



## Is there a pace-of-life syndrome linking boldness and metabolic capacity for locomotion in bluegill sunfish?



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### ARTICLE INFO

#### Article history:

Received 21 June 2016

Initial acceptance 13 July 2016

Final acceptance 21 July 2016

MS. number: A16-00548

#### Keywords:

aerobic capacity  
anaerobic capacity  
behavioural syndrome  
locomotor performance  
resting metabolic rate

The concept of behavioural syndromes (i.e. correlations between behavioural traits) has provided an important framework for understanding individual variation in animal behaviour and its link to individual variation in physiology and life-history traits. The pace-of-life syndrome concept posits that behavioural, physiological and life-history traits coevolve in response to correlated selection pressures, and therefore predicts a positive correlation between boldness (i.e. exploration and risk taking) and metabolic capacity for locomotor performance in individuals. We tested for a pace-of-life syndrome linking boldness and metabolic capacity for locomotor activity in juvenile bluegill sunfish, *Lepomis macrochirus*. Individual fish were screened and classified as bold or shy using an established refuge emergence test. Subsequently, the aerobic and anaerobic metabolisms of bold and shy individuals were quantified using respirometry and by measuring the metabolic by-products of white muscle anaerobic glycolysis following exhaustive exercise, respectively. Bold fish demonstrated 25% greater metabolic scope for activity (i.e. aerobic capacity) than shy fish, which was attributable to a 15% greater maximum metabolic rate. However, there was no significant difference in resting metabolic rate or anaerobic energy expenditure (i.e. anaerobic capacity) between bold and shy fish. These results partially support a pace-of-life syndrome linking boldness and aerobic metabolism in juvenile bluegill sunfish, but did not reveal a link between boldness and anaerobic metabolism. Our findings suggest that aerobic and anaerobic capacities may be subject to different selection pressures, and that physiological processes governing maximum anaerobic performance in fishes are independent from behavioural and physiological traits related to boldness.

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Understanding the origins and maintenance of interindividual variation in behaviour within populations has received considerable attention among behavioural and evolutionary ecologists over the past two decades (Foster & Endler, 1999; Wilson, 1998) because of its importance for the evolution of behavioural adaptations (Dingemanse & Réale, 2005; Smith & Blumstein, 2008). The concept of behavioural syndromes provides a conceptual framework for understanding individual variation in animal behaviour (Sih, Bell, & Johnson, 2004; Wilson & McLaughlin, 2007). Behavioural syndromes are suites of correlated behaviours that exist across

situations, such that the absolute expression of a particular behavioural trait (e.g. aggression) by individuals may vary across situations, but the relative expression (i.e. rank order) of that behaviour among individuals remains consistent (Sih et al., 2004). Behavioural syndromes can be expressed at the individual, population or species level (Atwell et al., 2012; Ingley, Rehm, & Johnson, 2014; Sih et al., 2004). Although behavioural syndromes can be influenced by experience (Bell & Sih, 2007; Brown, Burgess, & Braithwaite, 2007; Frost, Winrow-Giffen, Ashley, & Sneddon, 2007), be domain specific (e.g. feeding, mating, antipredator; Coleman & Wilson, 1998; Wilson & Stevens, 2005) and covary with ecological conditions (Dingemanse et al., 2007), persistence of individual behavioural differences across situations implies that behaviour is not infinitely plastic; that is, not all individuals are capable of expressing the optimum behaviour in all situations (Sih et al., 2004).

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One ecologically important axis of behavioural variation is the shy–bold continuum (Wilson, Clark, Coleman, & Dearstyne, 1994). Boldness may be defined as an animal's willingness to take risks and explore novel objects or environments, such that bolder individuals tend to behave normally or become actively exploratory when confronted with novel situations, whereas shyer individuals retreat or become vigilant (Wilson et al., 1994; Wilson & McLaughlin, 2007). Boldness has been linked to several ecologically important behaviours, including learning (Dugatkin & Alfieri, 2003; Guillette, Reddon, Hurd, & Sturdy, 2009; Sneddon, 2003), aggression (Huntingford, 1976; Riechert & Hedrick, 1993; Sundström, Petersson, Höjesjö, Johnsson, & Järvi, 2004), mate choice (Godin & Dugatkin, 1996), locomotor activity (Sneddon, 2003; Wilson & Godin, 2009) and dispersal or migratory tendency (Chapman et al., 2011; Fraser, Gilliam, Daley, Le, & Skalski, 2001). A number of studies (Ariyomo, Carter, & Watt, 2013; Brown, Burgess, et al., 2007; Mazué, Dechaume-Moncharmont, & Godin, 2015; van Oers, Drent, De Goede, & Van Noordwijk, 2004; Sinn, Apiolaza, & Moltschanivskyj, 2006) suggest that boldness has a genetic basis and may be a heritable trait in some species. However, which behavioural trait leads to greater fitness depends on the specific environment, with bold individuals typically faring better in low-risk, stable environments, and shy individuals faring better in high-risk, variable environments (Sih et al., 2004).

