

RESEARCH ARTICLE

Exercise training reveals trade-offs between endurance performance and immune function, but does not influence growth, in juvenile lizards

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ABSTRACT

Acquired energetic resources allocated to a particular trait cannot then be re-allocated to a different trait. This often results in a trade-off between survival and reproduction for the adults of many species, but such a trade-off may be manifested differently in juveniles not yet capable of reproduction. Whereas adults may allocate resources to current and/or future reproduction, juveniles can only allocate to future reproduction. Thus, juveniles should allocate resources toward traits that increase survival and their chances of future reproductive success. We manipulated allocation of resources to performance, via endurance exercise training, to examine trade-offs among endurance capacity, immune function and growth in juvenile green anole lizards. We trained male and female captive anoles on a treadmill for 8 weeks, with increasing intensity, and compared traits with those of untrained individuals. Our results show that training enhanced endurance capacity equally in both sexes, but immune function was suppressed only in females. Training had no effect on growth, but males had higher growth rates than females. Previous work showed that trained adults have enhanced growth, so juvenile growth is either insensitive to stimulation with exercise, or they are already growing at maximal rates. Our results add to a growing body of literature indicating that locomotor performance is an important part of life-history trade-offs that are sex and age specific.

KEY WORDS: Anole, *Anolis carolinensis*, Life history, Locomotion

INTRODUCTION

Energetic resources acquired by individuals can be allocated to a variety of structures and functions in the body, but once resources are allocated to some purpose they cannot be re-allocated to something else (Stearns, 1989, 1992; Roff, 1992), which results in phenotypic trade-offs. Any trait that requires energetic resources in some form, including those that are key to Darwinian fitness, such as growth, immunity, reproduction and physical performance, is potentially susceptible to trade-offs (Stearns, 1989, 1992; Zera and Harshman, 2001; Ricklefs and Wikelski, 2002; Demas et al., 2012; Hasselquist and Nilsson, 2012; Lailvaux and Husak, 2014). One of the most ubiquitous and well-studied trade-offs is that between survival and reproduction (Stearns, 1989), but this trade-off is not

necessarily manifested in the same way among individuals within a population, nor is it immediately evident for those incapable of reproduction (Mangel and Stamps, 2001). Instead, the relative importance of phenotypic traits that may be involved in trade-offs, and hence how much energy is being invested in those traits, can differ among demographic groups in a population. One example is the differential investment in current versus future reproduction by males and females driven by sex-specific fitness incentives (Wedell et al., 2006; Bonduriansky et al., 2008; Maklakov and Lummaa, 2013). Another example is the relative investment in a given trait among different age classes. For many species, growth is important early in life so that individuals can attain a body size that is at least adequate to successfully reproduce. Hence, a high proportion of resources go toward growth early in life, with a shift toward other traits later in life (Mangel and Stamps, 2001). This switch is pronounced in species that only become sexually mature after attaining some minimal body size, with resource allocation switching from predominantly growth to current reproductive success (Reznick, 1983; Stearns, 1989, 1992).

Juveniles of many species need to survive to reproductive maturity, yet still set themselves up for successful future reproduction (e.g. via large body size). In this scenario, juveniles are expected to invest heavily in those traits that enhance survival, such as immune function and whole-organism physical performance (e.g. endurance capacity or sprint speed; Carrier, 1996; Irschick and Garland, 2001) while also optimizing the rate and magnitude of growth. Experimentally enhanced immune function suppresses growth in birds (Soler et al., 2003), and physical performance and growth trade off in fishes and birds. In fishes, even when at the same body length, faster-growing animals swim less efficiently than slower-growing animals (Kolok and Oris, 1995; Gregory and Wood, 1998, 1999). Swordtail fish (*Xiphophorus helleri*) that had low food availability early in life, and hence reduced growth, subsequently had increased growth when given more resources; however, that compensatory growth resulted in their inability to improve performance with aerobic training (Royle et al., 2006). Transgenic coho salmon that grow twice as fast in length as control fish have critical swimming speeds that are half those of control fish of similar size (Farrell et al., 1997). Similarly, compensatory growth in zebra finches (*Taeniopygia guttata*) did not immediately decrease flight performance, but instead resulted in a greater decline in flight performance after the reproductive period (Criscuolo et al., 2011). Compensatory growth has also been shown to have other lasting effects, such as decreased reproductive output in guppies (*Poecilia reticulata*), the magnitude of which increases throughout adulthood (Auer et al., 2010). The take-home message from these studies of compensatory growth is that truncated investment in growth early in life can have profound detrimental effects on other traits that are important for survival,

