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ORIGINAL ARTICLE

Every breath you take: assessing metabolic costs of toxin resistance in garter snakes (*Thamnophis*)

Haley A. MONIZ,[1,2] Molly A. RICHARD,[3] C. M. GIENGER[3] and Chris R. FELDMAN[1,2]

[1]Department of Biology, University of Nevada, Reno, Nevada, USA, [2]Program in Ecology, Evolution and Conservation Biology, University of Nevada, Reno, Nevada, USA and [3]Department of Biology and Center of Excellence for Field Biology, Austin Peay State University, Clarksville, Tennessee, USA

Abstract

Trait specialization often comes at the expense of original trait function, potentially causing evolutionary tradeoffs that may render specialist populations vulnerable to extinction. However, many specialized adaptations evolve repeatedly, suggesting selection favors specialization in specific environments. Some garter snake (*Thamnophis*) populations possess specialized mutations in voltage-gated sodium channels that allow them to consume Pacific newts (*Taricha*) defended by a highly potent neurotoxin (tetrodotoxin). These mutations, however, also decrease protein and muscle function, suggesting garter snakes may suffer evolutionary tradeoffs. We measured a key physiological process, standard metabolic rate (SMR), to investigate whether specialized adaptations in toxin-resistant garter snakes affect baseline energy expenditure. In snakes, skeletal muscles influence metabolism and power ventilation, so inefficiencies of sodium channels in these muscles might impact whole-animal energy expenditure. Further, because sodium channels are membrane-bound proteins, inefficiencies of channel kinetics and performance might be exacerbated at suboptimal temperatures. We measured SMR in 2 species, *Thamnophis atratus* and *Thamnophis sirtalis*, that independently evolved tetrodotoxin resistance through unique mutations, providing replicate experiments with distinct underlying genetics and potential physiological costs. Despite our expectations, neither resistance phenotype nor sodium channel genotype affected metabolism and resistant snakes did not perform worse under suboptimal body temperature. Instead, *T. atratus* and *T. sirtalis* show nearly identical rates of mass-adjusted energy expenditure at both temperatures, despite differing eco-morphologies, life histories, and distant phylogenetic positions. These findings suggest SMR may be a conserved feature of *Thamnophis*, and that any organismal tradeoffs may be compensated to retain whole-animal function.

Key words: adaptation, standard metabolic rate, tetrodotoxin, toxin-resistance, tradeoff

INTRODUCTION

Natural selection acts on wild populations much like a tinkerer, using preexisting supplies to modify structure and function (Jacob 1977). Thus, altering a trait may be a constrained process where not all options on-hand will lead to an optimal or uncompromised result (Futuyma &

Correspondence: Haley A. Moniz, Department of Biology, University of Nevada, Reno, 1664 North Virginia Street (MS 0314), Reno, NV 89557, USA.
Email: haley.a.moniz@gmail.com

Moreno 1988; Weinreich *et al*. 2006; Storz *et al*. 2009; Feldman *et al*. 2012; Guillaume & Otto 2012; Storz 2016). Highly specialized traits are thought to impart evolutionary tradeoffs when the original function of the trait is still necessary or important in certain contexts but has been altered by natural selection for a novel purpose (Guillaume & Otto 2012; Vamosi *et al*. 2014; Day *et al*. 2016). For example, animals that have evolved fossorial habits possess short and stout limb structures adapted for digging, but at the expense of running speed and efficiency (Hildebrand 1962; Gans 1983). What remains less clear, however, is whether and how tradeoffs in underlying genes and their products (e.g. proteins) scale up to affect phenotypes. If changes in coding regions impair the original function of important proteins, we expect those molecular-level deficits will scale up, and affect the tissues where those proteins are expressed (e.g. muscle, heart, nerve, blood), ultimately impacting whole-organism performance (Storz *et al*. 2009; Storz 2016). Connecting specialization at the genetic and tissue level to whole-organism function is integral to understanding the limits of trait evolution, especially in the context of ecological specialization.

Functional tradeoffs are common in diet specialization (Schondube & del Rio 2003; Pauw *et al*. 2020) and successfully consuming toxic food, in particular, can provide many direct and indirect benefits, such as the ability to monopolize and radiate onto unexploited toxic hosts (Ehrlich & Raven 1964; Wheat *et al*. 2007), or sequester toxic dietary compounds for defense (Daly *et al*. 1997; Savitzky *et al*. 2012; Erb & Robert 2016; Petschenka & Agrawal 2016). Toxins often target specific essential proteins, disrupting molecular function and potentially interrupting important physiological pathways, causing harm or even death to consumers (Brodie 2009). Thus, selection should promote adaptations that allow consumers to avoid dangerous outcomes (Arbuckle *et al*. 2017). However, altering essential proteins may impose costs that constrain the path to toxin resistance (Feldman *et al*. 2012; Dobler *et al*. 2019), resulting in a tradeoff between the benefit of the new adaptive trait and maintenance of its original function (Maynard-Smith *et al*. 1985; DePristo *et al*. 2005).

