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Making Olympic lizards: the effects of specialised exercise training on lizard performance

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ABSTRACT

Exercise training is well known to affect a suite of physiological and performance traits in mammals, but effects of training in other vertebrate tetrapod groups have been inconsistent. We examined performance and physiological differences among green anole lizards (*Anolis carolinensis*) that were trained for sprinting or endurance, using an increasingly rigorous training regimen over 8 weeks. Lizards trained for endurance had significantly higher post-training endurance capacity compared to the other treatment groups, but groups did not show post-training differences in sprint speed. Although acclimation to the laboratory environment and training explain some of our results, mechanistic explanations for these results correspond with the observed performance differences. After training, endurance-trained lizards had higher hematocrit and larger fast glycolytic muscle fibres. Despite no detectable change in maximal performance of sprint-trained lizards, we detected that they had significantly larger slow oxidative muscle fibre areas compared to the other treatments. Treatment groups did not differ in the proportion of number of fibre types, nor in the mass of most limb muscles or the heart. Our results offer some caveats for investigators conducting training research on non-model organisms, and they reveal that muscle plasticity in response to training may be widespread phylogenetically.

KEY WORDS: Endurance, Exercise, Lizard, Locomotion, Performance, Sprint speed

INTRODUCTION

Exercise is generally defined as physical activity that increases contraction of skeletal muscle and energy expenditure above resting levels, resulting in improved performance that depends on the intensity and frequency of exercise (Booth et al., 2012). Exercise, and the molecular and physiological changes that follow, is simple to envision in the context of human activity, and hence has served as the basis for a huge literature on plasticity in skeletal muscle physiology (e.g., Trappe et al., 2006; Yan et al., 2011; Wilson, 2013), largely because of the numerous health benefits exercise has for humans (Deslandes et al., 2009; Hawley and Holloszy, 2009; van Praag, 2009; Church, 2011). Research on non-human mammals has added to our general understanding of how exercise training affects muscle and cardiovascular tissue (e.g., Gollnick and King, 1969; Guy and Snow, 1977; Constable et al., 1987; Evans and Rose, 1988; Bebout et al., 1993; Kim et al., 2013). Nevertheless, research on 'exercise' in non-human, especially non-mammalian, vertebrates has lagged behind with regards to how exercise might alter the phenotype and influence the evolution of morphology and performance (Garland et al., 1987; O'Connor et al., 2011; Sinclair et al., 2013; Lailvaux and Husak, 2014). Recent theoretical approaches (Nathan *et al.* 2008) and empirical data (Palstra and Planas, 2013; He et al., 2013), though, suggest that non-human animals are not likely fundamentally different in how skeletal muscle is altered by increased use. Thus, a fuller understanding of how physical performance can be altered by the intensity and frequency of use can give great insight into how selection might operate on performance and what the evolutionary response might be.

Locomotion has long been viewed as an important contributor to survival and reproductive success in a variety of animal taxa (Dickinson et al., 2000; Irschick and Garland,

2001; Husak and Fox, 2008), and recent ecomorphological research gives maximal locomotor abilities a central role as a target for selection (Arnold, 1983; Husak et al., 2006; Lailvaux and Irschick, 2006; Irschick et al., 2007; Husak and Fox, 2008; Byers et al., 2009). Maximal locomotor capacities seem intuitively essential during a high-speed chase between predator and prey, for example, and some studies have detected selection on maximal locomotor capacities in nature (reviewed in Irschick et al., 2008), but it is unclear how common such scenarios actually are for free-living individuals (Wilson et al., 2013), how often animals actually use maximal capacities (Irschick and Losos, 1999; Husak and Fox, 2006), and whether frequent use in these or other contexts alters muscle physiology to increase performance in subsequent situations that may impact fitness. In humans, both endurance running and sprint training can result in dramatic changes to skeletal muscle organization, from gene expression (Wang et al., 2004; Wilson, 2013) to contractile properties, hypertrophy, and power output (Trappe et al., 2006; Harber and Trappe, 2008; Lundberg et al., 2013). There are several studies on exercise effects in non-mammalian tetrapods demonstrating that exercise training can enhance endurance capacity in amphibians (Cummings, 1979; Miller and Camilliere, 1981), crocodilians (Owerkowicz and Baudinette, 2008; Eme et al., 2009) and birds (Gaunt et al., 1990), but such studies have been conducted on only a few very distantly related species. This, combined with equivocal results in some studies, makes it difficult to understand the origin and adaptive significance of skeletal muscle plasticity. Thus, studies of exercise in non-mammalian vertebrate species in which locomotion is an integral part of their daily activities will reveal if training effects are universal and potentially important to fitness. We studied the effects of endurance and sprint exercise in lizards, a group in which locomotion and the fitness consequences of poor locomotor performance have been well

studied (Le Galliard et al., 2004; Miles, 2004; Husak et al., 2006; Calsbeek and Irschick, 2007; John Alder et al., 2009).

Previous studies on lizard exercise were unable to demonstrate successful training of lizards to increase endurance capacity, and the reasons are likely varied. Gleeson (1979) found that 6-8 weeks of increasingly intense training in the iguanid lizard *Sceloporus occidentalis* had no significant effects on running performance or its presumed metabolic correlates. While these results were attributed to a potential fundamental difference between mammalian and saurian metabolic flexibility, Garland et al. (1987) pointed out that the training regimen used was likely not intense enough above baseline movement patterns in nature to elicit a response. Garland et al. (1987) compared *Amphibolorus nuchalis* lizards trained five days a week for eight weeks (with increased intensity) on a treadmill to a sedentary control group and found no differential effect on endurance. There was, however, evidence of joint degradation after the very strenuous training regimen, which the authors speculated was an effect of overly excessive training. O'Connor et al. (2011) used a training regimen that included training for treadmill endurance, maximum run time on a circular track, and maximum burst speed, finding no training effect on any one of the performance traits. Most likely, the simultaneous combination of both types of exercise resulted in a lack of locomotor specialisation due to interference in the cellular pathways involved with protein turnover in the types of exercise, as is the case in mammals (Coffey et al., 2009a, 2009b). Although these studies found no training effects on locomotor performance in lizards, because of the reasons described above, it is also possible that lizards simply do not respond to exercise as fishes and mammals do (e.g., Davison, 1997; Palstra and Planas, 2013; He et al., 2013). We used these previous studies as a guide to help design our training regimen to maximize the likelihood of success. Thus, we designed a training regimen that focused a lizard's

training specifically on either sprinting or endurance (or control), and the frequency of training was roughly intermediate to previous studies and with modest increases in intensity over the course of the experiment.