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such as resistance to disease and starvation, oxidative stress and over-winter mortality (Forsen et al., 2004; Dmitriew and Rowe, 2005; Johnsson and Bohlin, 2006; De Block and Stoks, 2008). Thus, even though many traits potentially contribute to the survival of juveniles, such as growth, endurance and immune function, they cannot all receive equal resource allocation simultaneously.

Re-allocation of resources to enhance performance may also suppress other traits important to survival and future reproduction, such as components of the immune system. Increased immune responses during infection or experimental manipulation may shunt energetic resources or critical macromolecules away from traits such as performance (Schall et al., 1982; Martin et al., 2003, 2012; Adamo et al., 2008; Adelman and Martin, 2009), resulting in reduced performance capacity. For example, in *Psammmodromus algirus* lizards, experimental induction of an immune response by injection of lipopolysaccharide significantly reduced sprint speed (Zamora-Camacho et al., 2015). In adult green anoles, diet restriction significantly decreased the swelling response to phytohemagglutinin (PHA), and training significantly exacerbated the detrimental effects of diet restriction on bacterial killing ability of plasma (Husak et al., 2016). Taken together, results to date suggest that traits contributing to performance capacity and components of the immune system share a common resource pool from which the two must compete for allocation.

We took an experimental approach to discern whether there are trade-offs in juveniles between growth and two traits important to survival, immune function and endurance performance. Instead of manipulating growth as others have done in previous studies, we manipulated potential allocation to performance in green anole lizards (*Anolis carolinensis* Voigt 1832) by training individuals to enhance endurance capacity. Adult green anoles respond strongly to endurance training, even when resources are severely limited, but training increased growth in adults, perhaps because of increased levels of endogenous growth factors (Husak et al., 2016). However, growth rates of adult *A. carolinensis* are significantly lower than those of juveniles (Lailvaux et al., 2004), so we predicted the opposite effect in juveniles: training may decrease growth as resources are shifted toward performance enhancement and away from growth. Furthermore, because large body size is potentially more important for reproductive success in males (Jenssen et al., 2005; Lailvaux et al., 2004), we tested whether trade-offs were more evident in females than males. Alternatively, because growth is so important to juvenile green anoles for future reproductive success, there may be no response to training.

MATERIALS AND METHODS

General husbandry

We obtained juvenile male ($n=20$) and female ($n=20$) green anoles from a commercial vendor (Candy's Quality Reptiles, LaPlace, LA, USA), and housed them in male–female pairs in 12-liter cages (medium Kritter Keepers, Lee's Aquarium & Pet Products, San Marcos, CA, USA). The small body size of all individuals made it clear that all were juveniles hatched in the late summer before the study was started and conducted (early fall). Lizards were housed at 28–31°C on a 12 h:12 h light:dark cycle for 4 weeks of acclimation before the beginning of the experiment (Husak et al., 2015, 2016). Training and all measures of performance were conducted within this temperature range. Lizards were fed *ad libitum* commercially obtained crickets (Fluker Farm, Port Allen, LA, USA) three times a week, with calcium and vitamin D supplemented once a week throughout the experiment. Cages were sprayed with tap water three times a day so that water was available

ad libitum, and humidifiers were kept on in the room to maintain humidity above 40%.

All research was conducted under approval of the University of St Thomas Animal Care and Use Committee.