The opportunity to investigate potential patterns in dietary specialization and the scaling of evolutionary costs across physiological levels exists for 2 species of garter snakes (*Thamnophis*) in the western United States (Fig. 1) that have independently evolved specialized sodium channel mutations (Geffeney *et al*. 2005; Feldman *et al*. 2009, 2010; Hague *et al*. 2017). These mutations allow some snake populations to prey on toxic Pacific newts (*Taricha*;

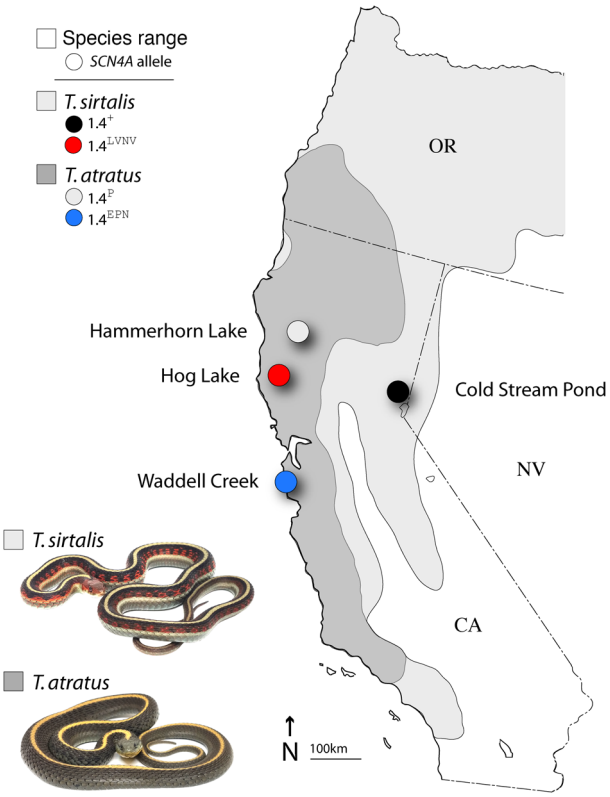

Figure 1 Geographic distribution of *Thamnophis atratus* and *T. sirtalis* in the western United States (after Stebbins 2003) with sampling localities and *SCN4A* allele denoted by colored circles (low resistance: black and grey; high resistance: blue and red). Detailed sample and locality information given in Table S1, Supporting Information. Snake images by HAM.

Brodie *et al*. 2002; Hanifin *et al*. 2008; Greene & Feldman 2009). Concentrated in newt skin is a potent neurotoxin (Tetrodotoxin; TTX) that, when ingested, binds to voltage-gated sodium channels, inhibiting action potentials in muscle tissue and in the nervous system (Fozzard & Lipkind 2010), typically leading to respiratory failure (Brodie 1968; Narahashi 2001). Novel mutations in the outer pore of skeletal muscle sodium channels ($Na_v1.4$) of TTX-resistant snakes (Fig. 2a) alter protein charge and conformation, reducing the binding ability of TTX and allowing for consumption of toxic prey without lethal effects (Geffeney *et al*. 2005; Feldman *et al*. 2009, 2012; McGlothlin *et al*. 2014, 2016).

However, the mutations that allow garter snakes to consume toxic newts occur in a highly conserved region of the voltage-gated sodium channels (Goldin 2001, 2002; Zakon 2012). Because these proteins are central to vital aspects of homeostasis and tissue function (Hille 2001), tinkering with sodium channels to reduce binding of TTX

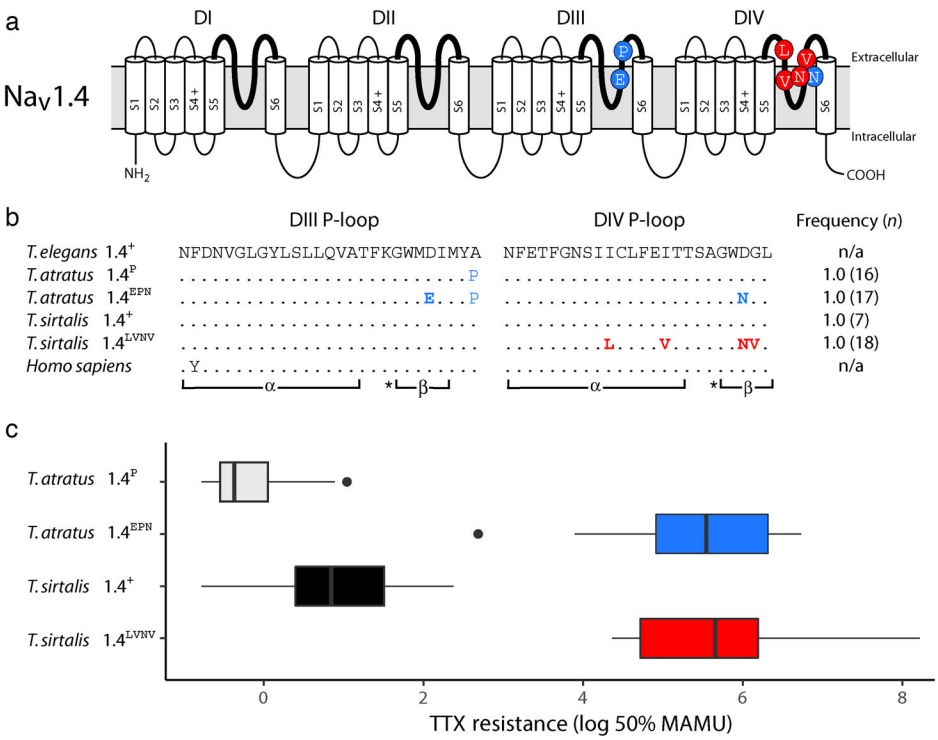

Figure 2 (a) Secondary structure of Na$_v$1.4, showing the membrane spanning P-loops (thick black lines) that create the outer pore that tetrodotoxin (TTX) binds to, along with the locations of functionally relevant mutations in the P-loops of garter snakes. Colors correspond to species, *SCN4A* genotypes, and level of TTX resistance, with the most extreme being triple mutant 1.4^{EPN} in *T. atratus* (blue) and quadruple mutant 1.4^{LVNV} in *T. sirtalis* (red). Adapted from Feldman *et al*. (2009). (b) Amino acid sequences of DIII and DIV pore loops for the *SCN4A* alleles of our focal garter snake populations, along with the sequence of ancestral 1.4^{+} in *T. elegans* and human for comparison (demonstrating the high conservatism of this locus). (c) Box-plots of log-transformed TTX resistance (mean 50% MAMU ± SD) in the 4 focal genotypes, demonstrating the significant difference between non-resistance genotypes (1.4^{+}, 1.4^{P}) and resistance genotypes (1.4^{EPN}, 1.4^{LVNV}) in phenotypic resistance for both *T. atratus* ($n = 32$; $P < 0.0001$) and *T. sirtalis* ($n = 22$; $P < 0.0001$).