Boldness and certain physiological traits in individual animals may be intercorrelated, thereby forming behaviour–physiology syndromes. Previous investigations of physiological correlates of boldness have focused primarily on sex differences (Ariyomo et al., 2013; Harris, Ramnarine, Smith, & Pettersson, 2010; Ingley et al., 2014), growth rate (Brown, Jones, & Braithwaite, 2007; Mas-Muñoz, Komen, Schneider, Visch, & Schrama, 2011; Ward, Thomas, Hart, & Krause, 2004), the glucocorticoid stress response (Atwell et al., 2012; Raynaud & Schradin, 2014; Thomson, Watts, Pottinger, & Sneddon, 2011) and resting metabolic rate (Careau, Thomas, Humphries, & Réale, 2008; Finstad, Forseth, Ugedal, & Næsje, 2007; Lantová, Zub, Koskela, Šíchová, & Borowski, 2011; Väättäinen, 2013). Resting metabolic rate (RMR; often used synonymously with basal metabolic rate in endotherms, or standard metabolic rate in ectotherms) is the minimum energy required for an animal to maintain physiological homeostasis (Careau et al., 2008). Two competing cause-and-effect models have emerged to explain observed relationships between boldness and resting metabolic rate (Careau et al., 2008). The 'performance model' states that a higher RMR is needed to maintain the digestive and metabolic machinery that supports boldness-related behaviours (e.g. higher levels of activity and aggression), and thus predicts that RMR will be positively correlated with boldness. In contrast, the 'allocation model' states that animals with finite energy resources must manage a limited energy budget, such that available energy should be allocated either to RMR or to boldness-related behaviours (Careau et al., 2008). Consequently, the allocation model predicts a negative correlation between RMR and boldness. To date, most empirical studies have favoured the performance model over the allocation model (Biro & Stamps, 2010), but depending on the system the two principles may act concurrently and cancel each other out, resulting in no net phenotypic correlation (Careau, Killen, & Metcalfe, 2014).

Related to the performance model described above, the pace-of-life syndrome concept posits that behavioural, physiological and life-history traits coevolve in response to correlational selection pressures (Réale et al., 2010), and thus, in addition to predicting a positive relationship between boldness and RMR, it also predicts a positive relationship between boldness (i.e. exploration and risk taking) and metabolism supporting aerobic and anaerobic locomotor activity (Le Galliard, Paquet, Cisel, & Montes-Poloni, 2013). Curiously, despite the observation that boldness has been linked to

increased locomotor activity (Sneddon, 2003; Wilson & Godin, 2010) and greater predation risk (Dugatkin, 1992; Milinski, Lüthi, Egger, & Parker, 1997), few studies have examined the relationship between boldness and locomotor performance (Careau & Garland, 2012; Farwell & McLaughlin, 2009; Le Galliard et al., 2013). Moreover, we are unaware of any studies that have directly tested for correlations between boldness and metabolic (aerobic and anaerobic) capacity to perform locomotor activities; although, Killen et al. (2014) have shown that aerobic capacity, but not anaerobic capacity, is correlated with dominance in damselfish, *Pomacentrus amboinensis*. In vertebrates, low-intensity, sustainable locomotor activity (e.g. foraging and migration) is fuelled by oxidative phosphorylation (aerobic metabolism) and is limited by an individual's metabolic scope for activity (MSA), the difference between maximum metabolic rate (MMR) and RMR (Beamish, 1978; Bennett, 1978). In contrast, high-intensity locomotor activity (e.g. prey capture and predator evasion) is fuelled primarily by anaerobic glycolysis (anaerobic metabolism), which results in a buildup of the metabolic by-product, lactate, and is only sustainable for several seconds to a few minutes (Beamish, 1978; Bennett, 1978). The pace-of-life syndrome concept predicts that both aerobic (i.e. MSA) and anaerobic capacity (anaerobic energy expenditure; AEE) should be greater in bold individuals than in shy ones.

In the current study, we tested for a pace-of-life syndrome linking boldness and metabolic capacity for locomotor activity in wild-caught juvenile bluegill sunfish, *Lepomis macrochirus*. Juveniles of this species are an excellent organismal model for our study because (1) they have been shown to exhibit strong and consistent individual differences in boldness (Wilson & Godin, 2009), (2) boldness in this species is correlated with higher levels of activity and greater use of fast undulatory swimming over slow labriform swimming (Wilson & Godin, 2010) and (3) many aspects of their behavioural ecology have been characterized (Spotte, 2007). We hypothesized that juvenile bluegill sunfish would exhibit a pace-of-life syndrome linking boldness to both aerobic and anaerobic metabolism, and therefore, we predicted that bold fish would exhibit greater RMR, greater aerobic capacity (MSA) and greater anaerobic capacity (AEE) than shy fish.

## METHODS

### Experimental Animals

Juvenile bluegill sunfish ( $N = 82$  in July 2009 and  $N = 56$  in July 2010) were angled from near-shore, shallow habitats in Lake Opinicon, Ontario, Canada ( $44^{\circ}33'32''N$ ,  $76^{\circ}19'41''W$ ), using small barbless hooks baited with a small piece (~1 cm) of earthworm. To minimize angling stress and injury, we used a standardized angling protocol that required all fish be landed, have the hook removed and be placed into a cooler with fresh lake water within 10 s of the hook being set (i.e. from when the fish bit down on the hook). Any angled fish with visible signs of injury (i.e. hooked somewhere other than upper or lower jaw) or disease, or that took longer than 10 s to land and free from the hook, were excluded from the study.