Pre-treatment measurements

Prior to the onset of training, we took several measurements from each lizard. We measured mass and snout–vent length (SVL) of each lizard, as well as endurance capacity on a motorized treadmill (PetRun model PR700 modified for lower speeds). Endurance was recorded as the time to exhaustion, determined by when lizards lost their righting response while running on a treadmill rotating at 0.3 km h⁻¹ (Perry et al., 2004; Cox et al., 2009; Husak et al., 2015, 2016). Treadmills were cleaned with ethanol between lizards during measurements and training bouts described below.

After pre-treatment measures, lizards were allowed to rest for 3 days, after which we randomly assigned each to one of two treatment groups: trained ($n=10$ males, 10 females) or untrained (control; $n=10$ males, 10 females). Some mortality in all groups reduced these sample sizes slightly, as indicated below and in the figures. The two treatment groups did not differ from each other in initial SVL (two-way ANOVA with sex and training as factors: $P>0.3$ for both main effects and interaction), mass (two-way ANOVA with sex and training as factors: $P>0.85$ for both main effects and interaction) or endurance (two-way ANCOVA with SVL as a covariate and sex and training as factors: $P>0.6$ for both main effects and interaction).

Training

Lizards were randomly assigned to either a control or trained group. Lizards were trained 2 days a week (Monday and Wednesday) for 7 weeks. Endurance training was conducted on the same treadmill on which we measured endurance capacity, but at a slower speed (0.18 km h⁻¹ instead of 0.3 km h⁻¹). Lizards were run each training session for an increasing amount of time and eventually with increasing incline (following a modified procedure in Husak et al., 2016). We began with no incline and increased training session time from 5 min (week 1) to 7 min (week 2), and then 10 min (week 3) before adding an incline of 9 deg (still 10 min for week 4), and then increasing time to 15 min for week 5. Finally, weeks 6–7 were at the increased incline of 13 deg. These reduced times from our previous adult training (Husak et al., 2015, 2016) are still realistic and relevant to green anoles, though at the higher end of activity, as is the goal of training (see Husak et al., 2016 for full justification). Travel time is a significant proportion of daily time (Jenssen et al., 1995) and energy budgets (Orrell et al., 2004) for adult male and female green anoles in nature. Although we do not know the time or energy budgets of juveniles, we assume they are comparable to those of adults outside of the breeding season when they spend more time and energy foraging and digesting food compared with during the breeding season (Orrell et al., 2004). This suggests that the rather modest time and energy that our lizards spent on the treadmill was not the major source of the resultant trade-offs, but rather the physical changes that occurred, as well as any molecular pathways turned on or off as part of the exercise response.

The control treatment lizards were handled once daily two 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 s. Any effect on trained individuals of being in an unfamiliar location during training is unlikely, because the treadmills eventually became familiar places, as they were trained on the

same treadmill by the same person, making the physiological stress of training the most important factor.

Post-treatment measurements

At the end of the experiment we re-measured endurance, mass and SVL for all individuals. Individuals were allowed to rest for 3 days after endurance measurements before PHA swelling assays (Huyghe et al., 2009; Martin et al., 2006; Husak et al., 2016), a standard assay to quantify a mitogen-induced swelling response. These were followed by 1 day of rest. Lizards were then trained one last time (to avoid a long gap between last training and the measuring of endpoints), and 4 days later were euthanized. To prevent changes in circulating corticosterone levels at the time of euthanization, lizards were removed from their cage and euthanized by rapid decapitation in less than 4 min. Microhematocrit tubes were used to immediately collect trunk blood at the time of decapitation for hematocrit (Husak et al., 2015) and hormone analysis. Blood samples were centrifuged and plasma was frozen at -80°C until assays were performed. Carcasses were fixed in 10% aqueous formalin solution, then rinsed and transferred to 70% ethanol. Hearts were removed and dissected to separate atria from the ventricle, dried, and weighed to the nearest 0.001 mg (Mettler Toledo UMX2).