may induce tradeoffs at the whole-organism level. Previous work on TTX-resistant voltage-gated sodium channels has established such tradeoffs at the biochemical level in the form of reduced sodium conductance and selectivity, and altered channel gating kinetics (Lee *et al*. 2011; Hague *et al*. 2018; del Carlo 2020; reviewed in Feldman *et al*. 2012). Work on the specific mutations in *Thamnophis* shows these protein-level tradeoffs scale up to the tissue level, such that snakes harboring these mutations display weaker and slower muscles (del Carlo 2020). However, it remains untested whether and how these changes affect whole-animal energy expenditure. We sought to test whether the link between molecular and muscular costs continues to scale to organismal tradeoffs by influencing metabolic processes. We examined 2 species [*Thamnophis atratus* Kennicott, 1860 and *Thamnophis sirtalis* (Linnaeus, 1758)] that have independently evolved TTX-resistance through distinct skeletal muscle sodium channel mutations (Feldman *et al*. 2009), providing 2 evolutionary replicates of a possible tradeoff.

In snakes, skeletal muscles impact several key aspects of whole-animal performance including processes that underlie metabolic rate, particularly ventilation (Rosenberg 1973; Lillywhite 2014). Specifically, ventilation occurs when snakes contract a suite of skeletal muscles that surround the body, thereby squeezing the ribs and expelling air, and then relaxing these muscles to expand the ribs and allow air intake (Rosenberg 1973). Because mutant Na$_v$1.4 are expressed in the muscles required for ventilation, we expect that any reduced protein and muscle performance in TTX-resistant snakes will impact muscular activity, and therefore standard metabolic rate (SMR). Specifically, the altered kinetics and conductance of mutant channels (Lee *et al*. 2011; Hague *et al*. 2018; del Carlo 2020) should change the shape and duration of action potentials and even lead to increased

propagation failures in muscle tissue, thus demanding more energy to complete processes contributing to SMR, such as the ventilatory cycle. We therefore predict that snakes with resistance-conferring mutations will have higher metabolic rates compared to TTX-sensitive snakes possessing the ancestral sodium channels.

As ectotherms, nearly all physiological functions are sensitive to environmental temperature (Taylor *et al*. 2021). Indeed, many aspects of performance in snakes show marked differences when examined at various temperatures (Arnold & Bennet 1984; Stevenson *et al*. 1985; Brodie & Russell 1999; Shine *et al*. 2000). Thus, differences in SMR between TTX-resistant and TTX-sensitive snakes may vary under different temperatures. For example, a metabolic cost might not be apparent under the ideal thermoregulatory opportunities of most laboratory conditions and will potentially be exposed under suboptimal temperatures. We therefore measured SMR at 2 ecologically relevant temperatures (20°C and 30°C) commonly experienced by these ectotherms in the wild (Gibson & Falls 1979; Avery 1982; Lillywhite 1987; Peterson 1987; Rosen 1991). Because $Na_v1.4$ are bound in a cell membrane that changes in flexibility across a thermal gradient, exposure to different temperatures alters the orientation and stiffness of the channel itself in ways that might impact the kinetics of channel opening, as well as sodium conductance and selectivity (Somero 1995; Fields 2001; Lundbaek *et al*. 2004). We predict that differences in SMR would be accentuated when measured at the lower temperature of 20°C (Chappell & Ellis 1987).

Here, we investigate whether the protein and muscular costs of toxin resistance impact SMR in 2 species of garter snakes (*T. atratus* and *T. sirtalis*). We predict that TTX-resistant snakes have higher metabolic rates, and suboptimal (cold) temperature will further impact SMR compared to TTX-sensitive snakes. Tradeoffs in metabolic rates during periods of inactivity may reduce whole-animal energy availability, and thus performance during activities required for survival and fitness. If unable to compensate for energetic losses, specialized adaptations may then impose long-term fitness consequences associated with energetic tradeoffs, such as limited growth and reproductive potential.

MATERIALS AND METHODS

Animal collection and housing

We collected adult aquatic garter snakes (*T. atratus*; $n = 33$) and common garter snakes (*T. sirtalis*; $n = 25$) from 4 watersheds (6 sites) in northern California (Fig. 1) representing 2 resistance phenotypes (TTX-sensitive, TTX-resistant) and harboring distinct $Na_v1.4$ genotypes (Table S1; Fig. 2b). We housed snakes in individual cages at 25°C with a 12L:12D photoperiod, in an appropriately sized hide box (based on size of snake), an under-tank heating pad, and water *ad libitum*. We fed snakes frozen-thawed fish or mice weekly. Tail tips for sodium channel gene sequencing were removed upon entry to the lab. Snakes were then allowed to acclimate to housing conditions for a minimum of 1 week prior to any assays, after which we conducted resistance phenotype assays and metabolic rate assays in a randomized fashion. All snakes were allowed 2 weeks of acclimation time between the first and second assay. Due to this, we do not expect that assay order impacted our results.