Captured sunfish were placed in a cooler (50.4 litres;  $56 \times 30 \times 30$  cm) containing lake water and transferred to shore-side laboratory facilities at the Queens' University Biological Station (Lake Opinicon; total transit time <30 min), where they were housed individually in shaded plastic tanks (39.5 litres;  $52 \times 40 \times 19$  cm) overnight for at least 16 h before being used in experiments the next day. Holding tanks were supplied continuously with fresh, aerated lake water, and artificial plants were added to each tank to provide refuge and minimize holding stress (Portz, Woodley, & Cech, 2006). Water temperature ranged from 20.6 to 24.4 °C in 2009 and from

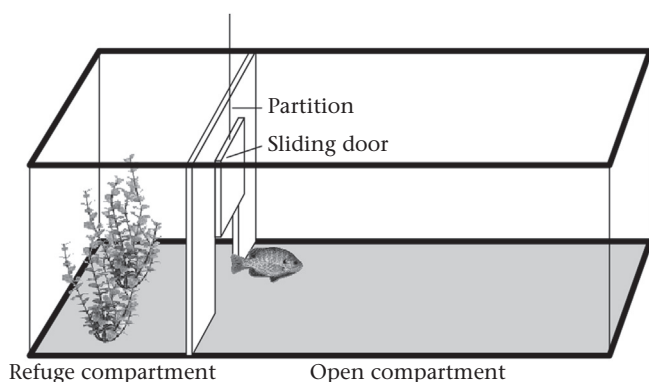
22.8 to 23.9 °C in 2010. Fish were not fed between the time of capture and the end of experiments.

### Boldness Behavioural Assay

On the day after capture, we assessed individual fish for their boldness using the established refuge emergence test, modified for use in juvenile bluegill sunfish (Wilson et al., 2011; Wilson & Godin, 2009) and which has been shown in this species to be highly repeatable within individuals, even after 1–3 months post-release in the wild (Wilson & Godin, 2009). The premise of the emergence test is that an individual fish's boldness is related to its willingness to leave refuge to explore a novel environment, such that a shorter latency to emerge from a refuge into a novel environment is inferred to represent greater boldness or risk taking. Notwithstanding its potential limitations (Beckmann & Biro, 2013; Carter, Feeney, Marshall, Cowlishaw, & Heinsohn, 2013), this test has been widely used to quantify individual boldness in diverse taxa, including fishes (Carter et al., 2013; Näslund, Bererhi, & Johnsson, 2015).

Test sunfish were carefully netted from holding tanks and transferred individually in water to one of two identical test aquaria (Wilson et al., 2011; 82-litre glass aquarium, 92 × 30 × 30 cm; Fig. 1). Each aquarium was divided into two compartments by an opaque white Plexiglas partition (located 25 cm from one side of the aquarium) equipped with a sliding door to allow movement of the fish between compartments. The smaller compartment had two artificial plants fixed to its substratum and served as a potential 'refuge' (occupying highly vegetated habitats reduces individual predation risk in sunfish; Spotte, 2007), whereas the larger compartment was left open and served as a 'novel environment'. The bottom of the test aquarium was covered with aquarium gravel, and the ends and back of the aquarium were covered with cardboard to minimize external disturbances. Both aquaria were uniformly illuminated overhead with fluorescent lights. Between trials, water in the aquaria was drawn down using an aquarium pump and replaced with fresh aerated lake water.

At the beginning of each trial, we gently placed the test subject into the refuge compartment of the test aquarium with partition door closed. After a 15 min acclimation period, we remotely raised the partition door with remote pulley system, allowing the fish free access to the larger open compartment. An observer sat quietly



**Figure 1.** Schematic representation of the behavioural arena used to assay juvenile bluegill sunfish for boldness. The aquarium was divided into two compartments, separated by an opaque white Plexiglas partition equipped with a sliding door (controlled remotely by a pulley) that allowed movement of the fish between compartments. The smaller compartment of the aquarium contained refuge in the form of two artificial aquarium plants anchored to the gravel substratum, whereas the larger compartment was open and served as a novel environment. Boldness was assigned based on the time it took individuals to leave shelter and swim into the open compartment.

behind a blind in front of the aquarium and recorded the time elapsed (=latency time) for the subject to leave the refuge and enter the novel open environment for up to a maximum of 20 min. Subjects that emerged from the refuge within 20 min were categorized as 'bold' and those that did not were categorized as 'shy'. To validate our use of this 20 min criterion for dichotomous assignment to shy and bold groups, we allowed 18 test subjects that did not exit the refuge within 20 min up to an additional 40 min (total 60 min maximum) to do so. The 20 min cutoff appeared to be valid, as all but two (89%) of the 18 shy fish that were left in the test tank for up to 1 h remained in the refuge for at least 40 min and 72% remained in the refuge for the full hour.

At the end of each refuge emergence trial, we carefully netted the test subject from the test aquarium and returned it to either its holding tank for later assessment of its aerobic metabolism (in 2009) or into an opaque Plexiglas 'black box' (to promote recovery from anaerobic disturbance during transfer and handling) for later assessment of its anaerobic metabolism (in 2010). Each box contained three equal-sized chambers (20 × 15 × 15 cm), with a 2.5 cm diameter hole in each partition to allow fresh lake water to flow through the box. Water depth in the boxes was 11 cm and fresh water flow ranged from 1.6 to 2.0 litres/min.