We conducted an experimental study of specialised exercise training in the green anole lizard (*Anolis carolinensis*) to test whether sprinting or endurance performance could be improved by training and what traits might be associated with any such performance enhancement. Although there are no selection studies in the wild to confirm that sprinting and endurance are important to individual fitness, green anoles use sprinting for predator escape and slow, sustained locomotion for territory patrolling and foraging (Jenssen et al., 1995; Irschick and Losos, 1996; Irschick et al., 2005). Thus, exercise in the form of sprinting and endurance are likely ecologically relevant to green anoles. By modifying the approach of previous training studies, we hoped to answer three main questions. First, will endurance and sprint training enhance those performance traits? Second, if there are performance enhancements, is the change due to muscle fibre hypertrophy or a shift in proportion of fibre types? Third, how does training alter other potential predictors of performance, such as blood hematocrit levels and limb muscle and heart masses? We expected to find significant changes in performance in both the sprinting and endurance groups. We predicted to see hypertrophy of fast glycolytic fibres in muscles of the sprint-trained lizards, resulting in increased muscle masses, compared to the endurance lizards, which we predicted would have higher proportions of slow oxidative fibres. Additionally, we hypothesized that the endurance group would have greater heart mass and blood hematocrit levels than the sprint-trained groups.

RESULTS

Paired t-tests revealed that endurance increased for all treatments (control: t = 3.85, P = 0.005; sprint-trained: t = 4.84, P = 0.003; endurance-trained: t = 3.25, P = 0.01), but the magnitude of increase was highest in the endurance trained group (Fig. 1). Sprint speed did not significantly change for any of the treatments (control: t = -0.30, P = 0.77; sprint-trained: t = -1.18, P = 0.28; endurance-trained: t = -2.22, P = 0.06). Results were similar when considering only post-training data. Sprint speed did not differ among treatments ($F_{2, 21}$ = 0.62, P = 0.55), but endurance did ($F_{2, 21}$ = 6.97, P = 0.005). Tukey's HSD comparisons revealed that endurance-trained individuals had greater endurance capacity than both sprint-trained (P = 0.04) and control individuals (P = 0.005), and sprint-trained and controls did not statistically differ from each other in endurance (P = 0.73; Fig. 1). There was no statistical difference in SVL ($F_{2, 21}$ = 253, P = 0.10) or mass ($F_{2, 21}$ = 0.70, P = 0.51) among treatments. Hematocrit differed among treatments ($F_{2, 21}$ = 4.74, P = 0.02), with endurance-trained individuals having significantly higher hematocrit than controls (P = 0.02) and a trend toward higher hematocrit than sprint-trained lizards (P = 0.06). Controls did not differ statistically from sprint-trained lizards in hematocrit (P = 0.97). There were no other statistical differences in hematocrit among treatments (P > 0.1 for all; Fig. 2). Forelimb length did not differ among treatments (ANCOVA: interaction, $F_{2, 18}$ = 0.90, P = 0.42; treatment, $F_{2, 20}$ = 0.61, P = 0.55), nor did hindlimb length (ANCOVA: interaction, $F_{2, 18}$ = 2.24, P = 0.14; treatment, $F_{2, 20}$ = 0.16, P = 0.86).

Of the eight muscles weighed, only the biceps showed a significant difference among treatments (ANCOVA: interaction, $F_{2, 18}$ = 0.18, P = 0.84; treatment, $F_{2, 20}$ = 10.89, P = 0.0006), with control and endurance-trained individuals not statistically differing (P = 0.22), but both endurance-trained (P = 0.02) and control (P = 0.0004) individuals having larger biceps than

sprint-trained individuals (Table 1). Heart mass did not differ among treatments (ANCOVA: interaction, $F_{2, 18} = 0.99$, $P = 0.39$; treatment, $F_{2, 20} = 1.38$, $P = 0.28$).

We characterized fibres as one of three fibre types on the basis of their staining characteristics (Bonine et al., 2001): fast glycolytic (FG), fast oxidative glycolytic (FOG), or slow oxidative (SO). The proportion of the number of muscle fibre types did not differ among treatment groups in the biceps (FG: $F_{2, 21} = 0.74$, $P= 0.49$; FOG: $F_{2, 21} = 0.58$, $P = 0.57$; SO: $F_{2, 21} = 1.89$, $P = 0.18$), gastrocnemius (FG: $F_{2, 21} = 1.96$, $P = 0.17$; FOG: $F_{2, 21} = 1.62$, $P = 0.22$; SO: $F_{2, 21} = 2.87$, $P = 0.08$), or iliofibularis (FG: $F_{2, 21} = 1.68$, $P = 0.21$; FOG: $F_{2, 21} = 1.98$, $P = 0.16$; SO: $F_{2, 21} = 3.34$, $P = 0.06$). However, there were significant differences among groups in fibre cross-sectional area for two of the three muscles examined (Table 2). For the iliofibularis there were differences in FG and SO FCSA, but not FOG. The sprint-trained group had larger FG fibres than the endurance-trained ($P = 0.0003$) and control ($P = 0.003$) groups (endurance-trained and control groups did not differ in FG fibres; $P = 0.44$), whereas the endurance trained group had larger SO fibres than the sprint-trained ($P = 0.003$) and control ($P = 0.002$) groups (sprint-trained and control groups did not differ in SO fibres; $P = 0.99$). For the gastrocnemius (Fig. 3) there were differences in FG and SO fibre CSA, but not FOG. The sprint-trained group had larger FG fibres than the endurance-trained ($P = 0.0003$) and control ($P = 0.004$) groups (endurance-trained and control groups did not differ in FG fibres; $P = 0.46$), whereas the endurance trained group had larger SO fibres than the sprint-trained ($P = 0.005$) and control ($P = 0.005$) groups (sprint-trained and control groups did not differ in SO fibres; $P = 0.97$). For the biceps there were no differences in FCSA for FG, FOG, or SO fibres.