Sodium channel gene sequencing

In garter snakes, variation in one particular Na_v paralog, the skeletal muscle sodium channel ($Na_v1.4$), appears largely responsible for physiological resistance to TTX at the whole-animal level (Geffeney *et al*. 2005; Feldman *et al*. 2010; McGlothlin *et al*. 2016; Hague *et al*. 2017). Thus, we examined functional variation in the gene that encodes the skeletal muscle sodium channel (*SCN4A*) for each snake. Specifically, we focused on DNA sequence variation in the domain 3 (DIII) and 4 (DIV) outer pore (P-loops) of $Na_v1.4$. Particular residues in these P-loops interact strongly with TTX (Terlau *et al*. 1991; Choudhary *et al*. 2003; Geffeney *et al*. 2005; Fozzard & Lipkind 2010) and display amino acid variation associated with TTX resistance within and among populations of *Thamnophis* (Geffeney *et al*. 2005; Feldman *et al*. 2009, 2010; Hague *et al*. 2017; Gendreau *et al*. 2020).

We extracted genomic DNA from tail-tips with the DNeasy Blood & Tissue Kit (Qiagen Inc., Germantown, MD, USA), and amplified fragments of genomic DNA corresponding to DIII and DIV P-loops by PCR using primers designed specifically for garter snakes (Table S2, Supporting Information). We cleaned PCR products with ExoSAP-IT (USB Corp., Cleveland, OH, USA) and performed cycle sequencing reactions with Big Dye 3.1 Chemistry (Applied Biosystems Inc., Foster City, CA, USA). We sequenced all samples in both directions using amplification primers and cleaned cycle sequencing reactions with Sephadex columns (Sigma-Aldrich, St. Louis, MO, USA). We resolved sequenced fragments on an ABI Prism 3730 DNA Analyzer (Thermo Fisher Scientific,

Waltham, MA, USA) at the Nevada Genomics Center (University of Nevada, Reno, NV, USA).

Whole-animal TTX resistance phenotype assays

To measure resistance at the level of the whole animal, we used a well-established resistance assay (Brodie & Brodie 1990; Ridenhour *et al*. 2004). Briefly, we sprinted each individual snake on a 3-m track equipped with infrared break-beam sensors (Adafruit, New York, NY, USA) every 20 cm to measure speed at pre- and post- injection time points after serially increasing mass-adjusted doses of TTX (Alomone Labs, Jerusalem, Israel) diluted with Ringer's solution (Sigma-Aldrich, St. Louis, MO, USA). This track was lit from above using natural-light lamps (Lavish Home, Lorain, OH, USA) and contained a top-mounted camera (Hero4 GoPro, San Mateo, CA, USA) to film all races (1060 linear video, 60 frames per second). We fasted snakes at least 3 days prior to sprint measures to dispel influence of digestion/gut load on performance (Garland & Arnold 1983). We placed snakes immediately from their enclosures onto the track and encouraged them to sprint forward by following them with a plastic-bristled duster or hand, and by tapping near their tails. Once we established a baseline pre-injection speed, we injected TTX into intraperitoneal space and left snakes in their cages to metabolize for 30 min prior to racing. We calculated doses using mass-adjusted mouse units (MAMU) where 1 MAMU = 0.01429 ug of TTX per gram of snake (Brown & Mosher 1963; Brodie & Brodie 1990; Ridenhour *et al*. 2004). Once an individual had been raced at 4–5 doses of TTX (i.e. 1, 5, 10, 20 MAMU), we calculated whole-animal resistance to TTX using regression to find the point at which their baseline speed is reduced to 50% (termed 50% MAMU). We performed regression on log-transformed dosages after correcting doses equal to one as 0.999, and those equal to 0 as 0.001 (Brodie *et al*. 2002; Ridenhour *et al*. 2004; Reimche *et al*. 2020). Some measures may underestimate the true 50% MAMU of highly resistant individuals, as volumes and cost of TTX required to assess extremely high dosages were not realistically obtainable.

Metabolism assays

We measured SMR at 30°C, a temperature that is considered within the preferred range for *Thamnophis*, and 20°C, a less optimal temperature well within habitat fluctuations (Stewart 1965; Gibson & Falls 1979; Rosen 1991). Previous studies support that temperatures nearing 20°C have measurable effects on whole-animal performance, including reduced sprint (Arnold & Bennet 1984; Brodie & Russell 1999), antipredator response (Shine *et al*. 2000), and tongue-flicking (Stevenson *et al*. 1985). We avoided temperatures too close to critical thermal minima as snakes would be subject to controlled temperatures for upwards of 24 h (Doughty 1994). To ensure a post-absorptive digestive state, we fasted individuals for a minimum of 3 days before beginning experimental trials. We randomly assigned order of testing and tested each individual at both temperatures.

We measured rates of carbon dioxide production ($\dot{V}CO_2$) using open-flow respirometry (Withers 1977; Lighton 2008). We used a Sable Systems CO_2 analyzer and Sable Systems MUX flow multiplexer (Sable Systems International, Las Vegas, NV, USA) to alternately measure 4 animals and 1 reference baseline (room air). An Ametek R-1 flow controller (Ametek Inc., Berwyn, PA, USA) pumped room air through a Drierite drying column, then through a manifold that split the air stream and directed to separate mass flow controllers (Sierra Mass Trak; Sierra Instruments, Monterey, CA, USA). Mass flow controllers maintained chamber airflow rates between 15 to 50 $mL \cdot min^{-1}$, depending on the experimental temperature and size of the snake (all snakes continuously received air flow, even if excurrent air from a particular snake was not being diverted to the analyzer). We constructed respirometry chambers from syringes and translucent plexiglass cylinders ranging from 140 mL to 1.2 L in volume to accommodate differences in snake size. Before pushing excurrent chamber air through the CO_2 analyzer, it was scrubbed of water vapor using a drying column packed with Drierite. We housed respirometry chambers in a Percival Scientific incubator (Percival Scientific Inc., Dallas, IA, USA) during trials to maintain experimental temperature and 12L:12D light cycle.