### Relationship Between Boldness and Aerobic Metabolism

In 2009, we randomly selected four individuals each day from the test subjects screened for boldness within the previous 2 days (and at least 18 h earlier) for measurement of their maximum metabolic rate (MMR) and resting metabolic rate (RMR). To achieve MMR, test subjects ( $N = 36$ ) were subjected to an established exhaustive exercise protocol before being placed into a respirometer chamber for measurement of oxygen consumption. Fish were chased individually to exhaustion in a 50 cm diameter annular tank containing fresh, aerated lake water. The chasing protocol involved manually chasing the subject while grasping at its tail to induce burst swimming. The subject was considered to be exhausted when it stopped swimming and failed to respond to tail grasping on three consecutive attempts. Once exhausted, the fish was quickly measured for body mass and total volume (determined by measuring water displacement), and was then placed into one of four identical respirometry chambers. Time between end of exhaustion protocol and placement in the respirometer was less than 60 s.

We measured the oxygen consumption of individual fish for 15 h using an automated, intermittent flow-through respirometry system (Loligo Systems, Tjele, Denmark), similar to that described in Herskin (1999). Four glass chambers (volume ~0.75 litres) were immersed in a 220-litre tank supplied with fresh, aerated water from Lake Opinicon. Water was recirculated through each chamber using a series of pumps controlled by AutoResp™ 4 software (Loligo Systems, Tjele, Denmark). The system alternated between a 5 min closed recirculating measurement phase and a 4 min open flushing phase, which were separated by a 30 s delay period during which water was recirculated through the chamber but no measurements were taken. Each glass chamber was outfitted with a fibre optic oxygen probe, which was connected to an OXY-4 oxygen meter (Loligo Systems, Tjele, Denmark) for measurement of changes in oxygen partial pressure ( $P_{O_2}$ ).  $P_{O_2}$  was recorded every second during the 5 min measuring phase, and a linear regression was calculated between  $P_{O_2}$  and time. The slope of the regression line ( $k$ ) was used to calculate oxygen consumption according to the equation:

$$M_{O_2} = k \times V_{\text{resp}} \times M^{-1} \times \alpha \quad (1)$$

where  $M_{O_2}$  is oxygen consumption rate (in mg of  $O_2$  per kg per h),  $V_{\text{resp}}$  is the volume of the respirometry chamber (in litres),  $M$  is fish



mass (in kg), and  $\alpha$  is the solubility of  $O_2$  in water at the experimental temperature (Cruz-Neto & Steffensen, 1997). All slopes used in the calculations were derived from regressions where  $r^2 > 0.95$ . This method yielded one  $M_{O_2}$  calculation every 9.5 min. MMR was the highest rate of oxygen consumption measured during the trial, RMR was the mean of the three lowest oxygen consumption rates measured during the trial, and metabolic scope for activity (MSA) was calculated as the difference between MMR and RMR.

In addition to the above metrics, we also calculated duration of post-exhaustion  $M_{O_2}$  elevation and excess post-exercise oxygen consumption (also known as EPOC). Excess post-exercise oxygen consumption is equal to the area under the curve relating post-exercise  $M_{O_2}$  over time to RMR (Scarabello, Heigenhauser, & Wood, 1991; Svendsen et al., 2010), and was calculated for each fish by taking the integral of the following double exponential equation:

$$M_{O_2} = ae^{k_1t} + be^{k_2t} + c \quad (2)$$

where  $a$ ,  $k_1$ ,  $b$ ,  $k_2$  and  $c$  are constants, and  $e$  is the base of the natural logarithm. All constants except  $c$ , which was the calculated RMR for each fish, were estimated using the nonlinear least squares regression package, 'nls2', in R (Grothendieck, 2013). To account for natural intraindividual fluctuations in RMR, the fish were considered to have reached their resting state once  $M_{O_2}$  decreased to RMR + 10% (Kaufmann, 1990; Svendsen et al., 2010).

#### Relationship Between Boldness and Anaerobic Metabolism

In 2010, half ( $N = 28$ ) of the fish screened for boldness underwent the exhaustion protocol described above and the other half ( $N = 28$ ) was used as control (i.e. euthanized without undergoing exhaustion) to establish baseline levels of anaerobic muscle metabolites. Immediately following exhaustion, we euthanized the treatment fish by cerebral percussion and excised samples of white muscle, which were wrapped in aluminium foil and transferred to liquid nitrogen. Time from end of exhaustion protocol to transfer of muscle sample into liquid nitrogen was less than 60 s. The control fish were similarly euthanized. Sixteen of 28 control muscle samples and three of 28 treatment (one bold, two shy) muscle sample were mistakenly thawed prior to metabolic assays, and consequently unusable in our analyses. Final sample size for anaerobic metabolite analysis was 12 for control individuals, 11 for bold individuals and 14 for shy individuals.