Growth was calculated as $\text{final SVL} - \text{initial SVL} / \text{initial SVL}$ to account for the initial body size of the lizards, which may influence growth rates. We examined growth in the same way using mass, though mass may be a less accurate measure of size because of current state of hydration or other factors.

We measured swelling responses after injection with PHA. Injection of PHA induces a series of cellular responses that includes both innate and acquired immune defenses, which results in localized swelling at the site of injection (Martin et al., 2006). We measured the thickness of each lizard's left and right hind foot to the nearest 0.01 mm at a standardized location (between the first and fifth digits) and then injected 0.05 mg PHA (PHA-P, L8754; Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.01 ml sterile phosphate-buffered saline (PBS) into the right foot. The left foot was injected with the same volume of sterile PBS but without PHA (Huyghe et al., 2009; Husak et al., 2016). We measured feet at 24 h post-injection and calculated swelling as the change in foot thickness between pre- and post-injection measurements (subtracting swelling from PBS injections).

Plasma concentrations of testosterone and corticosterone were measured by radioimmunoassay, following standard protocols (Wingfield and Farner, 1975). Each sample (5–30 μl of plasma) was mixed in 0.5 ml of ddH₂O and equilibrated overnight at 4°C with ~ 1000 cpm of $^3\text{H-T}$ (NET-370, 80 Ci mmol^{-1}) and $^3\text{H-CORT}$ (NET-399, 93 Ci mmol^{-1}) from PerkinElmer Life Sciences (Boston, MA, USA). Samples were extracted twice with 2 ml diethyl ether, dried under nitrogen gas and reconstituted in 0.5 ml of 10% ethyl acetate in isooctane for column chromatography. Columns consisted of a diatomaceous earth (Celpure P300, no. 525243, Sigma-Aldrich):ethylene glycol:propylene glycol upper phase (4:1:1 m:v:v) and a diatomaceous earth:ddH₂O (3:1 m:v) lower phase. Samples were placed onto columns and neutral lipids were eluted with 2.0 ml isooctane, followed by testosterone with 2.0 ml 20% ethyl acetate in isooctane, and corticosterone with 2.25 ml 52% ethyl acetate in isooctane. Samples were dried under nitrogen gas, resuspended in phosphate buffer and refrigerated overnight. Mean recoveries for testosterone and corticosterone were 85% and 84%, respectively. Radioimmunoassays were performed using tritiated steroid tracer, antisera from Research Diagnostics

(Flanders, NJ, USA) for testosterone (T-3003) and Sigma-Aldrich for corticosterone (C8784), and steroid standards from Sigma-Aldrich (testosterone, T1500; corticosterone, C2505). Standard curves (1.95 to 500 pg) were run in duplicate. Samples were run singly and corrected for individual-sample recovery efficiency and initial volume. The assay coefficient of variation, based on two standards for each steroid that were run with samples, was 14% for testosterone and 3% for corticosterone.

Statistical analysis

Because our treatment groups did not differ in initial morphology or performance, and we only had post-training data on hematocrit, heart mass and PHA swelling response, we examined all variables by comparing post-training measures. Post-training body size did not correlate with endurance (SVL, $P=0.15$; mass, $P=0.06$), so we compared treatments with ANOVA. We also used ANOVA to compare treatments in post-training growth in SVL and mass, as well as hematocrit (arcsine-square root transformed). Heart mass measures were compared with analysis of covariance (ANCOVA), with body mass as the covariate. Pairwise comparisons were performed for all significant ANOVAs or ANCOVAs using Tukey's HSD with $\alpha=0.05$.