We sampled individual snake chambers for 30-min intervals with a 15-min baseline sample of room air flowing through an empty chamber between samples, with CO_2 concentration recorded every 3 s. We measured chamber CO_2 concentrations 8 times per day (every 3 hours in a 24-hour period) and used the most level 15-min period (lowest sum of absolute differences from the interval mean) from each 30-min sample to calculate the rate of CO_2 production. At each temperature, we measured snakes for 72 h and considered the mean of the 3 lowest 15-min measurements (out of ≈24 measurement periods) the SMR. We used the mean of the 3 lowest measurements to eliminate artificially low estimates that may have resulted from routine periods of apnea that are common in snakes (Heatwole 1977). We calculated the volume of CO_2 produced ($\dot{V}CO_2$) using Warthog Systems

LabAnalyst (Warthog System, warthog.ucr.edu), implementing the respirometry equations of Withers (1977).

DATA ANALYSES

We performed statistical analyses in R version 3.6.1 (R Core Team 2020) using packages *lme4* (Bates *et al*. 2015), *lmerTest* (Kuznetsova *et al*. 2017), and *emmeans* (Lenth 2020), as well as *ggplot2* (Wickham 2016) to visualize regressions.

Sodium channel genotypes

We edited raw chromatograms in Sequencher v4.2 (Gene Codes Corp., Ann Arbor, MI, USA) or Geneious v9.1.4 (Biomatters Ltd., Auckland, New Zealand; Kearse *et al*. 2012), and aligned nucleotide sequences in Geneious using a full *SCN4A* contig of *T. atratus* (Feldman *et al*. 2009; GenBank FJ570810.1) and *T. sirtalis* (Hague *et al*. 2017; KY745662.1). We then translated nucleotides to amino acid sequences in Mesquite v3.61 (Maddison & Maddison 2019) to examine functional variation (structural changes) in the P-loops. We submitted all sequences to GenBank (accession numbers pending).

Our notation for *SCN4A* alleles follows that of Feldman *et al*. (2010) and Hague *et al*. (2017). Briefly, the allele name includes the numerical designation of the sodium channel family member (i.e. 1.4) followed by a superscript of one letter amino acid abbreviations given in the order those derived allelic substitutions occur in the locus. This nomenclature reflects the functional molecular differences between the ancestral garter snake gene sequence (here termed 1.4^{+}) and derived *SCN4A* variants, rather than putative phenotypic effects or dominance attributes of alleles.

Genotype-phenotype matching

We investigated the difference in TTX resistance between distinct *SCN4A* genotypes for all snakes for which we could gather 50% MAMU ($n = 54$). We used *t*-tests on *T. atratus* ($n = 32$) and *T. sirtalis* ($n = 22$) separately to assess the relationship between genotype and phenotype within species because they possess independently derived *SCN4A* genotypes.

Standard metabolic rate

We compared allometric relationships between body mass and $\dot{V}CO_2$ within and between species at the test temperatures (20°C, 30°C) using a generalized linear mixed model (GLMM), assuming normally distributed errors. We modeled each species separately, with $\dot{V}CO_2$ being predicted as a function of body mass, temperature, TTX resistance (all considered fixed effects), and individual (random factor). We followed overall comparisons with pairwise Tukey-Kramer HSD post hoc comparisons of marginal (model-adjusted) means. We determined the level of statistical significance at alpha = 0.05 and reported means as mean ± standard error (s.e.m.).

RESULTS

Genotypic variation in sodium channels

We confirmed the $Na_v1.4$ genotypes (*SCN4A* sequences) of all the snakes we measured for SMR (33 *T. atratus* and 25 *T. sirtalis*). We found 2 alleles in both *T. atratus* (1.4^{P}, 1.4^{EPN}) and in *T. sirtalis*: (1.4^{+}, 1.4^{LVNV}), which directly correspond to their homozygous genotypes: ancestral TTX-sensitive $1.4^{+/+}$ *T. sirtalis* ($n = 7$), TTX-sensitive $1.4^{P/P}$ *T. atratus* ($n = 16$), TTX-resistant $1.4^{LVNV/LVNV}$ *T. sirtalis* ($n = 18$), and TTX-resistant $1.4^{EPN/EPN}$ *T. atratus* ($n = 17$). We did not find any heterozygous animals, and each population was fixed (homozygous) for its respective $Na_v1.4$ allele.

Phenotypic variation in whole-animal TTX resistance

Of the entire dataset, we collected 50% MAMU phenotypes for 32 *T. atratus* and 22 *T. sirtalis*. Both species show a wide range of 50% MAMUs due to the inclusion of both TTX-sensitive and extremely TTX-resistant animals. The 50% MAMUs of *T. atratus* display a slightly less extreme range of 0.46 to 836.67 while 50% MAMUs of *T. sirtalis* ranges from 0.46 to 3690.55. Within genotype variation, however, we found no overlap in this range (Fig. 2c). *T. atratus* 1.4^{P} had a mean of 1.02 (SD = 0.73), and *T. atratus* 1.4^{EPN} had a mean of 342.27 (SD = 266.09). *T. sirtalis* 1.4^{+} had a mean of 3.46 (SD = 2.90), and *T. sirtalis* 1.4^{LVNV} had a mean of 766.29 (SD = 495.46).