We measured the concentrations of muscle lactate, adenosine triphosphate (ATP) and phosphocreatine (PCr) as described in Gingerich and Suski (2012). Metabolite extract was prepared as in Booth, Kieffer, Tufts, Davidson, and Bielak (1995). Briefly, ~0.5 g of frozen muscle tissue was ground to a fine powder under liquid nitrogen using a pre-cooled mortar and pestle. A volume of perchloric acid (PCA) solution (8% PCA, 1 mM of EDTA) equal to four times the ground tissue mass was added to each sample. The samples were then vortexed for 10 s, incubated at 4 °C for 10 min and then centrifuged for 5 min at 3000 rpm. The supernatant was neutralized to pH 7.5 ( $\pm 0.5$ ) with a neutralizing solution (2 M of KOH, 0.4 M of KCl, 0.3 M of imidazole), vortexed for 10 s and centrifuged a final time at 10 000 g. Final supernatant was stored at  $-80$  °C. Metabolite assays were adapted from Lowry and Passonneau (1972) for use in microwell plates. Briefly, we treated a 5  $\mu$ l of sample with 3 U of lactate dehydrogenase from bovine heart (Sigma catalogue number L3916; 1000 U/ml) over 4 h in 150  $\mu$ l of hydrazine solution (1.1 M of NAD<sup>+</sup>, 200 mM of hydrazine, pH 9.5). Absorbance was determined at 340 nm. We determined lactate concentration using a lactic acid standard curve (0–4.44 mM). ATP and PCr concentrations were determined using a coupled enzymatic assay (Lowry & Passonneau,

1972). Samples were diluted 1:10 v/v in 50  $\mu$ l of dextrose buffer (1 mM of dextrose, 1.3 M of MgCl<sub>2</sub>, 0.5 mM of NAD<sup>+</sup>, 0.5 U of glucose-6-phosphate dehydrogenase (1 U/ml; Sigma catalogue number G 5760)) and background absorbance was measured to reduce error from glucose dehydrogenase activity. ATP concentration was then determined through two stepwise reactions. First, 1.5 U of hexokinase (1500 U/ml; Roche catalogue number 11426362001) was added to promote the first reaction whereby glucose was converted to glucose-6-phosphate. Previously added glucose-6-phosphate dehydrogenase completed the second reaction of glucose-6-phosphate to 6-P-gluconolactone with release of NADH. Final absorbance of the ATP reactions was measured at 340 nm after 30 min and concentration was determined using ATP standard curve (0–1.25 mM). PCr concentration was determined by adding an additional 50  $\mu$ l of dextrose buffer and 1  $\mu$ l of glycine buffer (0.25 M of glycine, 0.10 U/ml of creatine kinase (350 U/mg; Roche catalogue number 10127566001), 5 mM of ADP) and measuring final absorbance of the PCr reaction at 340 nm after 1 h. PCr concentration was determined using PCr standard curve (0–4.0 mM).

We estimated anaerobic energy expenditure (AEE) in ATP equivalents (McDonald, McFarlane, & Milligan, 1998) for each fish using the following equation:

$$AEE = (\Delta \text{lactate}) \times 1.5 + \Delta \text{ATP} + \Delta \text{PCr} \quad (3)$$

where  $\Delta$  is the difference between post-exhaustion values for either bold or shy individuals and the mean baseline value from nonexhausted control fish. Among the 12 control fish samples (four bold, eight shy), there was no significant difference between bold and shy individuals ( $t$  tests: lactate:  $t_{10} = 0.219$ ,  $P = 0.831$ ; ATP:  $t_{10} = 0.149$ ,  $P = 0.885$ ; PCr:  $t_{10} = -0.669$ ,  $P = 0.519$ ); therefore, data were pooled to yield a single mean baseline value for each metabolite.

#### Statistical Analyses

All statistical analyses were performed in the R statistical environment (R Core Team, 2015). We compared fish mass, fish length, duration of elevated  $M_{O_2}$  and excess post-exercise oxygen consumption between bold and shy individuals using two-sample  $t$  tests (R Package 'stats', 't.test' function;  $\alpha = 0.05$ ). MMR, RMR and MSA were also compared between bold and shy fish using general linear models (hereafter, 'GLM'; R Package 'stats', 'glm' function;  $\alpha = 0.05$ ), with boldness (i.e. bold or shy) as a fixed factor and water temperature and fish mass as linear covariates. We compared differences in lactate, ATP, PCr and AEE among treatments (i.e. control, bold, shy) using analysis of covariance models (hereafter, 'ANCOVA'; R Package 'stats', 'aov' function;  $\alpha = 0.05$ ), with treatment group as a fixed factor and fish mass as a linear covariate. Where a significant difference among treatment groups was identified, we used the post hoc Tukey's test (R Package 'stats', 'TukeyHSD' function;  $\alpha = 0.05$ ) to identify which paired comparisons were significant. All means are reported with standard error (SE) where appropriate.