DISCUSSION

Phenotypic changes as a result of exercise training are well established in humans, yet these effects are poorly studied in non-mammalian tetrapods. Our results show that specialised training in green anole lizards can result in dramatic improvements in performance, but only for endurance-trained individuals. These performance enhancements were associated with changes in morphology and physiology. Even in the sprint-trained group, which did not show a significant improvement in maximal sprint speed, there were detectable changes in the muscle fibres. Although we found changes in fibre cross-sectional area specific to the type of training, we found no detectable differences in fibre type proportions (in terms of numbers) related to type of training. The diversity of responses to specialised training we observed are similar to those seen in humans and other mammals, as well as fishes, and represent the first example to our knowledge of training effects in a non-mammalian tetrapod. Indeed, previous attempts to train lizards failed to detect enhancement of performance or physiology (Gleeson, 1979; Garland et al., 1987; O'Connor et al., 2011). However, we had the benefit of designing our training regimen based on those previous studies, finding the appropriate intensity and frequency to elicit a response. Our findings suggest that performance enhancement and at least some aspects of physiological plasticity following exercise may be conserved across vertebrates.

The nearly 300% increase in endurance capacity, on average, was likely due to a number of changes during training. Our results cannot conclusively determine the precise mechanisms leading to increased endurance capacity, but the differences in endurance were associated with corresponding differences in hematocrit and SO fibre CSA in two key locomotor muscles. The higher hematocrit in endurance-trained lizards is consistent in its directional change to studies of humans and other mammals (reviewed in Bexfield et al., 2009; Connes et al., 2013), as well as

190 birds (Riera et al., 1983), crocodilians (Eme et al., 2009), and fishes (Beamish, 1978), and
191 locomotor endurance in lizards is related to hematocrit (Garland, 1984; Garland and Else, 1987).
192 The increased oxygen-carrying capacity that comes with higher hematocrit may explain a
193 significant portion of the increase in endurance. The role of the other correspondingly different
194 variables is less clear. The hypertrophic effects of aerobic training have been equivocal and
195 somewhat controversial (Harber et al., 2012; Lundberg et al., 2013), but several studies have
196 shown increased muscle mass and increased slow oxidative fibre size after aerobic exercise
197 training in mammals, including humans (Schwartz et al., 1991; Schluter and Fitts, 1994; Trappe
198 et al., 2006; Harber and Trappe, 2008; Harber et al., 2009; Konopka et al., 2010; Lovell et al.,
199 2010; Harber et al., 2012). However, while FCSA certainly contributes to force production, it is
200 less clear how a larger FCSA might increase endurance. The relatively small proportional
201 increase in FCSA for slow oxidative fibres in the iliofibularis (2.6%) and gastrocnemius (2.4%)
202 may be a byproduct of the training and not a causal factor in increasing endurance, and most
203 likely does not explain the dramatic increase in endurance capacity for the endurance-trained
204 group. The slightly greater habituation to handling, since training involved the same process used
205 to measure endurance, may have added to the differences in endurance among groups, but this
206 effect cannot account for the other differences seen among groups. There were undoubtedly
207 unmeasured molecular and metabolic changes to endurance-trained lizards that resulted in
208 increased endurance. For example, in the frog *Rana pipiens* increased endurance was due to
209 changes in aerobic mechanisms (Cummings, 1979), whereas in the frog *Xenopus laevis* training
210 altered glycolytic machinery (Miller and Camilliere, 1982). Future research can explore whether
211 mechanisms in lizards are similar to those in amphibians or mammals (Greiwe et al., 1999;

Coffey and Hawley, 2007; Yan et al., 2011; Atherton and Smith, 2012; Egan and Zierath, 2013; Wilson, 2013).

We detected no increase in maximal sprint speed for sprint-trained individuals, but we did find a response at the muscle level. Both the iliofibularis and the gastrocnemius showed hypertrophy in their FG fibres, which is expected if the training was effective. This finding agrees with the large literature on mammals which has unequivocally demonstrated that anaerobic sprint and resistance training result in hypertrophied fast-twitch muscle fibres (Tesch, 1988; Jones et al., 1989; Roig et al., 2009; Bruusgaard et al., 2010). Interestingly, the biceps muscle was significantly smaller in the sprint-trained group compared to the other two groups. Increasing incline, as we did in our endurance-training regimen, increases motor function of the biceps muscles in green anoles (Foster and Higham, 2014), but we did not change incline to increase intensity for our sprint-trained group. Perhaps the consistent use of the forelimbs on a constant incline during sprint training (resulting in no increase in motor function) resulted in more resources being put into the gastrocnemius, the primary propulsive muscle (Higham et al., 2011), instead of the biceps. Future experiments that change incline during sprint training would be very instructive to elucidate what training variables alter muscle properties in lizards. The lack of a training effect on sprint performance is, then, most likely due to lizards becoming habituated to handling by trainers. Thus, there may have been an increase in sprint speed, but we were unable to detect it. Although lizards were chased consistently to run up the dowel during training sessions and sprint trials, they did not appear as motivated to exert themselves to maximal capacity by the end of the training time period. That is, they hesitated and did not make smooth, continuous runs, as are typically seen in sprint trials without long-term handling prior to trials. The fact that all three treatment groups tended to decrease during the experiment (though

significantly; see Results) is consistent with a change in motivation, as is the fact that the two groups handled the most (the two trained groups) had the largest average decreases in sprint speed. Nevertheless, maximal sprint speed was still repeatable in the post-treatment trials (r = 0.92 for fastest and second-fastest speeds), suggesting that there was consistency in how motivated individuals were. This is an important finding, because if we had only examined performance endpoints we would have concluded that sprint training had no effect. The effects on FG muscle fibre CSA, though, showed that not to be true.