Genotype-phenotype matching

We found a significant difference in TTX resistance phenotype (50% MAMU) between *SCN4A* genotype ($Na_v1.4$ alleles; Fig. 2c) for both *T. atratus* ($t_{24.30} = 18.08$, $P < 0.0001$) and *T. sirtalis* ($t_{8.03} = 8.62$, $P < 0.0001$). These results are consistent with previous work

Table 1 Estimated marginal means (s.e.m.) of standard metabolic rate (SMR, mL $CO_2 \cdot h^{-1}$) measured at 20°C and 30°C for different TTX resistance genotypes of *Thamnophis* with means model-adjusted for body mass and temperature and statistically similar groups denoted with superscript letters (A–D)

Species	Mass (SD)(g)	Phenotype	Genotype	Temp (°C)	SMR (mL $CO_2 \cdot h^{-1}$)
Thamnophis atratus	74.25 (25.97)	TTX-sensitive	1.4^{P}	20	0.48 (0.08)^{A}
Thamnophis atratus	49.65 (22.47)	TTX-resistant	1.4^{EPN}	20	0.55 (0.07)^{A}
Thamnophis atratus	74.25 (25.97)	TTX-sensitive	1.4^{P}	30	1.68 (0.08)^{B}
Thamnophis atratus	49.65 (22.47)	TTX-resistant	1.4^{EPN}	30	1.76 (0.07)^{B}
Thamnophis sirtalis	44.33 (26.44)	TTX-sensitive	1.4^{+}	20	0.62 (0.07)^{C}
Thamnophis sirtalis	40.43 (14.43)	TTX-resistant	1.4^{LVNV}	20	0.40 (0.11)^{C}
Thamnophis sirtalis	44.33 (26.44)	TTX-sensitive	1.4^{+}	30	1.85 (0.07)^{D}
Thamnophis sirtalis	40.43 (14.43)	TTX-resistant	1.4^{LVNV}	30	1.63 (0.11)^{D}

showing that TTX resistance (50% MAMU) and genotype are tightly correlated in these 2 species (Feldman *et al*. 2010; Hague *et al*. 2017).

Metabolism assays

For *T. atratus*, mean body size of non-resistant genotype individuals was 59.3 g (SD = 17.3) and 45.8 (SD = 18.7) for resistant ($t_{32} = 2.18$, $P = 0.04$); for *T. sirtalis*, mean body size of non-resistant individuals was 42.3 g (SD = 26.0) and 39.4 (SD = 14.9) for resistant ($t_{19} = 0.34$, $P = 0.73$). There was a positive allometric effect of body mass (g) on rate of CO_2 production ($mL \cdot h^{-1}$) for both species (*T. atratus*, $\beta = 0.018$, $t_{31} = 6.4$, $P < 0.0001$ and *T. sirtalis*, $\beta = 0.018$, $t_{35} = 7.9$, $P < 0.0001$; Fig. 3).

After adjusting for the effect of body size, both species showed increased CO_2 production at 30°C relative to 20°C. Model-adjusted (marginal) means increased 1.20 $mL \cdot h^{-1}$ for *T. atratus* ($t_{33} = 15.9$, $P < 0.0001$) and 1.22 $mL \cdot h^{-1}$ for *T. sirtalis* ($t_{25} = 13.8$, $P < 0.0001$). After accounting for effects of body mass and temperature, there were only small (non-significant) differences in the rate of CO_2 production between non-resistant and resistant genotypes for either *T. atratus* (difference = 0.07 $mL \cdot h^{-1}$, $t = 0.70$, $P = 0.49$) or *T. sirtalis* (difference = 0.22 $mL \cdot h^{-1}$, $t = 1.93$, $P = 0.07$; Table 1).

DISCUSSION

Physiological tradeoffs

Trait specialization at the expense of the original or general function of that trait is expected to cause evolutionary tradeoffs (Futuyma & Moreno 1988; Storz *et al*. 2009; Feldman *et al*. 2012; Guillaume & Otto 2012; Storz 2016). Such tradeoffs are often studied at the ecological level (i.e. dietary or habitat specialization) but less is known about the outcomes of molecular specialization, where tradeoffs might inhibit function across physiological levels to impact performance and survival. We investigated whether whole-animal tradeoffs occur in 2 species of garter snakes that possess adaptive tetrodotoxin (TTX) resistance resulting from specialized sodium channel adaptations. Because resistant sodium channels are expressed in skeletal muscle that influences processes involved in SMR, such as ventilation, we expected that resistant snakes would differ in metabolic performance compared to their TTX-sensitive counterparts. Here, despite harboring mutations that reduce performance in an essential protein and the muscles where that protein is expressed, TTX-resistant snakes show nearly identical mass-adjusted SMR relationships to TTX-sensitive snakes at 2 different temperatures (Fig. 3; Table 1).