#### Ethical Note

The experimental protocols (treatment of fish) used in this study were approved by the Animal Care Committee at Carleton University (protocol number B10-26) and adhered to guidelines for the use and care of research animals of the Canadian Council on Animal Care and ASAB/ABS, and thus to the legal requirements of Canada. The exhaustive exercise protocol (i.e. chasing) used in this study has been widely used for studying swimming capacity in fishes. Studies on sunfish (Heath & Pritchard, 1962) and other fish species

(reviewed in Kieffer, 2000; Milligan, 1996) indicate that the physiological effects of forced exhaustive swimming, including acid–base and ion disturbance, buildup of lactate and elevation of the stress hormone cortisol, are similar to those that occur during angling and tend to return to normal levels within about 12 h. By necessity, fish used to assay for anaerobic capacity had to be euthanized so that white muscle could be excised. Every attempt was made to minimize the number of fish that were killed, while still retaining sufficient statistical power to detect a difference between bold and shy fish if present. All other fish were healthy and returned to the lake at the end of the study.

## RESULTS

### Boldness Behavioural Assay

A greater number of fish that underwent screening for boldness with our refuge emergence test were classified as shy than were classified as bold (Table 1). In 2009, 52 of 82 (1.7:1) fish were classified as shy, and in 2010, 32 of 56 (1.3:1) fish were classified as shy. Bold fish in 2009 were, on average, 3% greater in length ( $t$  test:  $t_{74} = 2.435$ ,  $P = 0.017$ ) and 11% greater in mass ( $t_{74} = 2.167$ ,  $P = 0.033$ ) than shy fish, but there was no significant difference in length ( $t_{54} = 0.371$ ,  $P = 0.712$ ) or mass ( $t_{54} = -0.111$ ,  $P = 0.912$ ) between bold and shy fish in 2010 (Table 1). Refuge emergence times among fish classified as bold were similar between the 2 years, ranging from 13 s to 19.8 min, and averaging ( $\pm$ SE)  $7.34 \pm 0.90$  min in 2009 and  $8.07 \pm 1.26$  min in 2010 (Table 1).

### Relationship Between Boldness and Aerobic Metabolism

Of 36 individuals randomly selected for respirometry, 22 were classified as shy and 14 as bold. Data from one shy individual was excluded from analyses because the  $R^2$  value for the regression lines used to calculate oxygen consumption rates was below 0.95 for 98 out of 106 measurements obtained. Bold and shy fish showed similar patterns of post-exhaustion oxygen consumption (Fig. 2a). As expected, oxygen consumption was highest during the period immediately following exhaustive exercise. For all bold fish, the first  $M_{O_2}$  measurement was always highest. However, for shy fish,  $M_{O_2}$  was highest during the first measurement in 81% (17 of 21) of individuals, during the second measurement in 9.5% (2 of 21) of individuals and during the third measurement in 9.5% (2 of 21) of individuals. In both groups, mean  $M_{O_2}$  decreased sharply during the first 20–30 min post-exhaustion, but remained elevated above resting level for many hours (Fig. 2a). Neither duration of elevated  $M_{O_2}$  ( $t$  test:  $t_{33} = 0.251$ ,  $P = 0.804$ ) nor excess post-exercise oxygen consumption ( $t_{33} = 0.706$ ,  $P = 0.485$ ) differed significantly between bold and shy fish. Bold fish had elevated  $M_{O_2}$  for  $8.68 \pm 0.54$  h and shy fish had elevated  $M_{O_2}$  for  $8.5 \pm 0.50$  h. Mean  $\pm$  SE excess post-exercise oxygen consumption during the period of elevated  $M_{O_2}$  totalled  $2.12 \pm 0.14$  mg of  $O_2/g$  for bold fish and  $2.00 \pm 0.11$  mg of  $O_2/g$  for shy fish.

On average, bold fish had 25% greater MSA than shy fish (GLM:  $t_{31} = -2.767$ ,  $P = 0.009$ ; Fig. 2b). Mean  $\pm$  SE MSA was  $0.375 \pm 0.024$  mg of  $O_2$  per g per h for bold fish and  $0.300 \pm 0.015$  mg

of  $O_2$  per g per h for shy fish ( $t_{31} = -2.866$ ,  $P = 0.007$ ; Fig. 2b). RMR, on the other hand, did not differ between the two groups ( $t_{31} = 0.414$ ,  $P = 0.682$ ; Fig. 2b). Neither temperature (which varied by less than 4 °C) nor mass was a significant covariate for any of the three metabolic measurements (GLM:  $df = 31$ ,  $P \geq 0.179$ , for all tests).

### Relationship Between Boldness and Anaerobic Metabolism

There was no evidence of a difference in anaerobic metabolism between bold and shy individuals. Exhaustive exercise increased muscle lactate (ANCOVA:  $F_{2,33} = 191.8$ ,  $P < 0.001$ ; Tukey's test:  $P < 0.001$  for pairwise comparison of bold and shy against control) and decreased ATP (ANCOVA:  $F_{2,33} = 36.4$ ,  $P < 0.001$ ; Tukey's test:  $P < 0.001$  for pairwise comparison of bold and shy against control) and PCr (ANCOVA:  $F_{2,33} = 12.3$ ,  $P < 0.001$ ; Tukey's test:  $P < 0.001$  for pairwise comparison of bold and shy against control), relative to controls. However, there was no significant difference in post-exhaustion muscle lactate levels (Tukey's test:  $P = 0.765$ ), ATP (Tukey's test:  $P = 0.952$ ), or PCr (Tukey's test:  $P = 0.999$ ) between bold and shy fish (Fig. 3). Similarly, there was no significant difference in white muscle AEE between bold and shy fish (ANCOVA:  $F_{1,22} = 0.411$ ,  $P = 0.528$ ; Fig. 3). Estimated anaerobic capacity (i.e. AEE) was similar for bold (mean  $\pm$  SE =  $23.4 \pm 0.9$   $\mu$ mol of ATP/g) and shy fish (mean  $\pm$  SE =  $24.4 \pm 1.1$   $\mu$ mol of ATP/g). Mass was not a significant covariate for any of the anaerobic metabolite measurements (ANCOVA: lactate:  $F_{1,33} = 0.041$ ,  $P = 0.841$ ; ATP:  $F_{1,33} = 3.746$ ,  $P = 0.062$ ; PCr:  $F_{1,33} = 1.261$ ,  $P = 0.269$ ; AEE:  $F_{1,22} = 0.966$ ,  $P = 0.336$ ).