Our lack of detecting a training effect on sprinting at the whole-animal level brings up several problems that future investigators will face when studying exercise in non-model organisms. First, looking only at performance may be misleading. Several endpoints should be examined to ensure that there was or was not a response to training. Second, it is difficult to know at first what an appropriate exercise regimen should be for non-model species (see Booth et al., 2010 and associated commentaries). We had available several previous studies on lizards that failed to find a training response to help define our training regimen, even though these previous studies were on different species, and our design worked. Thus, those interested in exercise effects on non-model species will likely have to experiment with intensities and frequencies of training, finding a balance between adequate exercise and damaging or detrimental intensities. This is a large barrier to comparative exercise research and likely explains why there are so few studies on non-traditional study species. Finally, one must understand the relevance of exercise, and the response to it, in the species being studied. Does it have any ecological relevance to individuals' fitness such that a response to training is expected? How might the plastic response to training be beneficial? Are there tradeoffs when investing resources to enhance performance? This latter question may help better understand the adaptive

nature of training effects in general, and future studies on training effects in non-model species would benefit by framing their questions in a life-history perspective (Lailvaux and Husak, 2014). Along these lines, there is the possibility that the lizard species studied previously (Gleeson, 1979; Garland et al., 1987; O'Connor et al., 2011) truly do not respond to exercise, but green anoles do, and response to exercise can be gained or lost like other phenotypic traits, depending on selective pressures specific to each species. Either way, even though the details of what changed or not during training in green anoles is not conclusive in our study, it is an important first step to better understand the evolutionary history and conserved nature of performance physiology.

Conclusions

Because of its beneficial effects on humans, exercise has been the subject of thousands of studies. A search of PubMed (U. S. National Institutes of Health, accessed October 2014) for "human exercise" returned over 230,000 citations, revealing how much we know about the effects of exercise on a number of systems in the body. However, our knowledge about exercise effects does not extend far beyond humans, and not much beyond mammals. Our results, combined with perspectives from studies of exercise in fishes (Davison, 1997; He et al., 2013; Palstra and Planas, 2013), amphibians (Cummings, 1979; Miller and Camilliere, 1981), crocodilians (Owerkowicz and Baudinette, 2008; Eme et al., 2009), and birds (e.g., Gaunt et al., 1990) suggest that much of what we know about exercise effects on performance may extend to all vertebrates. However, the different mechanistic changes apparent within these groups makes more comparative studies imperative. Adding lizards to the list of higher vertebrate taxa showing

a response to exercise is an important step toward a fuller understanding of muscle plasticity. When more comparative data on mechanisms of training effects (or the lack thereof) across more vertebrates are available, we can begin to understand how general training effects are, as well as what factors lead to the evolutionary maintenance, gain, or loss of training responses, or whether such responses are evolutionarily labile.

MATERIALS AND METHODS

General husbandry

We obtained adult male green anoles (n = 30) from a commercial vendor (Candy's Quality Reptiles, LaPlace, LA, USA) that caught lizards from the wild and shipped them the next day. We housed lizards individually in 28-L plastic bins with mesh lids. Paper was attached to the outside walls of each cage to eliminate visual contact among lizards. Lizards were housed at 28-31° C on a 12:12 hr light:dark cycle for two weeks of acclimation before the beginning of the experiment. Full spectrum bulbs over the cages provided UV light. Humidity was maintained with a humidifier in the room and by misting each cage two times a day. Lizards were fed consistent diets of crickets and mealworms three times a week, with calcium and vitamin D dusted on crickets and mealworms once a week.

Pre-treatment measurements

Prior to the onset of training, we took several measurements from each lizard. We measured maximal sprint speed and endurance capacity (see below), as well as mass (to the nearest 0.1 g) and several morphometrics (snout-vent length [SVL], hindlimb length, forelimb length to the nearest 0.01 mm). Sprint speed was measured with each lizard being run three times in one day (trials being separated by two hours). The racetrack was a 2-m long, 5-cm diameter dowel covered in cork (for traction), and was equipped with vertically paired infrared photocells (Trackmate Racing, Surrey, British Columbia, Canada) at 0.25-m intervals so that a running lizard broke the beams sequentially and the elapsed time (msec, then converted to m/sec) for each interval was recorded by a computer. The track was placed at a 45° angle to simulate natural conditions (Cox *et al.* 2009). The fastest 0.25-m speed was considered maximal. This is a highly repeatable, standard procedure for *Anolis* lizards (Cox et al., 2009; Foster and Higham, 2012). We measured endurance by running lizards to exhaustion (loss of righting reflex) on a motorized treadmill (PetRun model PR700 modified for lower speeds) rotating at 0.3 km/h (Perry et al., 2004; Cox et al., 2009). Lizards were placed on the moving treadmill and encouraged to run by gentle tapping of the tail and hindlimbs with a small fan paintbrush. Lateral movements were constrained by confining lizards within a small open-bottom plexiglass box resting on the surface of the treadmill. A lamp with a 100-W bulb suspended over the treadmill maintained the temperature between 29-31° C during the trials. Duration from initiation of the trial to loss of righting reflex was recorded as the measure of endurance. Endurance was measured 24 hours after sprint speed trials were completed. Training began four days after endurance was measured.

