

RESEARCH ARTICLE

Physiological adjustments to high foraging effort negatively affect fecundity but not final reproductive output in captive zebra finches

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ABSTRACT

Foraging at elevated rates to provision offspring is thought to be an energetically costly activity and it has been suggested that there are physiological costs associated with the high workload involved. However, for the most part, evidence for costs of increased foraging and/or reproductive effort is weak. Furthermore, despite some experimental evidence demonstrating negative effects of increased foraging and parental effort, the physiological mechanisms underlying costs associated with high workload remain poorly understood. To examine how high workload affects haematology, oxidative stress and reproductive output, we experimentally manipulated foraging effort in captive zebra finches, *Taeniopygia guttata*, using a previously described technique, and allowed individuals to breed first in low foraging effort conditions and then in high foraging effort conditions. We found that birds upregulated haematocrit and haemoglobin concentration in response to training. Birds subjected to increased workload during reproduction had lower fecundity, although final reproductive output was not significantly different than that of controls. Offspring of parents subjected to high workload during reproduction also had higher oxidative stress when they were 90 days of age. Total antioxidant capacity and reactive oxygen metabolites of birds responded differently in the two breeding attempts, but we did detect an overall increase in oxidative stress in response to training in either attempt, which could explain the lower fecundity observed in birds subjected to increased workload during reproduction.

KEY WORDS: Exercise physiology, Workload, Oxidative stress, Reproduction, *Taeniopygia guttata*

INTRODUCTION

Many behaviours crucial for survival and reproductive success in free-living animals such as foraging and parental care involve elevated levels of activity or ‘workload’ (Halsey, 2016; Sinclair et al., 2014; Yap et al., 2017a). Foraging at elevated rates to provision offspring is thought to be an energetically costly activity (Caro et al., 2016; Maurer, 1996; Piersma, 2011; but see Williams, 2018) and it has been suggested that there are potentially physiological costs associated with the high workload involved

(Yap et al., 2017a). Indeed, there is some evidence suggesting that experimentally increased foraging and parental effort (e.g. via increased brood size, clutch size, etc.) adversely affects body condition (Veasey et al., 2001; Wiersma, 2005), survival (Briga et al., 2017; Daan et al., 1996), and reproduction (Deerenberg and Overkamp, 1999; Simons et al., 2014). However, for the most part consistent, unequivocal evidence for costs of increased foraging and/or reproductive effort is weak (Santos and Nakagawa, 2012; Williams, 2012; Zhang and Hood, 2016), one reason being that these studies have only looked at short-term costs and ignored the fact that costs can be deferred to later life-stages. Furthermore, despite some experimental evidence demonstrating negative effects of increased foraging and parental effort, the physiological mechanisms underlying costs associated with high workload remain poorly understood. A number of studies suggest that the carry-over effect of hard work on reproduction might not be purely energetic and that other ‘hidden costs’ (i.e. physiological costs) might be involved (Harrison et al., 2011; Nilsson, 2002; Simons et al., 2014; Veasey et al., 2001).

Given that foraging and provisioning offspring involves a significantly elevated level of activity for an extended period of time (Drent and Daan, 1980; Piersma, 2011), it seems intuitive that animals would exhibit a suite of behavioural and physiological adjustments in order to cope with the high workload (Sinclair et al., 2014; Yap et al., 2017a). For instance, Husak et al. (2015) found that green anole lizards (*Anolis carolinensis*) that were trained using an endurance training regime had higher haematocrit (Hct) and larger fast glycolytic muscle fibres. Similarly, pectoralis muscle citrate synthase activity and fatty acid transporters increase in response to exercise training in house sparrows (*Passer domesticus*) (Zhang et al., 2015). However, most of the aforementioned studies only investigated transient, short-term effects of exercise training. It is possible that animals are capable of upregulating physiology (e.g. metabolic enzyme activity, Hct, muscle mass) in the short term but would also downregulate physiology eventually when the costs of activity become too high (e.g. during reproduction) and maintaining energy balance becomes more difficult. The subsequent downregulation of physiology could be interpreted as a physiological cost of activity. Therefore, it is important to consider both short- and long-term physiological adjustments to increased foraging effort.

Although drastic changes in behaviour in response to increased foraging effort have been reported in a model passerine (zebra finch, *Taeniopygia guttata*), these studies found few physiological adjustments to training except an increase in masses of metabolic organs such as the flight muscle in females (Yap et al., 2017b; Zhang et al., 2018a), and a decrease in basal metabolic rate (Koetsier and Verhulst, 2011). Oxidative stress has been proposed as a general physiological mechanism mediating a diverse range of life-history trade-offs, such as survival and reproduction (Monaghan et al., 2009; Selman et al., 2012; Speakman and Garratt, 2014; Stier et al., 2012; Yap et al., 2017a; Zhang and Hood,

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2016). Some studies have shown that high workload (e.g. forced exercise training, increased foraging effort, increased flight costs) increases oxidative stress (Costantini et al., 2012; Fowler and Williams, 2017; Skrip et al., 2016; Yap et al., 2017b). In contrast, other studies that manipulated workload during reproduction (e.g. brood manipulation and wing clipping studies) had mixed findings, with some studies showing no change in oxidative stress (Wegmann et al., 2015a,b) and others showing an increase in oxidative stress (Christe et al., 2012; Losdat et al., 2011; Wiersma et al., 2004) in response to experimentally increased workload. These seemingly paradoxical findings could potentially be attributed to either differences in life-history strategies (Harrison et al., 2011; Zhang and Hood, 2016) or oxidative shielding during reproduction (Blount et al., 2016; Naviaux, 2012; Viblanc et al., 2018). However, many of these studies also failed to tease apart the effects of increased workload and the effects of reproduction.

To examine how physiological adjustments to high workload affect subsequent reproductive output, we experimentally manipulated foraging effort in non-breeding zebra finches using a previously described technique (Koetsier and Verhulst, 2011; Yap et al., 2017b). We then allowed individuals to breed first in low foraging effort conditions and then in high foraging effort conditions. We predicted that individuals subjected to experimentally increased foraging effort would have: (1) higher Hct and haemoglobin concentration [Hb] in the short term but would eventually decrease Hct and [Hb] during reproduction, when foraging costs become too high and maintaining energy balance becomes more difficult; (2) lower antioxidant defense and higher reactive oxygen metabolite production (i.e. higher oxidative stress overall); and (3) consequently, lower reproductive performance. We also predicted that amongst the individuals subjected to experimentally increased foraging effort, oxidative stress would be higher in the second (high foraging effort) breeding attempt compared with their oxidative stress levels in the first (low foraging effort) breeding attempt. Consequently, reproductive performance would also be lower in the second breeding attempt compared with the first breeding attempt in birds subjected to increased foraging effort.