RESULTS

Performance enhancement and cardiovascular effects

Training significantly increased endurance capacity (main effect of training: $F_{1,32}=16.82$, $P=0.0003$; Fig. 1), but there was no significant sex \times training interaction ($P=0.32$) or main effect of sex ($P=0.38$). Hematocrit was significantly increased by training ($F_{1,29}=524.83$, $P<0.0001$), but there was no sex difference ($P=0.91$) or sex \times training interaction ($P=0.69$). Heart ventricle mass (analyzed with ANCOVA) was significantly increased by training ($F_{1,28}=8.40$, $P=0.007$), but there was no effect of sex ($P=0.47$) or a sex \times training interaction ($P=0.89$). Heart atria mass (analyzed with ANCOVA) was unaffected by training or any interactions ($P>0.06$ for all), but females had larger atria than males ($F_{1,28}=4.25$, $P=0.049$).

Growth

Males grew in SVL more than females ($F_{1,32}=6.05$, $P=0.02$; Fig. 2), but training did not significantly affect growth in SVL ($P=0.30$), and

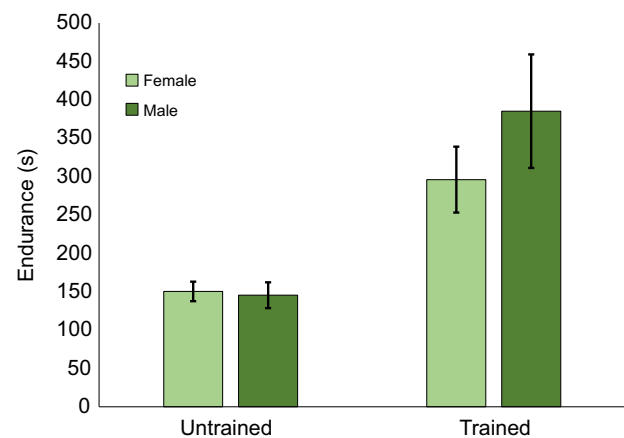


Fig. 1. Training enhanced endurance capacity of male and female green anole lizards. Mean (\pm s.e.m.) endurance capacity for lizards trained for endurance and untrained control lizards. For comparisons with two-way ANOVA: trained, $n=9$ males, 10 females; untrained, $n=8$ males, 9 females.

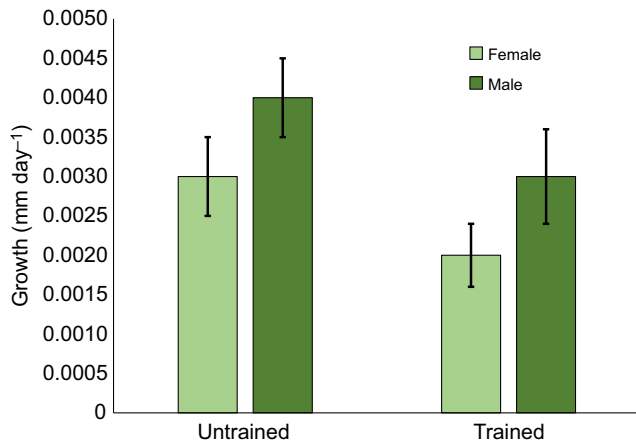


Fig. 2. Endurance training had no effect on growth of juvenile green anoles, but males grew more than females. Mean (\pm s.e.m.) relative growth [final snout–vent length (SVL) – initial SVL/initial SVL] shown for each treatment group and sex. For comparisons with two-way ANOVA: trained, $n=9$ males, 10 females; untrained, $n=8$ males, 9 females.

there was no sex \times training interaction ($P=0.77$). Males had significantly greater growth in mass than females ($F_{1,32}=6.13$, $P=0.02$), and untrained individuals increased mass more than trained individuals ($F_{1,32}=4.19$, $P=0.049$), but there was no sex \times training interaction ($P=0.38$).

Immune function

There was a significant sex \times training interaction in swelling response to PHA ($F_{1,29}=5.73$, $P=0.02$; Fig. 3). Examination of simple effects revealed that untrained females had a greater swelling response than untrained males ($P=0.046$). There were no other significant differences among treatment groups ($P>0.19$ for all).