We randomly assigned individual lizards to one of three treatment groups described below. The three groups did not differ from each other in SVL ($F_{2, 21} = 1.25$, $P = 0.31$), mass

($F_{2, 21}$ = 2.33, P= 0.12), hindlimb length ($F_{2, 21}$ = 0.44, P = 0.65), or forelimb length ($F_{2, 21}$ = 0.23, P = 0.80). Before training sprint speed was not related to SVL (r = 0.01, P = 0.96), but endurance was (r = 0.44, P = 0.03). The treatments did not initially differ in sprint speed (ANOVA: $F_{2, 21}$ = 0.46, P = 0.64), or endurance (ANCOVA with SVL as a covariate: slope, $F_{2, 18}$ = 0.10, P = 0.91; intercept, $F_{2, 20}$ = 0.91, P = 0.42). Ten lizards were assigned to each treatment group, but mortality during the experiment resulted in n = 9 for controls, n = 8 for endurance-trained, and n = 7 for sprint-trained treatments. Although there was some mortality over the course of the experiment, we do not feel that it was due to a decline in overall health of lizards, especially those being trained, as there was no difference between pre-treatment and post-treatment SVL or mass (paired t-tests, P > 0.06 for all).

Training

Lizards in the trained treatments were trained three days a week (Monday, Wednesday, Friday) for 8 weeks. In each of the trained groups, training increased in intensity twice for a total of three 'phases' of training. They were trained for three weeks in the first phase, three weeks in the second phase, and two weeks in the third phase.

The sprint speed training consisted of lizards being run up the same racetrack used to measure pre-treatment sprint speed, with intensity increasing by increasing the number and frequency of runs per day. In the first phase lizards were run three times per day with two hours of rest between runs. In the second phase lizards were run four times per day with two hours of rest between runs. In the third phase lizards were run four times per day with one hour of rest between runs.

Endurance training was conducted on the same treadmill on which we measured endurance capacity, but at a slower speed (0.18 km/h instead of 0.3 km/h). Lizards were run each training session for 30 min or until exhaustion. In each phase, lizards that were initially unable to go the full 30 min eventually did complete the full session before increasing intensity in the next phase. We increased intensity by increasing the angle of the treadmill. It went from flat (0°) in the first phase to 9° in the second phase, and then 13° in the third phase.

The control treatment lizards were handled once daily three times per week. This was meant to stimulate any stress response that may have resulted just from handling the trained lizards. Control lizards were captured, removed from their cage, and held for approximately 30 seconds. Although this is not the same amount of time spent training for endurance, it is approximately the amount of time for a sprint trial. In addition, corticosterone levels of another lizard species (*Eulamprus heatwolei*) were comparable after sprint trials compared to 30 min after being handled and chased in a cage for 30 sec (Langkilde and Shine, 2006). Further, endurance-trained lizards would have been the most stressed, but they had a significant increase in performance and did not have the highest mortality. Thus, we feel that our control treatment adequately served as a handling control.

Post-treatment measurements

After training ended we again took several measurements from each lizard. We measured maximal sprint speed and endurance capacity (as described above), as well as mass and the same morphometrics (SVL, hindlimb length, forelimb length) with measurers blind to the treatment of the lizard being run. Sprint speed was again measured with each lizard being run three times in

one day, with the fastest 0.25-m speed considered maximal. Endurance was again measured by running lizards to exhaustion on a motorized treadmill lying flat (0° incline) and rotating at 0.3 km/h. We took post-training blood samples four days after the end of training. Three days after blood collection sprint speed was measured, and endurance was measured the day after sprint speed trials. This timing was used to prevent changes in circulating corticosterone levels, which were being determined for a different project. Prior to beginning this study, we tested whether performance would be negatively affected three days after blood sampling in a different (not trained; $N = 10$) group of lizards, and results showed that neither sprint speed ($t = -1.16$, $P = 0.28$) nor endurance were significantly reduced ($t = -0.73$, $P = 0.48$). After centrifuging blood samples, we determined hematocrit for each lizard by using a Hamilton syringe to measure the volume of plasma and red blood cells to obtain the percentage of total blood volume that was red blood cells.

Lizards were then euthanized by rapid decapitation, and carcasses were processed for preservation. After euthanization, the right forelimb and hindlimb were immediately removed with scissors to ensure that all limb muscles were removed intact with part of the pelvis in the hindlimb and part of the pectoral girdle in the forelimb removed with the limbs. The limbs were then positioned at 90° angles with pins on foam blocks and immersed in isopentane cooled in liquid nitrogen (Bonine et al., 2001). After freezing, limbs were wrapped in foil and put in dry ice until moved to storage at -80°C.

The body and left limbs of the carcasses were preserved for muscle dissections. Lizards were positioned with limbs at right angles and fixed in 10% aqueous formalin solution for 24 hours, then rinsed and transferred to 70% ethanol (Huyghe et al., 2010). We dissected muscles following Herrel et al., (2008), with the muscle anatomy of *Anolis carolinensis* being most

similar to *A. valencienni*. We removed the following muscles: M. pectoralis pars superficialis (humeral retraction), M. biceps (elbow flexion), M. triceps brachii (elbow extension), M. caudofemoralis longus (femoral retraction), M. ambiens (knee extension), M. puboischiotibialis (knee flexion), M. iliofibularis (knee flexion), and M. gastrocnemius pars fibularis (ankle extension). Once removed, we patted each muscle dry and weighed it to the nearest 0.001 mg (Mettler Toledo UMX2). We also removed and weighed the heart of each lizard.

