experimental trial, birds were trained twice in the same manner as during the experimental trial. Each bird was released on a perch situated at 20 cm from the ground, at the base of a transparent vertical plastic tube. At the top of the flight tube (120 cm from the first perch), there was a perch where the bird could be collected after each flight. For the training trial as well as for the experimental trial, birds were released five times in the tube and were allowed to rest for 30 s between each flight. During the experimental trial, all flights were video recorded with a video camera (Casio Highspeed EX-H100, 420 frames s^{-1}). To determine flight speed, we analysed the videos and calculated the time it took the bird to cover a distance of 40 cm. This measure was made by counting the number of video frames (each frame covering 0.002 s) between two marks on the tube at 40 and 80 cm height. Flight speed was calculated with this formula: $V=d/[(F–I)/420]$ where d is the distance between the two marks (40 cm), I is the number of frames when the bird has crossed the first mark and F is the number of frames when the bird has crossed the second mark. All videos were analysed with Avidemux 2.5®. We used the fastest of the five flights as a measurement of the bird's escape flight ability (Kullberg et al., 2002).

Oxidative stress measurements

Oxidative stress measurements were done during and at the end of the growth period (10 and 30 days), as well as 1 year after the experiment (360 days) and evaluated in two steps: antioxidant levels and oxidative damage. Antioxidant levels were assessed in plasma samples (stored at –20°C until analysis), with the Oxy-Adsorbent test (Diacron International, Grosseto, Italy), as previously described in birds (Costantini et al., 2007; Costantini et al., 2008b; Beaulieu et al., 2010; Beaulieu et al., 2011) and mammals (Stier et al., 2012). The Oxy-Adsorbent test measures the total antioxidant capacity of the plasma using a colorimetric determination to quantify the ability of the antioxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl). Measurements are expressed as µmol l^{-1} HOCl ml^{-1} neutralised. For the Oxy-Adsorbent assay, mean intraplate coefficient of variation was of 2.2% and mean interplate coefficient of variation was of 7%.

We determined oxidative damage on DNA by measuring 8-hydroxy-2-deoxyguanosine (8-OHdG), using the 8-OHdG EIA kit (StressMarq Biosciences Inc., Victoria, BC, Canada) as previously described in birds (Stier et al., 2014). DNA was first extracted from blood cells (with DNeasy Blood and Tissue kit, Qiagen) and then genomic DNA was totally digested following previous instructions (Quinlivan and Gregory, 2008).

8-OHdG is the by-product of oxidative damage on DNA due to the deleterious effects of ROS and increased levels of this marker have been associated with the ageing process (Shen and Abate-Shen, 2007). DNA damage measurements are expressed in pg ml^{-1}. The mean coefficient of intraplate variation was of 3.4%.

Telomere length measurement

Telomere length was measured at 10 days, 30 days and 1 year after the experiment. Telomere length was measured on DNA extracted from red blood cells (stored for 3 months at – 20°C until analysis) using DNeasy Blood and Tissue kit (Qiagen). Blood is an appropriate tissue to measure telomere length in this case as erythrocytes are nucleated in birds. Moreover, sampling is not invasive, and blood telomere length is correlated with telomere length in other tissues (Reichert et al., 2013). Telomere length was assessed by the quantitative real-time amplification (qPCR) procedure (Cawthon, 2002) previously used in zebra finches (Criscuolo et al., 2009). Relative telomere length is expressed as the ratio (T/S) of telomere repeat copy number (T) to a control single gene copy number (S). We used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a non-variable control gene (Smith et al., 2011). Forward and reverse primers for the GAPDH gene were 5′-AACCAGCCAAGTACGATGACAT-3′ and 5′-CCATCAGCAGCAGCCTTCA-3′, respectively. Telomere primers were: Tel1b (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCC-TTACCCT-3′). qPCR for both telomeres and GAPDH was performed using 5 ng of DNA with sets of primers Tel1b/Tel2b (or GAPDH-F/GAPDH-R), each used at a concentration of 200 nmol l^{-1}/200 nmol l^{-1}, in a final volume of 10 µl containing 5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems). Telomere and GAPDH real-time amplification were performed on two different plates. qPCR conditions for telomeres were 10 min at 95°C followed by 30 cycles of 1 min at 56°C and 1 min at 95°C. PCR conditions for GAPDH were 10 min at 95°C followed by 40 cycles of 1 min at 60°C and 1 min at 95°C. Each plate (telomere and GAPDH) included serial dilutions (10, 5, 2.5, 1.25 ng) of DNA of the same reference bird. This was used to generate a reference curve to control for the amplifying efficiency of the qPCR (mean efficiencies for GAPDH and telomere plates were between 95% and 105%). Samples were run in duplicate on each plate, on a total of four plates. To take into account the slight variation of efficiency between telomere and GAPDH amplifications, we calculated relative telomere length using the method suggested by Pfaffl (Pfaffl, 2001). The mean values were used to calculate the relative T/S ratios using the formula: [(1+E telomere)^ΔCt telomere (control–sample)/(1+E GAPDH)^ΔCt GAPDH (control–sample)].