Hormone levels

There was no sex \times training interaction on testosterone levels ($F_{1,28}=0.005$, $P=0.95$). There was also no significant sex ($F_{1,28}=2.83$, $P=0.10$) or training ($F_{1,28}=0.0001$, $P=0.99$) effect. Testosterone levels scaled to SVL in males ($R^2=0.59$, $P=0.0008$), but not females ($R^2=0.12$, $P=0.18$). ANCOVA on testosterone

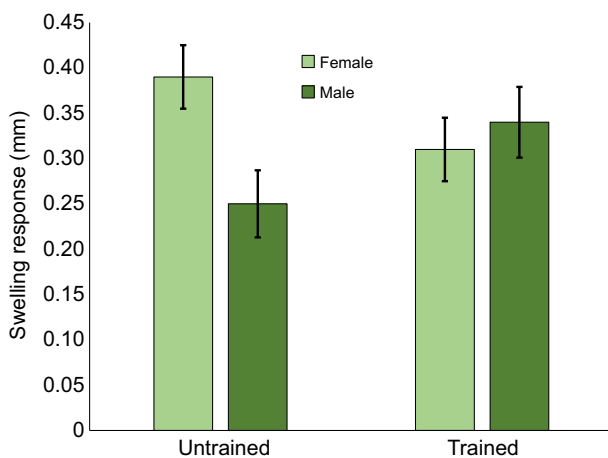


Fig. 3. Training suppressed immune function in female green anoles, such that the sex difference in controls disappeared in trained individuals. Mean (\pm s.e.m.) swelling response to phytohemagglutinin (PHA) shown for each treatment group and sex. For comparisons with two-way ANOVA: trained, $n=9$ males, 10 females; untrained, $n=8$ males, 9 females.

levels with SVL as a covariate and sex and treatment as factors revealed no difference in slope for testosterone in relation to training ($F_{1,26}=1.17$, $P=0.29$), but a significant difference in slope between males and females ($F_{1,26}=11.46$, $P=0.002$), with males having a higher slope. Thus, some males clearly became sexually mature during the experiment, and had higher testosterone, but this was not dependent on training or the lack thereof.

There was no sex \times training interaction on corticosterone levels ($F_{1,28}=0.0002$, $P=0.99$). Trained lizards had higher levels of corticosterone than untrained lizards ($F_{1,28}=4.74$, $P=0.038$; Fig. 4), but there was no significant sex effect ($F_{1,28}=3.91$, $P=0.058$).

DISCUSSION

Training had multiple effects on the phenotype of juvenile green anole lizards. Training significantly increased endurance capacity, though there was variation among individuals in the magnitude of the response. Trained individuals increased endurance performance, hematocrit and heart ventricle mass, as well as baseline corticosterone levels. Males grew more than females, which is expected in a species with strong male-biased sexual size dimorphism (Stamps, 1983; Cox et al., 2003), but trained lizards did not gain as much weight as untrained individuals. There was no effect of training on growth in body length, and growth in the two sexes was not affected differently by training. The only difference in training effects seen between the sexes was that training appeared to suppress immune function of females so that the sex difference evident in untrained individuals disappeared with training. These results, taken together, show that enhancing physical performance results in trade-offs for some, but not all, traits important to survival and reproduction in juvenile green anoles. Further, the phenotypic effects of enhanced endurance performance were not all the same as in adults, as predicted.

Many of the responses to training were as expected given how we predicted juveniles should allocate resources to increase future reproductive success. Juveniles need to grow to be successful as adults, and this is especially true for males, where larger body size affords a significant advantage in mating success. Consistent with this prediction, growth in body length (SVL) was unaffected by training, whereas in adults training enhanced growth in SVL (Husak et al., 2016). There are two potential explanations for this age difference: (1) the importance of growth to juveniles has resulted in