MATERIALS AND METHODS

Animal husbandry

Zebra finches [*Taeniopygia guttata* (Vieillot 1817)] were maintained in controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant light schedule, 14 h:10 h light:dark, lights on at 07:00 h). All birds were provided with a mixed seed diet (Panicum and white millet, 1:3, 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit (coral sand) and cuttlefish bone (calcium) *ad libitum*, and received a multi-vitamin supplement in the drinking water once per week. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (No. 1231B-94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

Experimental timeline and protocol

A total of 36 male and 36 female zebra finches were randomly selected from our colony at Simon Fraser University to be included in the experiment. Half of the birds from each sex were randomly assigned to either a high foraging effort group (HF) or control group. For the HF group, foraging effort was experimentally manipulated using a previously described training protocol (Koetsier and Verhulst, 2011; Yap et al., 2017b; Zhang et al., 2018a). Briefly, food (mixed seed) was provided in transparent Plexiglas containers (L×W×H: 40×10×13 cm) suspended from the roof of the cage

(L×W×H: 122×46×41 cm), with feeding holes low on the front panel to allow access to seeds. Perches made of wooden pencils (diameter 0.8 cm) were fitted adjacent to feeding holes to allow birds to perch while foraging for 21 days prior to the start of the experiment (similar to the standard feeders in control cages). Over a 14 day period perches were gradually shortened (0.5 cm every 2 days) and eventually removed completely to train birds to modify their foraging behaviour and obtain seeds in the high foraging cost condition. Birds in control foraging conditions were not trained and were given standard feeders (seed fountains) with perches adjacent to them throughout the experiment. All birds were kept in their respective foraging condition for a further 21 days until the end of the training period. All birds in the HF group acclimated to the training condition and therefore, no birds were removed from the study during the training period. Previous studies using this technique have found that HF birds adjusted their foraging behaviour and made significantly more trips to the feeder (Koetsier and Verhulst, 2011; Yap et al., 2017b).

At the end of the training period, HF birds were switched to control conditions and paired for breeding (HF males with HF females, control males with control females), i.e. in ‘common garden’, low foraging effort breeding conditions (i.e. all HF and control birds were given regular feeders). The breeding protocol followed previously described methods (Tissier et al., 2014; Yu et al., 2016), with slight modification. Briefly, birds were paired and housed in individual breeding cages (51×39×43 cm), each with an external nest box (14×14.5×20 cm). During the first breeding attempt, to avoid washing out the effects of high foraging effort, egg food supplement (eggs, breadcrumbs and cornmeal: 20.3% protein, 6.6% lipid) was only provided until the first egg was laid, after which only mixed seed diet was provided to all breeding pairs. Nest boxes were monitored daily between 08:00 h and 14:00 h for eggs laid, and new eggs were weighed (±0.01g) and numbered in consecutive order. Nest boxes were monitored after clutch completion until all the eggs have hatched. On fledging day (21 days of age), chicks were weighed (±0.01 g) and tarsus and wing length were measured (±0.01 mm). Chicks were reared by their parents until they reached 30 days of age, at which time they were separated and maintained in nonbreeding juvenile groups. Once birds could be sexed by the appearance of bill color and sexually dimorphic plumage, they were separated into sex-specific groups. Meanwhile, parents were returned to single-sex cages with regular feeders and were allowed to rest for 60 days, at the end of which they were subjected to the same training protocol again before making a second breeding attempt with the same partner. During the second breeding attempt, instead of breeding in common garden, low foraging effort conditions as in the first attempt, birds were paired and bred in their respective foraging treatment (i.e. HF birds breeding in HF condition, control birds breeding in control condition).

We collected blood samples for physiological measurements at six time points throughout the experiment: (1) prior to the start of the 14 day perch shortening period before the first breeding attempt (pre-training 1); (2) 14 days after complete removal of perches before the first breeding attempt (post-training 1); (3) when the chicks were 21 days old during the first breeding attempt (chick-rearing 1); (4) prior to the start of the 14 day perch shortening period before the second breeding attempt (pre-training 2); (5) 14 days after complete removal of perches before the first breeding attempt (post-training 2); and (6) when the chicks were 21 days old during the second breeding attempt (chick-rearing 2). A summary of the experimental timeline is provided in Fig. 1.

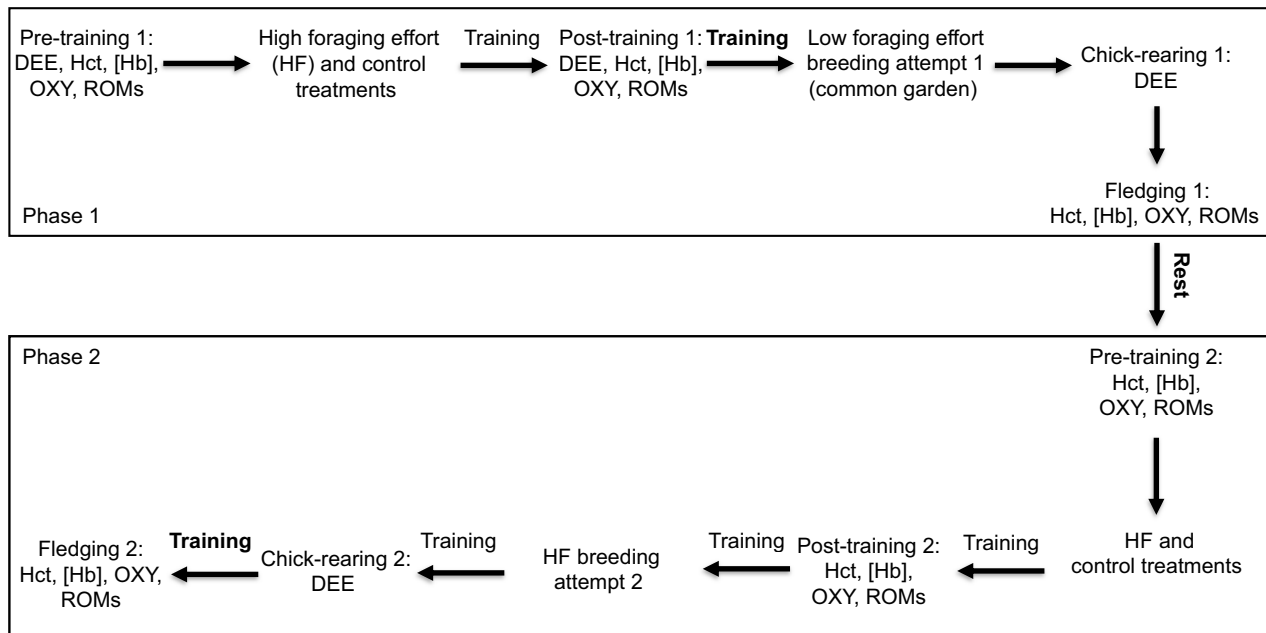


Fig. 1. Summary of the experimental timeline. DEE, daily energy expenditure; Hct, haematocrit; [Hb], haemoglobin concentration; HF, high foraging effort; OXY, total antioxidant capacity; ROMs, reactive oxygen metabolites.