We found that TTX-resistant and TTX-sensitive groups of both species experienced nearly the same reduction in SMR at lowered temperatures (1.20 $mL \cdot h^{-1}$ for *T. atratus* and 1.22 $mL \cdot h^{-1}$ for *T. sirtalis*). Overall, the SMR rates here fall within the range of those reported in other snake species, including viperids (Beaupre 1993; Spencer *et al*. 2020), other colubrids (Lelièvre *et al*. 2010), natricines (Blem & Blem 1990; Hopkins *et al*. 2004), and other garter snakes (Britt *et al*. 2006; Robert & Bronikowski 2010). It is interesting to note that both *Thamnophis* species displayed nearly identical SMR at both temperatures, despite differences in overall body form, foraging techniques and feeding ecology. For example, *T. atratus* are more heavy-bodied, highly aquatic, and forage by ambushing fish in cold streams or hunting amphibians in ponds and lakes (Lind & Welsh

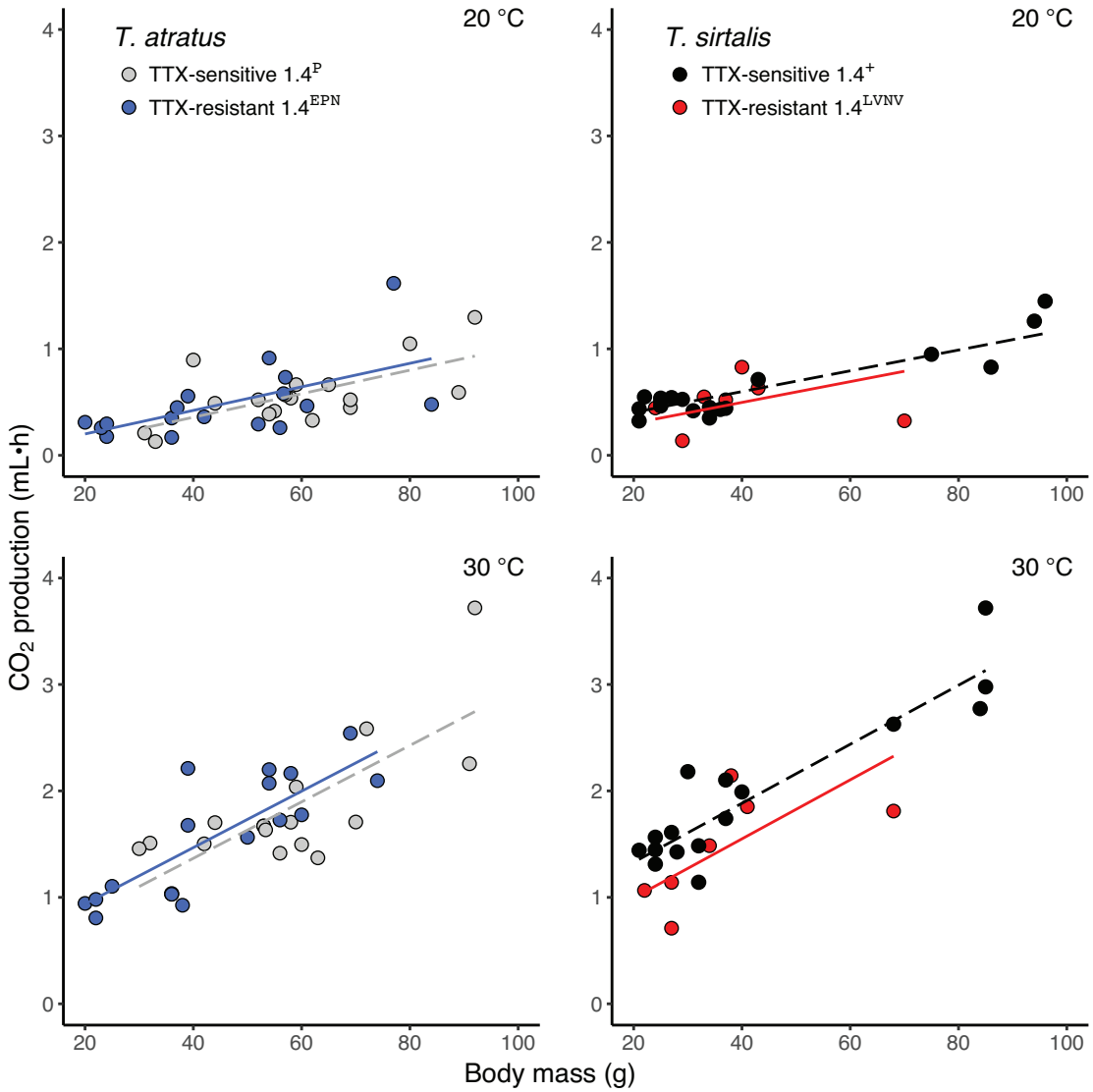

Figure 3 Generalized linear mixed model results for standard metabolic rate (SMR, as measured by CO_2 production) in TTX-sensitive *T. atratus* ($n = 16$; grey) and *T. sirtalis* ($n = 18$; black) and TTX-resistant *T. atratus* ($n = 17$; blue) and *T. sirtalis* ($n = 7$; red) with distinct *SCN4A* genotypes. After adjusting for the allometric effect of body mass (regression lines), both species showed increased CO_2 production at 30°C relative to 20°C (*T. atratus*: $P < 0.0001$; *T. sirtalis*: $P < 0.0001$). After accounting for effects of body mass and temperature, there was no significant difference between snakes with differing TTX resistance phenotypes (and *SCN4A* genotypes) for either *T. atratus* (difference = 0.07 $mL \cdot h^{-1}$, $P = 0.49$) or *T. sirtalis* (difference = 0.22 $mL \cdot h^{-1}$, $P = 0.07$; Table 1).