Histochemical techniques

We sectioned frozen muscles on a cryostat and stained them with two different procedures. Frozen hindlimbs were cut in half just distal of mid-thigh, and the proximal portion of the thigh was mounted on cryostat chucks using Tissue Tek embedding medium. Using a cryostat microtome (Leica CM3050 S), we sectioned hindlimb muscles at mid-thigh in a plane perpendicular to the femur in 20 μm sections at -20°C. Serial sections, captured on glass coverslips and air-dried for at least 30 min, were used for histochemical identification of fibre types, as done previously for green anoles (described in Rosen *et al.* 2004). We stained for myosin ATPase (mATPase; pH 9.4, 60 min) and succinic dehydrogenase (SDH; 37 min) to identify fibre types. We characterized fibres as one of three fibre types on the basis of their staining characteristics (Bonine et al., 2001): fast glycolytic (FG), fast oxidative glycolytic (FOG), or slow oxidative (SO). FG fibres stain dark with mATPase and light with SDH (Bonine et al., 2001). Muscles with higher oxidative capacities stain more darkly with SDH, whereas muscles with low oxidative capacities are lighter in colour (Gleeson and Harrison, 1988).

Glass slides containing cross sections of lizard hind limbs were photographed (using Neurolucida, ver. 9, MBF Bioscience, Williston, VT) using a camera mounted on a compound microscope (Nikon Instruments). A stage micrometer was photographed at each magnification for later use in determining muscle fibre areas. The iliofibularis (IF), gastrocnemius (G), and biceps of lizards from each treatment group were later analyzed for fibre composition and fibre size. These muscles were selected because of the importance in the stance (G) and swing (IF) phases of locomotion (Higham et al., 2011), as well as mass differences among treatments found in the biceps (see Results). Neurolucida software (MicroBrightField, Inc., Williston, VT) was used to count the number of fibres of each type to determine the proportion of each within a muscle. Then, the outline of approximately 20% of fibres for each fibre type (i.e., FG, FOG, and SO) were chosen within each muscle, and cross-sectional area (FCSA) was calculated using Scion Image (Scion Corp., Frederick, MD, USA). We averaged the areas for each fibre type within an individual for analysis. All measurements were made blind to the treatment for section being measured.

Statistical analysis

We first used paired t-tests to determine if individuals in each treatment group (separately) had significantly different sprint speed or endurance. Since our treatment groups did not differ in initial morphology or performance, and we only had post-training data on hematocrit, heart and muscle masses, and muscle fibre characteristics, we examined all variables by comparing post-training measures. Post-training body size did not correlate with sprint speed (SVL, $P = 0.06$; mass, $P = 0.19$) or endurance (SVL, $P = 0.90$; mass, $P = 0.66$), so we compared treatments with

analysis of variance (ANOVA). We also used ANOVA to compare treatments in post-training SVL, body mass, and hematocrit (arcsine-square root-transformed). We used analysis of covariance (ANCOVA), with SVL as the covariate, to compare treatments for post-training limb morphology, and we used ANCOVA with body mass as the covariate to compare muscle masses and heart mass. Analysis of muscle fibre proportions (arcsine-square root transformed) and cross-sectional area were compared among treatments for each muscle (IF, G, B) separately using analysis of variance (ANOVA). Fiber cross-sectional areas did not significantly scale to body size ($P > 0.22$ for all), likely due to the intentionally small range of body sizes in the study. Pairwise comparisons were performed for all significant ANOVAs using Tukey's HSD with $\alpha = 0.05$.

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Competing interests

The authors declare no competing financial interests.

Author contributions

J. F. H., A. R. K., and B. N. W. designed and ran the experiment. J. F. H. wrote the manuscript with input from all the authors.

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Table 1. Mean (± SEM) muscle masses (mg) from green anole lizards either trained for endurance or sprinting or not trained (control). Bolded values were significantly different among treatment groups.

	Pectoralis	Biceps	Triceps	Ambiens	Puboischiotibialis	Iliofibularis	Caudofemoralis	Gastrocnemius
Control	17.20±2.2	**7.36±0.4**	10.97±.08	14.29±0.9	9.36±0.6	4.84±0.5	38.04±2.8	8.11±0.5
Endurance	17.70±2.3	**7.04±0.9**	10.46±1.4	13.90±1.8	8.78±1.1	4.59±0.6	35.78±4.6	8.31±1.1
Sprint	17.33±2.2	**6.72±0.8**	11.59±1.4	14.37±1.8	9.94±1.3	4.75±0.6	41.98±5.3	8.89±1.1

Table 2. Results of analysis of variance (ANOVA) analyses on muscle fibre type cross-sectional areas in the biceps, gastrocnemius, and iliofibularis compared among the three treatment groups. A significant result means that the average cross-sectional area of the given fibre type differed among treatment groups. Pairwise comparisons with Tukey's HSD tests are summarized for significant ANOVAs (*P*-values are given in the text). Endurance = endurance-trained group; sprint = sprint-trained group; control = control group. Significant ANOVAs are shown in bold.