Reproductive success and offspring effect

For both breeding attempts, laying interval (days to lay first egg), clutch size, egg mass, brood size at hatching (BSH), and brood size at fledging (BSF; number of chicks that fledged from hatched eggs) were monitored. Additionally, for both breeding attempts when the chicks were 21 days old, we also measured their body mass, and wing and tarsus length. Then, when the chicks were 90 days old, we collected blood samples for physiological measurements, in addition to morphological trait measurements.

Physiological measurements and assays

All blood samples (~100 μ l) were obtained from the brachial vein following puncture with a 26 G needle and blood was collected using a 75 μ l microhaematocrit tube. Haematocrit (% packed cell volume) was measured with digital callipers (± 0.01 mm) following centrifugation of whole blood for 3 min at 13,700 g (Autocrit Ultra 3; BD Diagnostic Systems, Sparks, MD, USA). [Hb] (g dl⁻¹ whole blood) was measured using the cyanomethaemoglobin method (Drabkin and Austin, 1932) modified for use with a microplate spectrophotometer (BioTek Powerwave 340; Bio-Tek Instruments, Winooski, VT, USA), using 5 μ l whole blood diluted in 1.25 ml Drabkin's reagent (Sigma-Aldrich Canada, Oakville, Ontario, D5941) with absorbance measured at 540 nm. Intra- and inter-assay coefficients were 4.0% (triplicate) and 3.8% ($n=8$), respectively.

Blood samples were also assayed for total antioxidant capacity (μ mol HClO ml⁻¹, OXY) and reactive oxygen metabolites (mg H₂O₂ dl⁻¹; ROMs). All plasma samples were analyzed using a microplate spectrophotometer (BioTek Powerwave X340, Bio-Tek Instruments, Inc., Winooski, VT, USA) and 96-well microplates. Analyses of oxidative stress were carried out according to established protocols as described in Costantini et al. (2011), with slight modification. Specifically, we measured ROMs and OXY using the commercial kits dROMs and OXY Adsorbent Test (Diacron International, Grosseto, Italy) respectively. Intra-assay coefficient for OXY and dROMs were 6.2% (triplicate) and 8.5% (duplicate), respectively. Inter-assay coefficient for OXY and dROMs were 5.9%

($n=11$) and 6.8% ($n=10$), respectively. In addition, we calculated an index of overall index of oxidative stress (OS) by taking the ratio between ROMs and OXY and multiplying it by 1000 ($OS=ROMs/OXY \times 1000$) (Costantini et al., 2008, 2011).

Statistical analyses

Analyses were carried out using R version 0.99.467 (<https://www.r-project.org/>). Data were first examined for normality using Shapiro–Wilk test and non-normally distributed data were log-transformed prior to analysis (but plotted using untransformed values in figures). All measures of reproductive success (i.e. laying interval, clutch size, egg mass, brood size at hatch, brood size at fledging, offspring morphology at 21 days and 90 days of age, and physiological measures of offspring at 90 days of age) were analyzed with breeding attempt and treatment as main effects, female mass as covariate, and individual female ID as a random factor. For analyses of brood size at hatching and fledging, we analyzed the data with failed nests included (i.e. BSH ≥ 0 and BSF ≥ 0) and with failed nests excluded (i.e. BSH > 0 and BSF > 0). Proportions of nest failures were analyzed using Fisher's Exact chi-square test.

To link physiological measures of parents to the observed reproductive performance in each attempt, breeding attempt 1 and breeding attempt 2 were analyzed separately. First, to look at if, and how, training for increased foraging effort affected physiology, and whether the physiological effects of training were similar across both periods of training attempts, we tested the effect of treatment on body mass and all physiological metrics (i.e. Hct, [Hb], OXY, ROMs and OS) using general linear model (GLM) using post-training values as the dependent variable, with treatment as the main effect, and pre-training values the covariate. Sex was initially included in all models but was removed because we did not detect any interactions between sex and other variables ($P > 0.05$ in all cases; Table S1).

To investigate if physiological effects of training persisted through breeding in both breeding attempts, all physiological metrics were analyzed using repeated measure with chick-rearing

data as the dependent variable, sex, time and treatment as main effects, body mass and pre-training values as covariates, and individual bird ID as a random factor. Males and females were analyzed separately if interactions between sex, time and treatment were found. Additionally, sex was removed from the model if there were no interactions between sex, time and treatment. *F*- and *t*-statistics and *P* values were generated using the lmerTest package (<https://cran.r-project.org/web/packages/lmerTest>) and Tukey's HSD (package multcomp; <https://cran.r-project.org/web/packages/multcomp/>; Hothorn et al., 2008) was used to evaluate pairwise comparisons between treatments and breeding attempts following a significant mixed model. We also reported the least-squared means and standard errors of all physiological metrics from all time points in a separate table (Table 1). Repeatability of all physiological metrics was estimated from generalized linear mixed-effects models fitted by restricted maximum likelihood using the rptR package (<https://cran.r-project.org/web/packages/rptR/vignettes/rptR.html>; Stoffel et al., 2017). Repeatability is estimated over all time points and without correcting for any fixed effects. Additionally, detailed statistical output showing all variables and statistical models are presented in Table S1.

RESULTS

Repeatability of physiological traits

Moderate but significant repeatability was found for Hct ($R=0.575$, $P<0.001$, 95% CI=0.447–0.669). Weak but significant repeatability was found for [Hb] ($R=0.174$, $P=0.002$, 95% CI=0.045–0.324). Neither OXY ($R=0.10$, $P=0.051$, 95% CI=0–0.224) nor ROMs ($R=0.013$, $P=0.468$, 95% CI=0–0.143) were repeatable, likely because of the low sample sizes of these measures during the second breeding attempt.

Does training for increased foraging effort affect body mass and physiology?