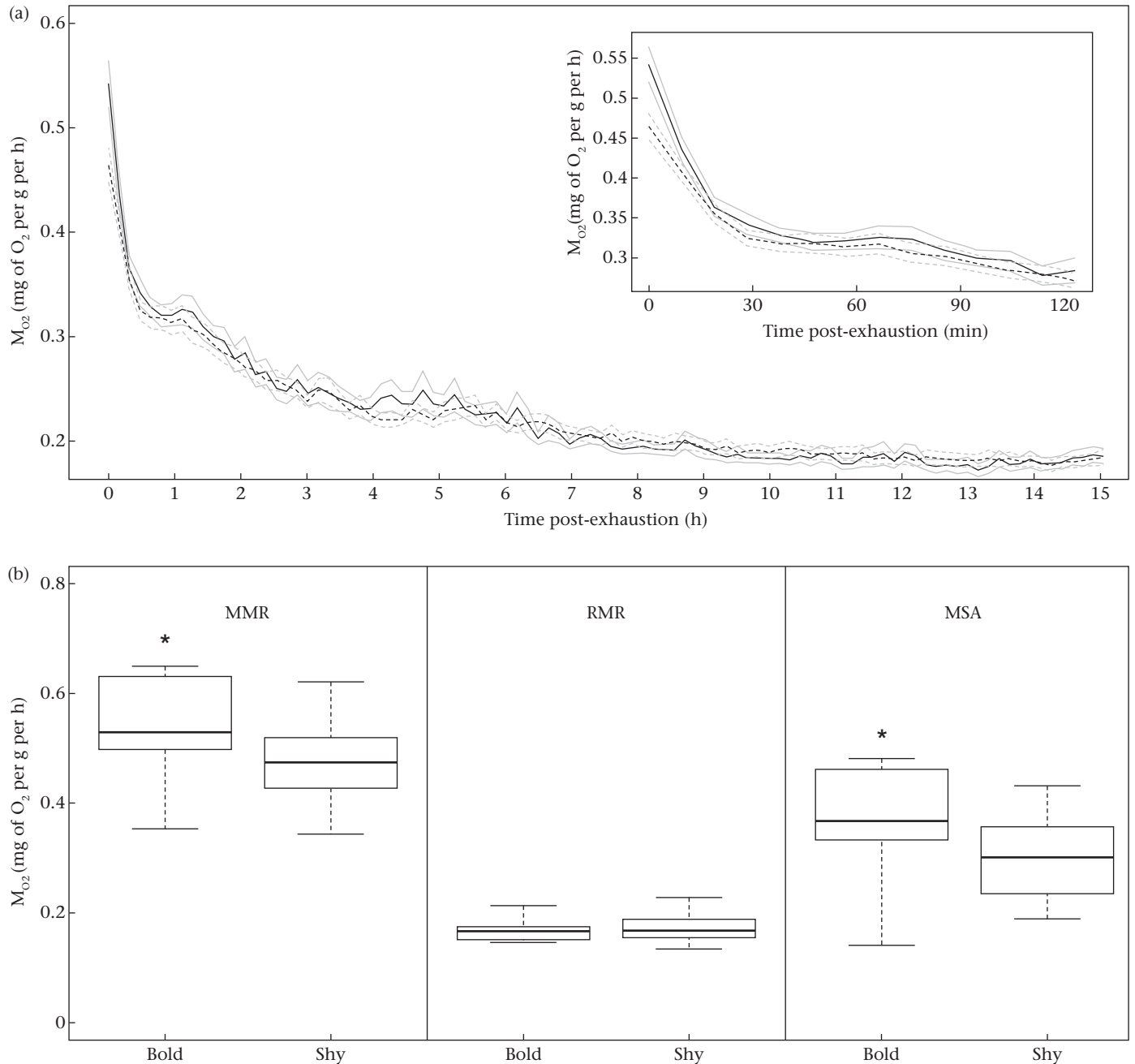
## DISCUSSION

Bold juvenile bluegill sunfish exhibited a significantly greater aerobic capacity for locomotor activity than shy bluegill sunfish, with the observed 25% greater mean metabolic scope (MSA) in bold fish reflecting a higher maximum metabolic rate (MMR) rather than a lower resting metabolic rate (RMR). We did not test in our study whether this relationship persists over time; however, based on results from previous studies on fishes, we predict that observed interindividual differences in both boldness and aerobic metabolism of juvenile bluegill sunfish should be consistent over time. Indeed, Wilson and Godin (2009) showed previously that boldness in juvenile bluegill sunfish, as determined by a refuge emergence test, is repeatable over a period of at least 1–3 months. No comparable data on repeatability of metabolic rate are available for juvenile sunfish, but studies on a variety of fish species have demonstrated temporal repeatability of both resting metabolic rate (Cutts, Adams, & Campbell, 2001; Maciak & Konarzewski, 2010; Marras, Claireaux, McKenzie, & Nelson, 2010; McCarthy, 2000) and maximum metabolic rate (Marras et al., 2010). In fact, Nespolo and Franco (2007) performed a meta-analysis of studies that addressed repeatability of whole-animal measures of metabolic rate and found that repeatability was nearly ubiquitous, irrespective of taxa (i.e. insects, fish, reptiles, birds and mammals), metabolic measurement (e.g. RMR, SMR, MRM), or time between measurements. However, repeatability of metabolic rate can

**Table 1**

Mean  $\pm$  SE body mass, body length and emergence latency for juvenile bluegill sunfish screened for boldness in 2009 and 2010 using an established refuge emergence test

Year	Shy/bold	N	Body mass (g)	Body length (mm)	Emergence latency time (min)
2009	Bold	30	23.6 $\pm$ 0.8	113 $\pm$ 1	7.34 $\pm$ 0.90
	Shy	52	21.3 $\pm$ 0.5	110 $\pm$ 1	NA
2010	Bold	24	21.0 $\pm$ 0.9	108 $\pm$ 2	8.07 $\pm$ 1.26
	Shy	32	21.1 $\pm$ 0.8	108 $\pm$ 1	NA

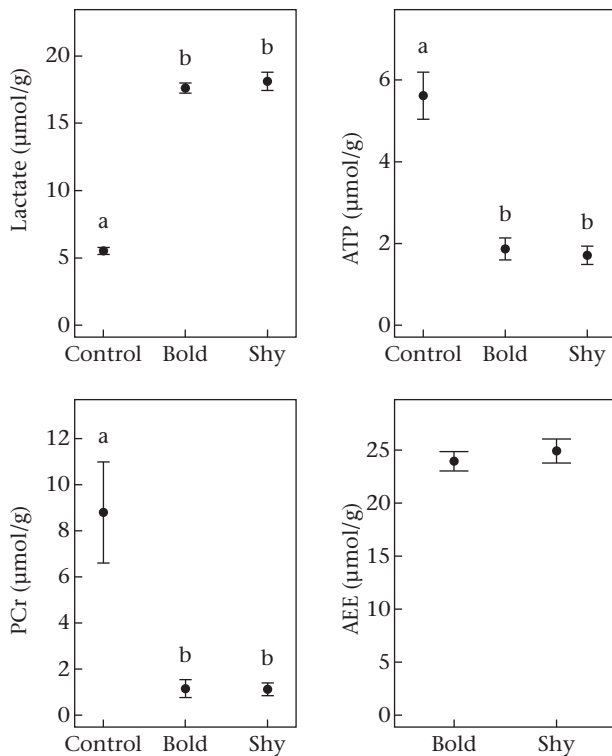