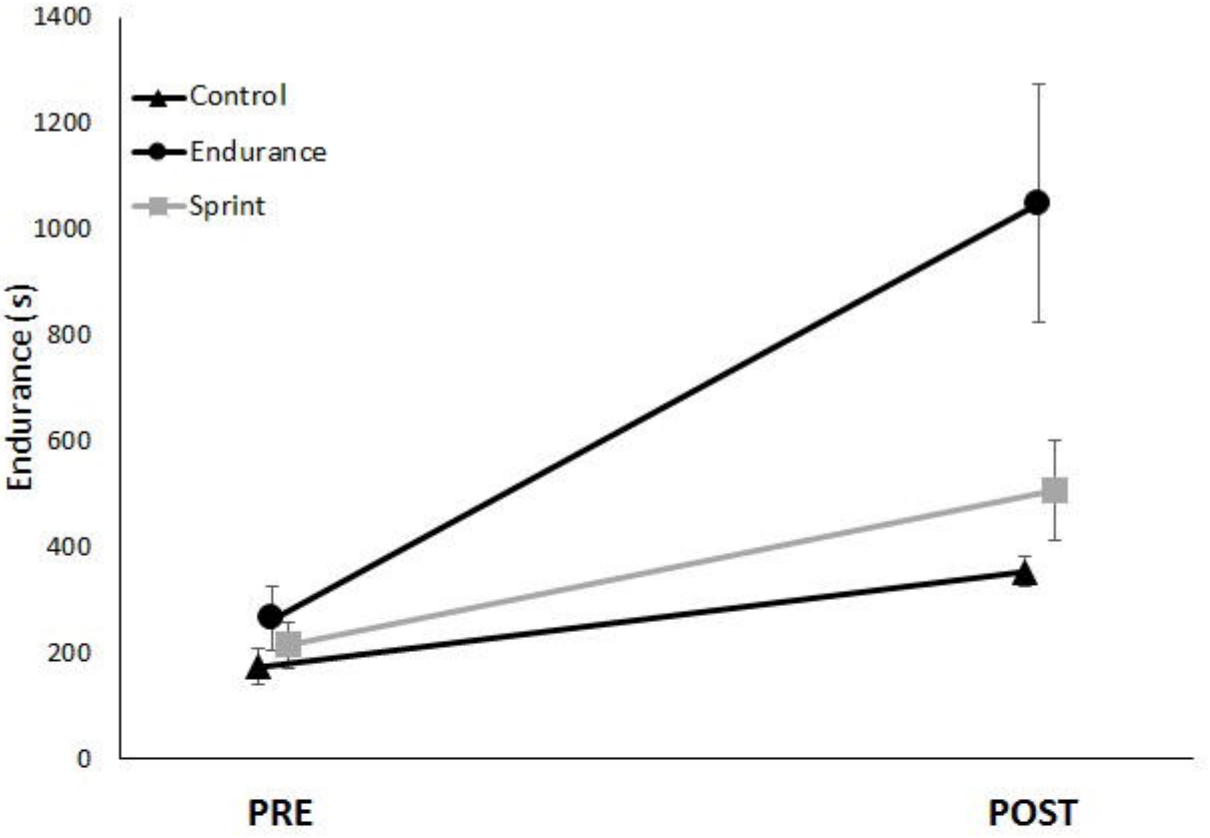
Muscle	Fast glycolytic	Fast oxidative glycolytic	Slow oxidative
Biceps	$F_{2, 21} = 2.34$, $P = 0.12$	$F_{2, 21} = 0.54$, $P = 0.59$	$F_{2, 21} = 1.40$, $P = 0.27$
Gastrocnemius	**$F_{2, 21} = 12.12$, $P = 0.0003$** Sprint > endurance = control	$F_{2, 21} = 2.50$, $P = 0.11$	**$F_{2, 21} = 8.56$, $P = 0.002$** Endurance > sprint = control
Iliofibularis	**$F_{2, 21} = 12.28$, $P = 0.0003$** Sprint > endurance = control	$F_{2, 21} = 2.42$, $P = 0.11$	**$F_{2, 21} = 10.43$, $P = 0.0007$** Endurance > sprint = control

Figure Legends

Fig. 1. Mean (± SEM) endurance capacity (A) and sprint speed (B) for lizards trained for sprint speed (S) and endurance (E), as well as control lizards (C), which were not trained.

Fig. 2. Mean (± SEM) hematocrit (proportion of blood that was red blood cells) for control, sprint-trained, and endurance-trained lizards.