We first asked how training for increased foraging effort affected body mass and physiology, and whether the physiological responses were consistent across both training phases prior to breeding. Regardless of sex, training had a consistent effect on traits reflecting aerobic capacity: birds upregulated Hct and [Hb] during both the first (Hct: $t_{39}=3.15$, $P<0.01$, Fig. 2B; [Hb]: $t_{30}=2.73$, $P=0.01$, Fig. 2C) and second training phase (Hct: $t_{47}=2.44$, $P=0.02$; Fig. 2B; [Hb]: $t_{49}=3.35$, $P<0.01$; Fig. 2C), but there was no effect of experimental treatment on body mass ($P>0.30$, Fig. 2A).

Training did not affect total antioxidant capacity of birds prior to their first breeding attempt ($t_{38}=0.35$, $P=0.72$, Fig. 2D) but HF birds had significantly lower post-training total antioxidant capacity prior to their second breeding attempt ($t_{46}=-2.24$, $P=0.03$, Fig. 2D). In contrast, plasma reactive oxygen metabolites were higher when birds were subjected to training prior to their first breeding attempt ($t_{25}=3.06$, $P<0.01$, Fig. 2E) but did not change in response to a second bout of training prior to their second breeding attempt ($t_{29}=1.32$, $P=0.20$, Fig. 2E). Therefore, overall, oxidative stress was consistently higher in birds subjected to training prior to both breeding attempts (first attempt: $t_{24}=2.44$, $P=0.02$, Fig. 2F; second attempt: $t_{25}=2.27$, $P=0.03$, Fig. 2F) but for different reasons. These findings were not affected by the sex of the birds (i.e. there were no interactions between sex, time, and treatment for measures of oxidative stress; $P>0.05$ in all cases, Table S1).

Do physiological effects of training for increased foraging effort persist through reproduction?

Having established that training for high foraging effort did affect physiological state we then asked how post-training state was

Table 1. Zebra finch measurements of body mass, Hct, Hb, OXY, ROMs and OS values for all 6 timepoints.

	Phase 1						Phase 2					
	Pre-training 1		Post-training 1		Chick-rearing 1		Pre-training 2		Post-training 2		Chick-rearing 2	
	Control	HF	Control	HF	Control	HF	Control	HF	Control	HF	Control	HF
Mass (g)	15.56±0.28	15.69±0.28	15.29±0.26	15.01±0.23	13.28±0.37	13.23±0.29	15.54±0.27	14.62±0.26	15.91±0.27	14.81±0.26	14.28±0.47	14.72±0.48
Hct (%)	50.55±1.19	50.47±1.13	50.09±1.13	52.06±0.94	49.01±1.47	50.81±1.18	51.76±1.18	52.64±1.01	50.76±1.19	54.89±1.04	48.33±1.80	48.47±1.85
Hb (g dl ⁻¹)	14.81±0.57	14.72±0.56	14.10±0.44	15.28±0.37	14.14±0.57	14.58±0.46	12.90±0.47	11.72±0.42	12.76±0.48	13.79±0.43	13.10±0.70	13.56±0.88
OXY (nmol l ⁻¹ HOCl neutralized)	203.88±5.66	195.88±6.02	184.15±4.84	184.57±4.26	193.51±6.87	207.97±5.46	182.89±5.17	196.69±5.12	203.62±5.27	186.29±5.02	218.57±8.16	194.92±10.39
log ROMs (mmol l ⁻¹ H ₂ O ₂ equivalent)	0.42±0.05	0.45±0.05	0.43±0.04	0.50±0.04	0.26±0.06	0.28±0.05	0.51±0.05	0.56±0.04	0.40±0.05	0.49±0.04	0.30±0.07	0.27±0.08
log OS (ROMs×1000/OXY)	1.10±0.05	1.16±0.05	1.16±0.04	1.23±0.04	0.96±0.06	0.95±0.05	1.24±0.05	1.28±0.04	1.10±0.05	1.23±0.04	0.98±0.07	0.99±0.08

Data shown are least-squared means±s.e.m.

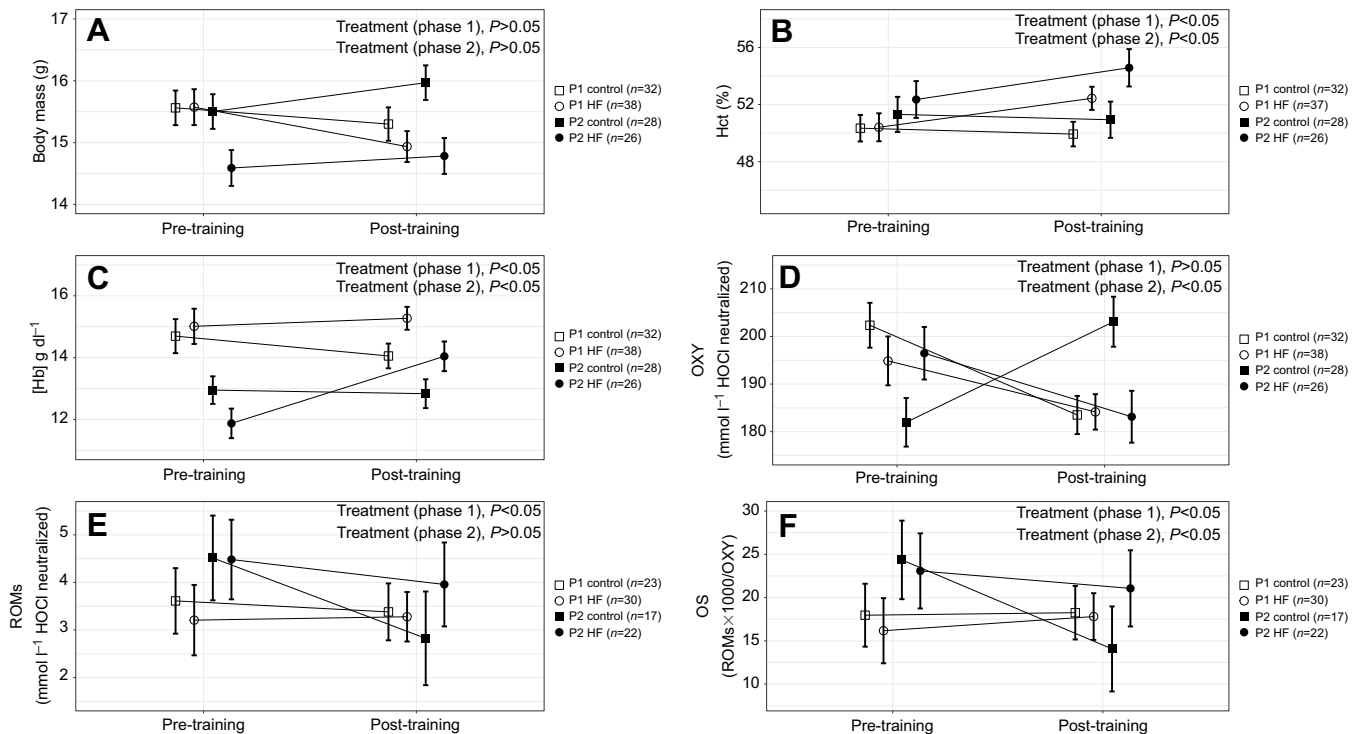


Fig. 2. Effects of training for increased foraging effort on zebra finch (*Taeniopygia guttata*) physiology during both training phases prior to breeding. (A) Body mass. (B) Hct. (C) [Hb]. (G) OXY. (H) ROMs. (I) Overall index of oxidative stress (OS). Data shown are least-squared means \pm s.e.m. Note that ROMs and OS were analyzed using log-transformed values but plotted using untransformed values. Circles and triangles represent control and high foraging effort (HF) birds, respectively. P1, phase 1; P2, phase 2.