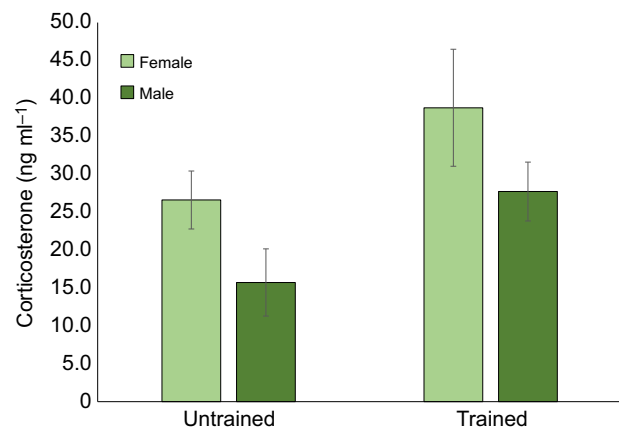


Fig. 4. Training increased corticosterone levels in both male and female green anoles. Mean (\pm s.e.m.) baseline corticosterone levels shown for each treatment group and sex. For comparisons with two-way ANOVA: trained, $n=9$ males, 10 females; untrained, $n=8$ males, 9 females.

reduced plasticity from external stimuli (e.g. training or increased activity); and (2) growth is already at a maximum and cannot physiologically be increased with further stimulation. Growth continues, but slows, in adult lizards (Stamps, 1983; Cox et al., 2003), and training likely increases growth factor production, which leads to increased growth (e.g. in humans: Kanaley et al., 1997; Gustafsson et al., 1999). If juveniles are already secreting high levels of growth factors (Licht and Hoyer, 1968; Rousseau and Dufour, 2007), they may not be able to produce more, or receptors may be near saturation such that higher concentrations have little to no effect. All of these possibilities warrant further investigation.

The PHA swelling response was significantly affected by training, but it was sex-specific, with only females suppressed compared with untrained individuals. This is in contrast to adults, where training resulted in only a marginal decrease in PHA swelling in both sexes (Husak et al., 2016). In untrained adults, males had a larger PHA swelling response than females, but females had a greater response in untrained juveniles. Adult males are, in general, predicted to have lower immune function than females (Rolff, 2002), because females, but not males, are assumed to maximize their fitness by investing in survival and longevity, which should enhance future reproduction (Nunn et al., 2009). Our results in juveniles match this pattern, even though this pattern apparently does not remain in adults (Husak et al., 2016). Nevertheless, training caused juvenile females to reduce immune function while they increased performance, a different trait that may be key for survival and future reproductive success (Irschick et al., 2008). It is possible that other aspects of the immune system were compromised or compensated for shifted resource allocation. Alternatively, some males had elevated testosterone levels by the end of the study. Given the immunosuppressive effects of testosterone (Folstad and Karter, 1992; Wedekind and Folstad, 1994; Braude et al., 1999; Fuxjager et al., 2011), these males may have already had reduced immune function such that training effects on immunity were only evident in females. Future studies can help elucidate this. In adults, bacterial killing ability of plasma was significantly reduced by training, but there was no difference between the sexes (Husak et al., 2016). We did not have enough plasma to conduct such assays on juveniles, but because the complement immune system involved with bacterial killing is heavily dependent on protein availability (Venesky et al., 2012), we predict similar results. Future studies will reveal whether bacterial killing responds in the same way in juveniles as adults.

Our results suggest alternative routes to optimize survival in juveniles. Allocating resources to one whole-organism trait important to survival (endurance performance) trades off with investment in another (immune function), but there is likely selection to prioritize one over the other depending on the environment. Endurance has been shown to predict survival in juvenile lizards (reviewed in Husak, 2015), and such selection may explain why the response to increased activity (i.e. exercise) persists even under significant caloric restriction (Husak et al., 2016). Depending on local abundance and diversity of pathogens, enhanced performance may be more beneficial to survival in the short-term to reach sexual maturity and reproduce than maintaining a high-quality immune response. Which survival-important trait takes priority for resource allocation may depend on what the biggest threat is to survival, predators or pathogens. More work is needed to disentangle these possibilities.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.F.H. and J.C.R. designed and ran the experiment. M.B.L. ran hormone assays. J.F.H. wrote the manuscript with input from all the authors.

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