Mean intraplate coefficient of variation was 1.1% for the Ct values of the GAPDH assays and 3.0% for the Ct values of the telomere assays. Interplate coefficient of variation (calculated on four samples repeated on the different plates) was 1.6% for the Ct values of the GAPDH assays, 2.9% for the Ct values of the telomere assays and 8.5% for the relative T/S ratios. On each plate, a negative control (water) and a melting curve (primer–dimer) were run to check for the absence of non-specific amplification.

Statistical analysis

Effects on nestling development, fledging age and flight performance

General linear mixed models with time (day 10, 20, 30, 40, 60 and 360) as a repeated variable, nest identity as a random factor, and brood manipulation, sex and the interaction between brood manipulation and time as fixed factors, were used to analyse body mass changes during growth.

Body mass gain was also analysed using these same linear mixed models, with time period (0–20 days versus 20–40 days versus 40–60 days) as a repeated variable, nest identity as a random factor, and sex, brood manipulation and the interaction between brood manipulation and time period as fixed factors. Initial body mass of each time period was added to the model to control for any gap of growth stages between individuals [i.e. effect of tissue maturation on growth rate (Ricklefs, 1979)]. The first time point corresponds to the period when nestlings are most dependent on the parents. The second time point corresponds to the period when they are less dependent as they fledged at ~20 days but are still in the parental cage and partially fed by their parents. The last time point corresponds to the period when the nestlings are totally independent as they were separated from their parents around 35 days old.

The growth rate constant (K) was analysed using linear mixed models, with nest identity as a random factor, and sex, brood manipulation and the interaction between brood manipulation and time as fixed factors.

Fledging dates (in days) were analysed using linear mixed models with nest identity as a random factor, and sex and brood manipulation as fixed factors. For flight performance analysis, we used linear mixed models with time period (45 versus 360 days) as a repeated variable, nest identity as a random factor, body mass as a covariate, and sex, brood manipulation and the interaction between brood manipulation and time period as factors in the model. *Post hoc* analyses were conducted using LSD tests.

Effects on oxidative stress, telomere length and survival

Antioxidant levels, oxidative damage and telomere length were analysed with linear mixed models with time period (10 days versus 30 days versus 360 days) as a repeated variable, nest identity as a random factor, and sex, brood manipulation and the interaction between brood manipulation and time as fixed factors. Given the relationships that exist among these variables, when testing for differences in oxidative damage among treatment groups, antioxidant levels were used as a covariate, while when testing for the impact on telomere length, DNA oxidative damage levels were used as a covariate. To test the effects of growth rate on these variables, we also included body mass as a covariate. *Post hoc* analyses were conducted using LSD tests. Determinants of chick survival 1 year after the experiment between groups (control versus enlarged versus reduced) were evaluated using a Generalised Linear Modelling procedure (GzLM) with a logistic

binary distribution of the dependent variable (survival or not). The experimental group and sex were entered as fixed factors, and telomere length, DNA oxidative damage and antioxidant levels at 30 days were entered as covariates. All statistical analyses were performed using SPSS v. 18.0. Means are indicated ±s.e.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
The experiment was conceived and designed by S.R., S.M., F.C. and P.B. Data were acquired by S.R., S.M., F.C. and S.Z.; analysis and interpretation of the data and writing were done by S.R., S.M., F.C. and P.B.

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