affected by subsequent reproduction. Regardless of sex and experimental treatment, birds decreased body mass from post-training to chick-rearing (first attempt: $F_{1,20}=48.83$, $P<0.01$; second attempt: $F_{1,11}=22.77$, $P<0.01$, Fig. 3A). Despite observing a training-induced increase in Hct and [Hb], both Hct (first attempt: $F_{1,19}=7.84$, $P=0.01$; second attempt: $F_{1,10}=1.71$, $P=0.22$; Fig. 3B) and [Hb] (first attempt: $F_{1,14}=6.15$, $P=0.02$; second attempt: $F_{1,10}=4.94$, $P=0.05$, Fig. 3C) were not different between treatment groups during chick-rearing at the end of both reproductive attempts, even when HF birds were maintained in HF conditions during breeding in the second breeding attempt.

Total antioxidant capacity of both sexes was not affected by reproduction in the first breeding attempt ($F_{1,16}=1.45$, $P=0.25$, Fig. 3D). Training resulted in lower total antioxidant capacity and total antioxidant capacity stayed low until the end of reproduction in the second breeding attempt ($F_{1,45}=4.69$, $P=0.03$, Fig. 3D). Although training increased ROMs, ROMs were not different in relation to training in chick-rearing birds at the end of both reproductive attempts (first attempt: $F_{1,14}=6.15$, $P=0.02$; second attempt: $F_{1,10}=4.93$, $P=0.05$, Fig. 3E), even when HF birds were maintained in HF conditions during breeding in the second breeding attempt. Overall oxidative stress remained the same from post-training to the end of reproductive bout in the first attempt ($F_{1,7}=3.44$, $P=0.10$, Fig. 3F). However, overall oxidative stress was higher in HF birds at post-training but was similar between both treatment groups during chick-rearing at the end of the second reproductive attempt ($F_{1,2}=29.60$, $P=0.03$, Fig. 3F), even though they were still being kept in HF condition during breeding. Findings pertaining to changes in oxidative stress (or lack thereof) were not affected by the sex of the birds ($P>0.05$ in all cases, Table S1).

Do physiological adjustments to training affect reproductive output?

Finally, we asked how post-training physiological state affected reproductive performance under ‘common garden’ low foraging effort conditions and high foraging effort conditions. When birds were trained and bred in HF conditions, laying interval was similar to that in birds in control conditions ($F_{1,9}=3.90$, $P=0.08$, Fig. 4A). Birds that were trained and bred in HF conditions had lower fecundity: lower egg mass ($F_{1,244}=5.38$, $P=0.02$; Fig. 4B) and smaller clutch size ($F_{1,8}=11.82$, $P=0.01$; Fig. 4C). During the first breeding attempt when birds were bred under common garden conditions, nest failure rate was significantly higher in the control group than HF group ($P=0.046$). Specifically, 42.86% (6 of 14) of control nests and 23.53% (4 of 17) of HF nests failed (i.e. birds laid eggs and hatched chicks but failed to fledge chicks). During the second breeding attempt, when birds were trained and bred in HF conditions, nest failure rate was not significantly different between the two groups ($P=0.99$). Specifically, 45.45% (5 of 11) of control nests and 50% (3 of 6) of HF nests failed. Despite showing differences in fecundity, final reproductive output, including both BSH ($F_{1,8}=0.25$, $P=0.63$, Fig. 4D) and BSF ($F_{1,9}=0.93$, $P=0.36$, Fig. 4E) were not affected by training for increased foraging effort. These findings hold true even when failed nests were excluded from our analyses (BSH: $F_{1,4}=1.37$, $P=0.31$; BSF: $F_{1,2}=0.16$, $P=0.73$).

Do physiological adjustments to training affect offspring's morphology and physiology?

We did not detect any difference in body mass and tarsus length of fledglings at 21 days of age in relation to parental training treatment in either breeding attempts ($P>0.15$ for all, Fig. 5A,B). However,