1994; Rossman *et al*. 1996; Preston *et al*. 2012), while western *T. sirtalis* tend to be more slender, riparian generalists that actively forage for a broad array of prey (Gregory 1978; Kephart & Arnold 1982; Rossman *et al*. 1996). Differences in these natural history traits have been shown to influence metabolic rates in other snakes, especially through alternate foraging strategies (Dupoué *et al*. 2017). Because terrestrial foraging and aquatic hunting expose snakes to different temperatures and require specific prey acquisition strategies (Secor & Nagy 1994; Stuginski *et al*. 2018), *T. atratus* and *T. sirtalis* should experience different abiotic constraints (Hailey & Davies 1986; Dupoue *et al*. 2017) that may ultimately influence activity rates, thermal physiology, and temperature-sensitive physiological processes (such as sodium channel performance dynamics). Furthermore,

dietary differences might lead to differences in energetics and assimilation efficiency (Britt & Bennett 2008; Reguera *et al*. 2011) that could impact SMR. Yet despite differences in feeding ecology and life history, and regardless of the fact that *T. atratus* and *T. sirtalis* occupy opposite ends of the garter snake phylogeny (de Quieroz *et al*. 2002; McVay *et al*. 2015), both species displayed almost the same SMR, suggesting that metabolic rates may be a conserved aspect of all *Thamnophis* (Rosen 1991).

Even though both *T. atratus* and *T. sirtalis* exhibit reduced sodium channel conductance, which leads to weakened muscle strength and speed (del Carlo 2020), these costs did not scale up to impact whole-animal energy expenditure (during periods of inactivity). This result was consistent even with a compounding stressor of lower (suboptimal) body temperature; both TTX-sensitive and TTX-resistant snakes exhibited similar losses in metabolic rate at the low temperature. The lack of an apparent tradeoff in whole-animal metabolism, despite reduced sodium conductance in the ion channels that activate muscles that directly contribute to SMR suggests that costs do not scale from protein and muscle to impart whole-animal costs (at least at the magnitude of effects seen in lower levels). It may also be that the lower temperature we used was not a significant enough stressor to reveal a tradeoff during periods of inactivity, or that membrane rigidity does not exacerbate sodium channel costs, which may instead be revealed at temperatures above the optimal range. Although we expect suboptimal temperature to decrease many aspects of whole-animal performance in ectotherms (Arnold & Bennet 1984; Stevenson *et al*. 1985; Brodie & Russell 1999; Shine *et al*. 2000), perhaps a cost will not be evident until snakes are exposed to more physically demanding stressors such as extreme exertion or prolonged activity. This is an important avenue for future studies, as it might more closely approximate real ecological interactions such as fleeing a predator, swimming for long periods, or wrestling with difficult prey. This approach could be accomplished by obtaining field metabolic rates (FMR; e.g. Nagy 2005), or using maximal performance measures that capture ecologically relevant activities, such as sprint and endurance.

Additionally, it is possible that snakes have evolved compensatory genetic mechanisms or adopted behavioral modifications to overcome costs at the sodium channel and muscle level. Behavioral compensation has been documented in response to muscle weakness or fatigue, as well as other physical limitations (McMahon 1984; Otten 1989; Gans & Gaunt 1991). For example, TTX resistant snakes might only be active under narrow environmental conditions that permit optimal sodium channel and muscle function. However, in holding snakes at a consistent temperature, we removed the opportunity for active thermoregulation, which is the most likely first avenue for behavioral compensation. Potential compensation at the genetic level remains to be explored, but may be similar to mechanisms seen in other toxin-resistant animals, whereby gene duplication circumvents the molecular costs of toxin resistance because one copy maintains normal gene function (and remains toxin sensitive) while the second copy evolves toxin resistance (Dobler *et al*. 2012; Zhen *et al*. 2012; Ujvari *et al*. 2015; Dalla & Dobler 2016; Petschenka *et al*. 2017; Dobler *et al*. 2019; Karageorgi *et al*. 2019). Though we lack evidence for such duplications in garter snakes (McGlothlin *et al*. 2016; Perry *et al*. 2018; Gendreau *et al*. 2020), more focused genomic work on TTX-resistant populations of *Thamnophis* is needed. Additionally, post-transcriptional modification (through RNA editing or alternative splicing) is known to create sodium channel variants with functional differences in invertebrates (Liu *et al*. 2004; Song *et al*. 2004; Lin *et al*. 2009); thus, it seems plausible that the same mechanism could operate in TTX-resistant snakes, such that snakes can express both TTX-resistant and TTX-sensitive sodium channel isoforms, or simply produce a greater number of sodium channels to compensate for their weaker performance. Other forms of compensation might involve changes in the upstream or downstream pathways of muscle activation, serving to rescue some degree of hypofunctional sodium channels.

Despite the potential costs, many specialized traits persist, indicating that the negative effects of specialization are often balanced by novel benefits (Futuyma & Moreno 1988; Kassen 2002; Bolnick *et al*. 2003). Understanding the nature of functional tradeoffs and how tradeoffs are overcome remains a challenge in evolutionary biology (Agrawal 2020). In this system, adaptive specialization to a toxic diet has led to measurable costs at molecular and tissue levels (del Carlo 2020), yet these costs are not emergent at the whole-organism level, suggesting mechanisms of compensation yet to be discerned.

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CONFLICT OF INTEREST

The authors declare no competing or financial interests.

DATA AVAILABILITY STATEMENT

We deposited all animals as voucher specimens in University of Nevada, Reno (UNR). SMR data are available on the Open Science Framework digital repository (link pending). All DNA sequences data are available on GenBank (accession numbers pending).

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Detailed sample and locality information from two focal species (33 *Thamnophis atratus* and 25 *T. sirtalis*) harboring distinct $Na_v1.4$ genotypes (*SCN4A* mutations)

Table S2 Primers used on *Thamnophis* for PCR amplification and Sanger sequencing of the pore loops involved in TTX resistance in the skeletal muscle voltage-gated sodium channel gene (*SCN4A*, *SCN8A*, *SCN9A*)

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