Fig. 3. Mean (± SEM) cross-sectional area (μm^2) of fast glycolytic (FG), fast oxidative glycolytic (FOG), and slow oxidative (SO) muscle fibers in the gastrocnemius muscles of control, sprint-trained, and endurance-trained lizards.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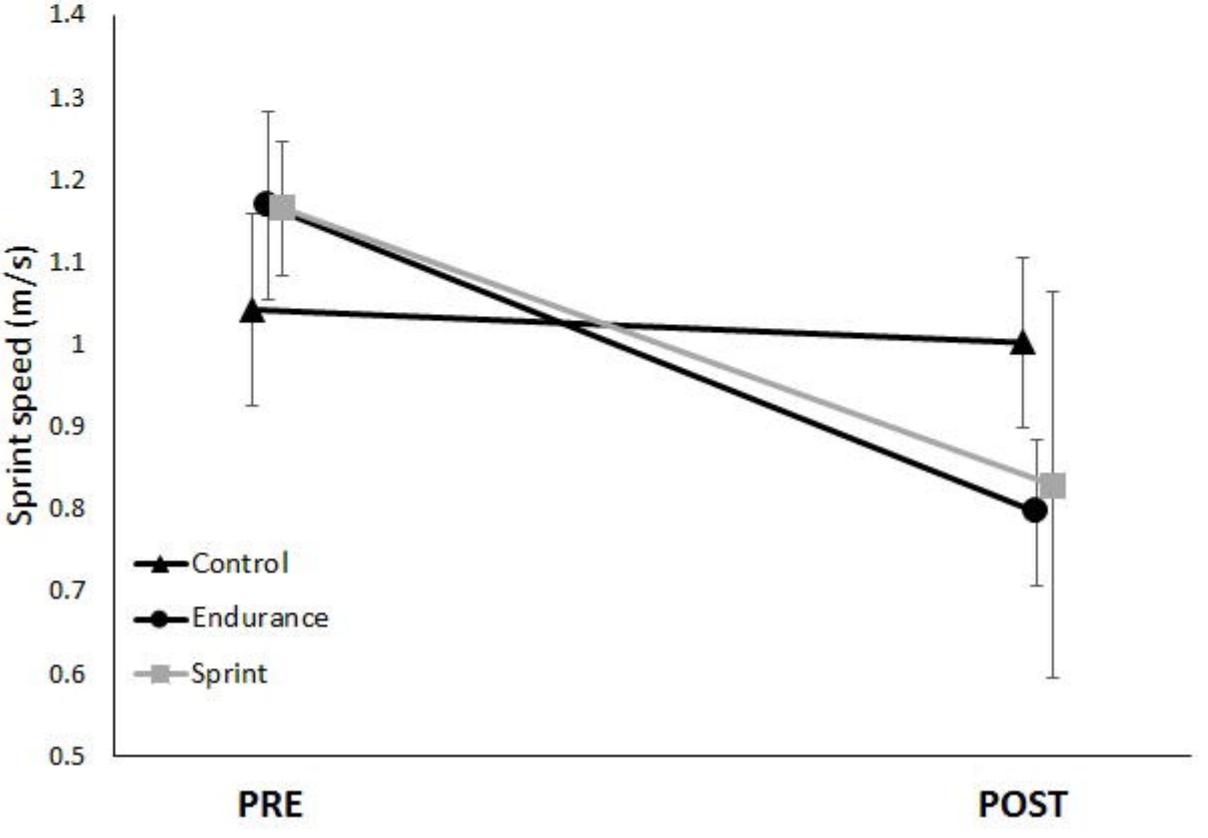
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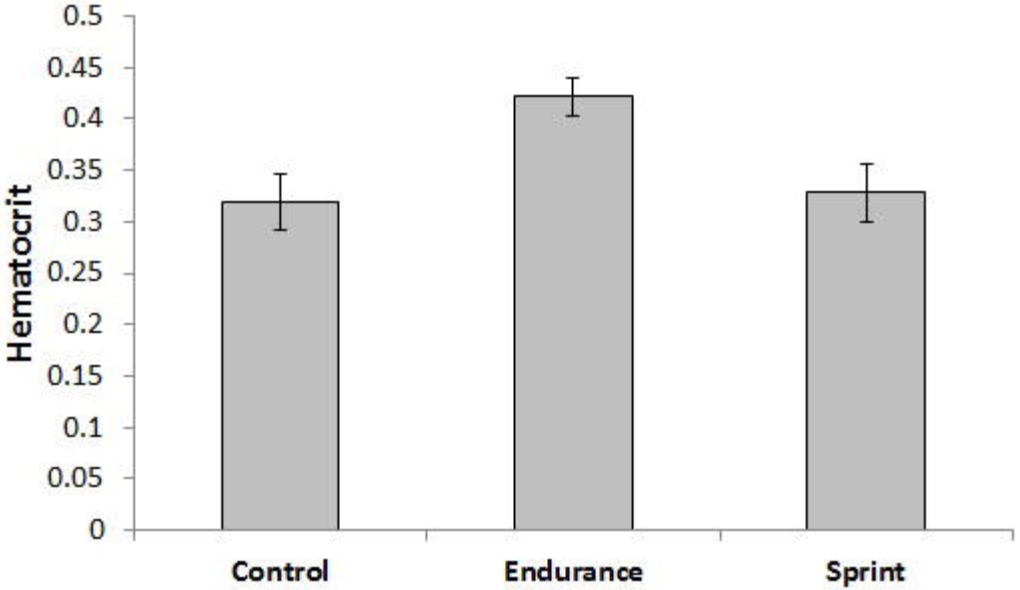
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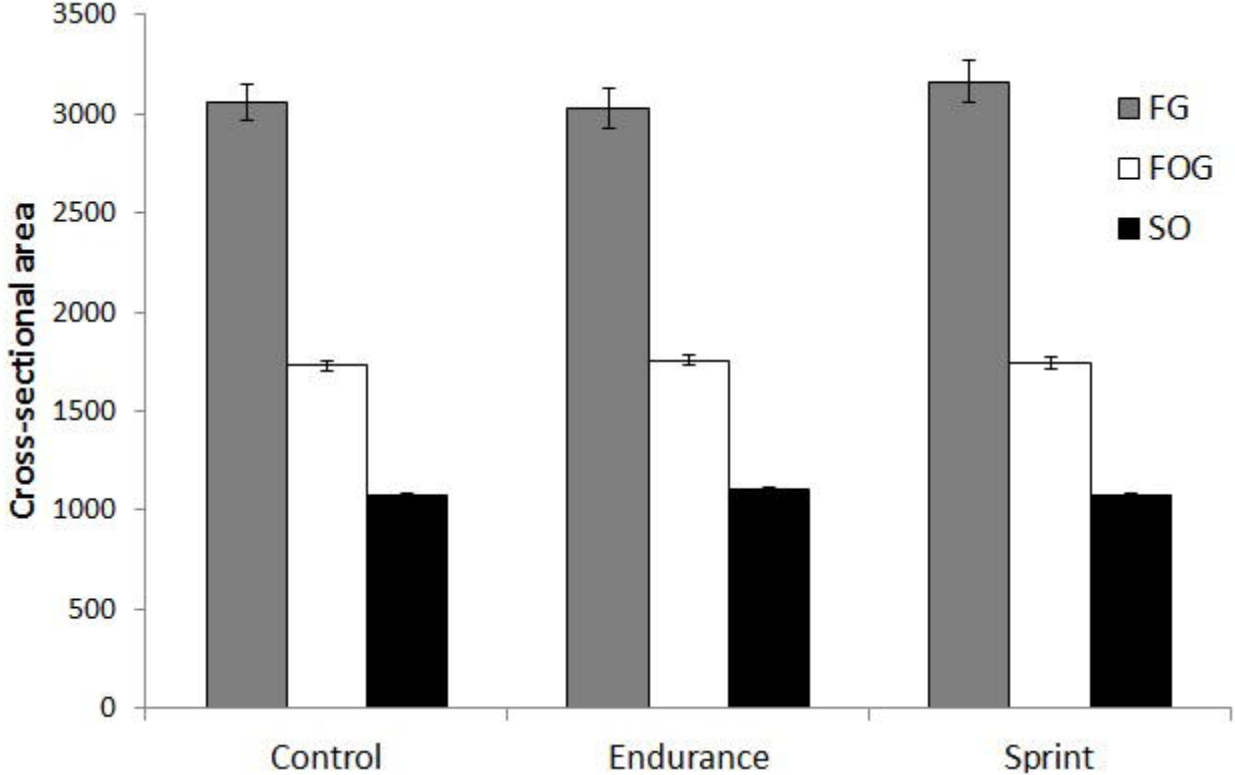
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