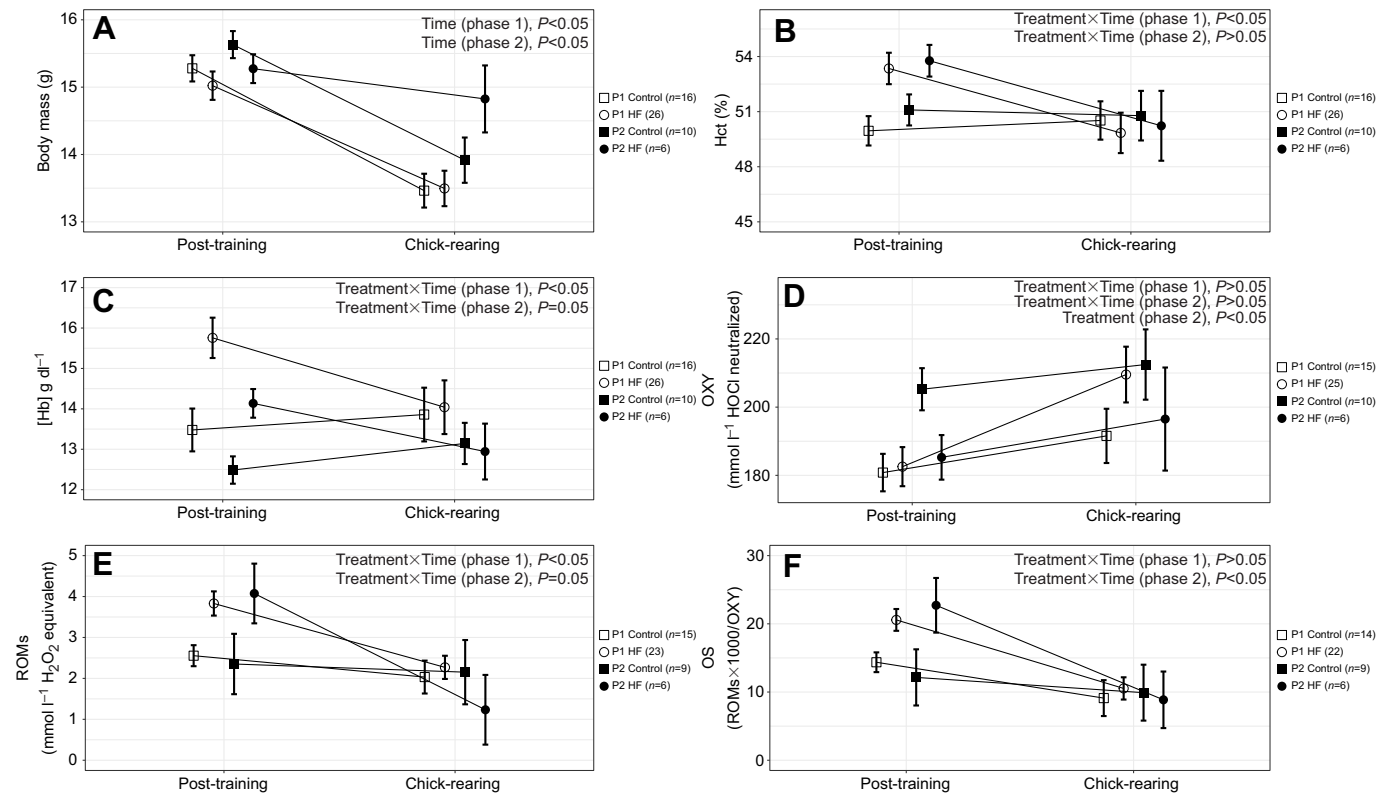


Fig. 3. Adjustments of physiological characteristics of zebra finch from post-training to chick-rearing in both breeding attempts. (A) Body mass. (B) Hct. (C) [Hb]. (G) OXY. (H) ROMs. (I) OS. Data shown are least-squared means \pm s.e.m. Note that ROMs and OS were analyzed using log-transformed values but plotted using untransformed values. Circles and triangles represent control and HF birds, respectively.

fledglings from HF parents at 21 days of age had shorter wing length in both breeding attempts ($F_{1,22}=5.79$, $P=0.02$, Fig. 5C).

Similarly, chicks from both parental training treatment groups had similar body mass ($F_{1,32}=1.17$, $P=0.29$, Fig. 5D), tarsus length ($F_{1,32}=0.05$, $P=0.82$, Fig. 5E) and wing length ($F_{1,32}=0.04$, $P=0.85$, Fig. 5F) at 90 days of age. At 90 days of age, with the exception of higher reactive oxygen metabolites production in chicks from HF parents in the second breeding attempt ($F_{1,24}=12.83$, $P=0.002$, Fig. 5J), we did not detect any difference in other physiological traits, including Hct ($F_{1,31}=3.19$, $P=0.08$, Fig. 5G), [Hb] ($F_{1,32}=0.34$, $P=0.56$, Fig. 5H) and OXY ($F_{1,24}=1.66$, $P=0.21$, Fig. 5I).

DISCUSSION

We experimentally manipulated foraging behaviour and workload in zebra finches using a previously described technique (Koetsier and Verhulst, 2011; Yap et al., 2017b) and investigated how physiological adjustments to ‘exercise’ (*sensu* Halsey, 2016) affected subsequent reproductive performance and offspring quality. We trained birds to high foraging effort twice, with an intervening reproductive attempt prior to the second training period. Training-induced physiological responses were mostly consistent across both training phases, with HF birds exhibiting increases in Hct, [Hb] and oxidative stress. However, these physiological changes did not carry over through reproduction. Additionally, breeding under high foraging effort condition negatively impacted fecundity but not final reproductive output.

We found no effect of training on body mass, confirming results of a previous study (Yap et al., 2017b; but see Briga et al., 2017). However, unlike Yap et al. (2017b), we found that birds increased Hct and [Hb] in response to training, providing evidence for

physiological responses to training. The discrepancies in findings despite both studies employing identical training technique could be due to a difference in timing of Hct and [Hb] measurements (day 3 post-training in Yap et al., 2017b versus day 14 post-training in the current study). This suggests that 3 days of training might not be sufficient to cause upregulation of Hct and [Hb], as physiological processes such as erythropoiesis typically take place over several days (Rosse and Waldmann, 1966; Williams et al., 2012). Training affected antioxidant capacity and reactive oxygen metabolite production differently in the two, repeated training phases, although overall oxidative stress was consistently higher in trained birds prior to both breeding attempts, which is similar to the results of Yap et al. (2017b). Previous studies suggested that antioxidant capacity and reactive oxygen metabolite production are not necessarily coupled (Costantini and Verhulst, 2009; Skrip and McWilliams, 2016) and that there is not always repeatability of antioxidant capacity over time, although there is substantial repeatability in overall oxidative stress (Beamonte-Barrientos and Verhulst, 2013). These findings together indicated that it is important to look at both antioxidant capacity and reactive oxygen metabolites together when evaluating oxidative stress, rather than a simple ratio of these two metrics.

Most of the physiological adjustments in response to training did not persist through reproduction. Interestingly, despite not being affected by training, body mass of birds decreased from post-training to chick-rearing, suggesting an effect of reproduction and/or chick-rearing on mass that is independent of training. Although post-training Hct and [Hb] were higher in HF birds, these traits returned to pre-training level at fledging in the first breeding attempt, when birds were bred in low foraging effort conditions.

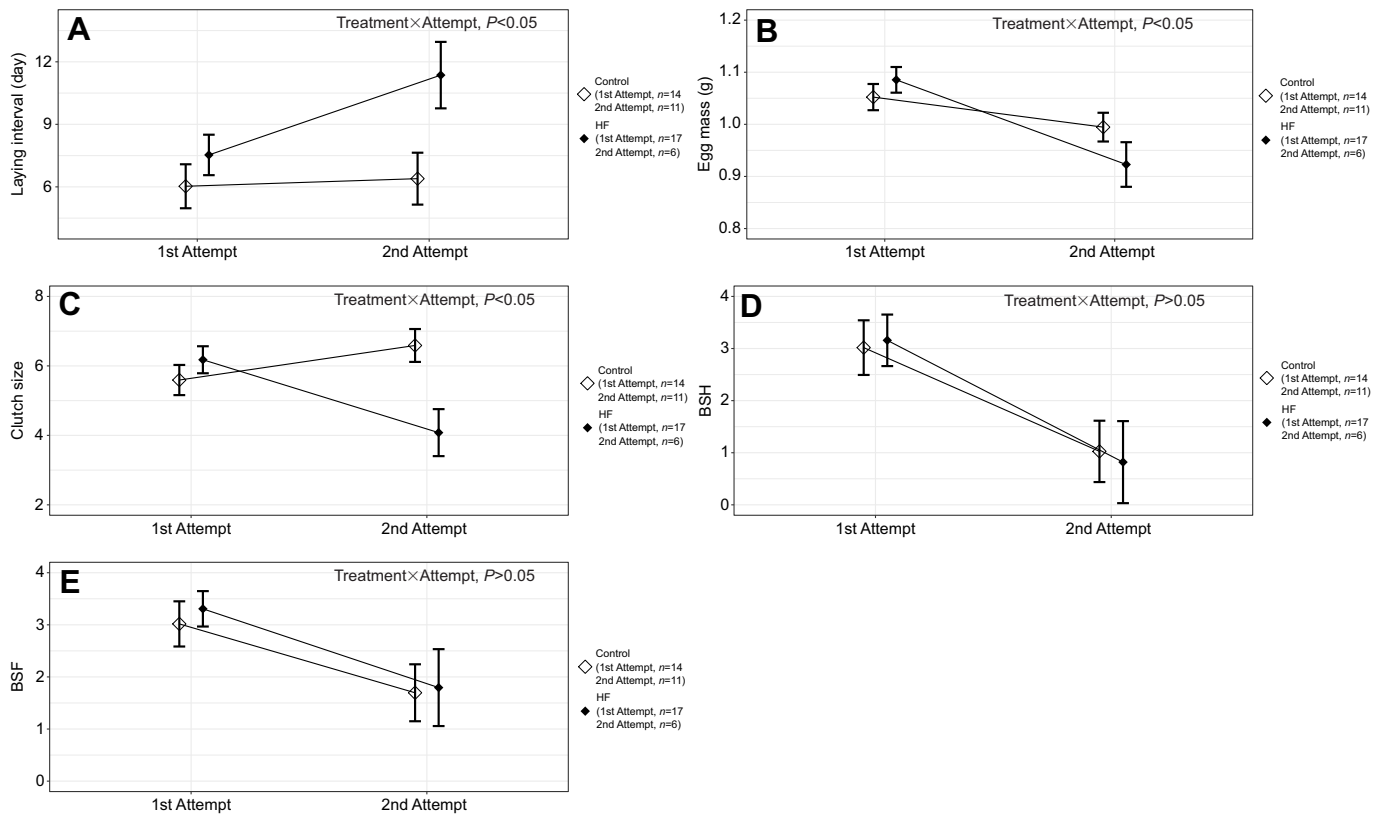


Fig. 4. Effects of increased foraging effort on reproductive output in zebra finch in both breeding attempts. (A) Laying interval. (B) Egg mass. (C) Clutch size. (D) Brood size at hatching (BSH). (E) Brood size at fledging (BSF). Data shown are least-squared means \pm s.e.m. Open diamonds represent control birds, while filled diamonds represent HF birds.

This suggests that high Hct and [Hb] were required to sustain the high workload of increased foraging costs, but the high levels were no longer maintained when foraging conditions become easier. However, Hct and [Hb] also returned to pre-training levels at fledging in the second attempt, even though birds were still being kept in the HF condition. Downregulation of Hct and [Hb] in this case possibly represents a cost of high workload, where birds could no longer maintain high Hct and [Hb] when the combined effort of high foraging costs and reproduction (parental care) became too high and maintaining energy balance became more difficult. In contrast, the effect of training on antioxidant capacity persisted until the chicks fledged. Reproduction did not affect total antioxidant capacity in the first attempt, likely because birds were breeding in relatively easy (i.e. low foraging effort) conditions. It should be noted that although HF birds had lower antioxidant capacity relative to control birds in the second breeding attempt, this was mostly due to increased antioxidant capacity in control birds. This suggests that the observed higher antioxidant capacity in control birds was due to the effects of oxidative shielding (Blount et al., 2016; Naviaux, 2012; Viblanc et al., 2018) in the first attempt being carried over to the second attempt. HF birds were not able to maintain high antioxidant capacity because they were breeding in hard (i.e. high foraging effort) conditions throughout the second attempt. Similarly to other studies that demonstrated that animals tend to upregulate antioxidant defense during reproduction to minimize oxidative stress (i.e. oxidative shielding) (Blount et al., 2016; Naviaux, 2012; Viblanc et al., 2018), our study showed that oxidative stress was not affected by reproduction or the combined effort of training and reproduction in the case of the second breeding attempt.

As expected, the two breeding regimes (i.e. breeding under low foraging effort 'common garden' condition versus breeding under high foraging effort condition) yielded different results regarding reproductive output of birds. Training for increased foraging effort did not affect subsequent reproduction in the first breeding attempt. Although some studies investigating the effects of increased workload on reproduction generally found a delay in the timing of reproduction (Deerenberg and Overkamp, 1999; Simons et al., 2014; Wiersma, 2005), many other studies failed to find evidence of impaired reproduction due to increased workload (Schmidt-Wellenburg et al., 2008; Tomotani et al., 2018). Additionally, some studies also found that increased workload during reproduction did not affect final reproductive output, but negatively impact body condition of the parents (Casagrande and Hau, 2018; Fletcher et al., 2013). A few studies in rodents even found positive effects of high activity level on reproduction (Vega et al., 2015; Zhang et al., 2018b), which is possibly due to mitochondrial hormesis (Zhang and Hood, 2016; Zhang et al., 2018c). The absence of a treatment (i.e. training) effect on reproductive output in the first breeding attempt is not surprising considering that most of the time, trade-offs and 'costs of reproduction' can only be detected when environmental conditions are poor (Stearns, 1989, 1992).

Contrary to the findings in the first breeding attempt and unlike other studies that showed no effects of increased workload on reproduction (Schmidt-Wellenburg et al., 2008; Tomotani et al., 2018), findings from the second breeding attempt of the current experiment showed reduced fecundity (i.e. smaller clutch size and egg mass) when birds were subjected to increased foraging effort

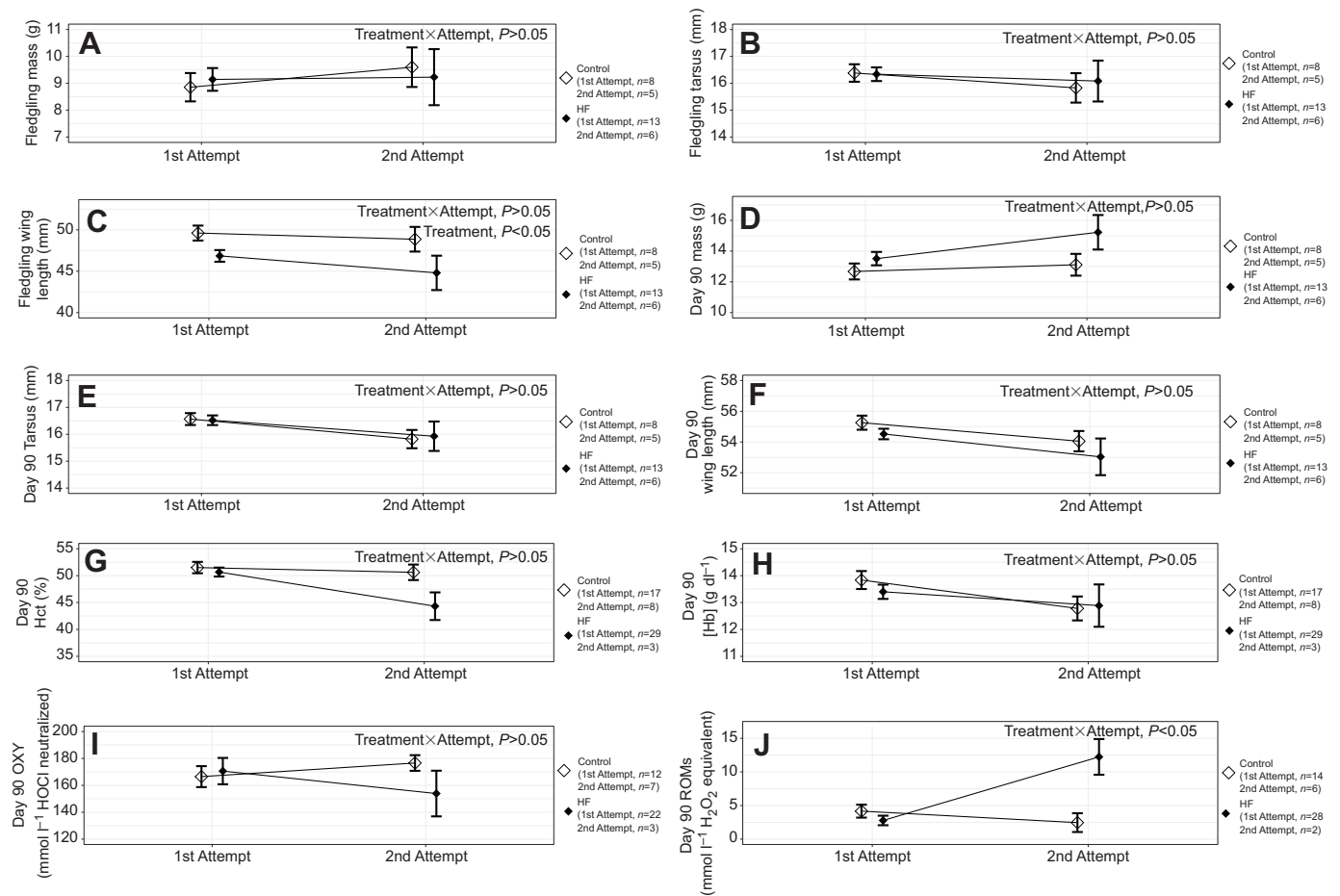


Fig. 5. Effects of increased foraging effort on zebra finch offspring morphology and physiology. (A) Mass, (B) tarsus length and (C) wing length at 21 days of age. (D) Mass, (E) tarsus length, (F) wing length, (G) Hct, (H) [Hb] (I) OXY and (J) ROMs at 90 days of age. Data shown in are least-squared means \pm s.e.m. Note that ROMs and OS were analyzed using log-transformed values but plotted using untransformed values. Open diamonds represent control birds, while filled diamonds represent HF birds.

and breeding at the same time. Despite finding lower fecundity in birds subjected to increased foraging effort and breeding at the same time, we did not find any evidence for a reduction in final reproductive output. Our finding of reduced fecundity is similar to results from a previous study by Briga (2016), where birds exposed to long-term high foraging costs had smaller clutch sizes. However, contrary to findings from the present study, Briga (2016) found that high workload during reproduction reduced brood sizes, fledgling numbers and post fledging survival. The disparity in findings could be explained by individual parents optimizing reproductive investment by investing fewer resources in egg production and more resources for chick rearing (Linhares et al., 2014; Schwarzkopf and Andrews, 2012; Williams, 2012). However, we did have some evidence that offspring produced by HF parents in the second breeding attempt were of lower quality, as indicated by the higher oxidative stress observed when they were 90 days old, although other indicators of quality including multiple morphological traits and haematology were not significantly different between offspring of HF parents and control parents. It should be noted that many of the metrics for reproductive success such as BSH, chick mass and BSF are lower than previously reported in the same species from the same colony (Tissier et al., 2014; Yu et al., 2016), probably due to a lack of egg food supplementation during breeding. However, the values observed in our study are similar to other studies in which captive

zebra finches reproduced under poor diet quality conditions (i.e. mixed seed diet only) (Criscuolo et al., 2011; Griffith et al., 2017).

In summary, our study has shown that birds exhibited consistent physiological adjustments to training (e.g. increased Hct and [Hb]), but these physiological responses were subsequently affected by reproduction (e.g. decreased Hct and [Hb]), even when birds were maintained in high foraging effort conditions. Findings from our study also suggested that experimentally increased workload during reproduction can lead to physiological costs in the form of increased oxidative stress, potentially to a high enough level to negatively affect reproductive performance, as evident from the lower fecundity observed in HF birds in the second breeding attempt, as well as the poorer offspring quality produced by HF parents in the second breeding attempt. It is unclear whether training would confer any benefits to subsequent reproduction, and whether reproduction can in turn modulate any subsequent response to training. Some studies have suggested that moderate increases in ROS due to increased activity level should lead to increased respiratory capacity of tissues and physiological functions, and consequently improved reproductive performance (Zhang and Hood, 2016; Zhang et al., 2018b,c). Although our study did not document any positive effects of training on reproduction, future studies should repeat the training and breeding protocol described above and investigate whether different levels of activity (i.e. shorter duration, lower training

intensity) would affect subsequent reproduction differently, as well as whether reproduction can in turn modulate any subsequent physiological responses to training.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.N.Y., T.D.W.; Validation: K.N.Y.; Formal analysis: K.N.Y., M.L.V.; Investigation: K.N.Y., D.R.P., M.L.V., O.T.; Writing - original draft: K.N.Y.; Project administration: K.N.Y., T.D.W.

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