**Figure 2.** Relationship between boldness and aerobic metabolism in juvenile bluegill sunfish. (a) Mean metabolic rate ( $\pm$ SE in grey) of bold (solid line) and shy (dashed line) fish, measured for 15 h after being subjected to an exhaustive exercise protocol. Inset shows the first 2 h in greater detail. (b) Box plots comparing maximum metabolic rate (MMR), resting metabolic rate (RMR) and metabolic scope for activity (MSA) between bold and shy fish. An asterisk signifies a significant difference ( $\alpha = 0.05$ ) between bold and shy individuals.

decrease over long periods (White, Schimpf, & Cassey, 2013), particularly if environmental conditions (e.g. food availability) change (Norin & Malte, 2011).

The observed relationship between boldness and metabolic scope in the current study is consistent with the pace-of-life syndrome concept (Réale et al., 2010), which predicts a positive correlation between boldness and aerobic capacity for locomotion (Le Galliard et al., 2013). Killen et al. (2014) similarly observed a positive correlation between aerobic capacity and dominance in damselfish, *P. amboinensis*. However, while both studies suggest a relationship between behaviour and aerobic capacity, we cannot differentiate whether these behavioural traits

and aerobic metabolism are linked through inherent differences in physiology or reflect phenotypic plasticity in aerobic metabolism. Le Galliard et al. (2013) observed a weak positive relationship between boldness and both maximum sprint speed and endurance in juvenile common lizards (*Zootoca vivipara*), but these relationships were not present during the younger juvenile stage, which suggests that divergence in locomotor performance occurred during the first year of life. Le Galliard et al. (2013) were sceptical that differences in locomotor performance in their study were due to biomechanical or physiological differences among individuals, and instead attributed differences in locomotor performance to temperament-related differences in motivation to run. Our results



**Figure 3.** Relationship between boldness and anaerobic metabolism in juvenile bluegill sunfish. Mean  $\pm$  SE post-exercise muscle lactate, adenosine triphosphate (ATP) and phosphocreatine (PCr) concentrations were determined for bold and shy fish subjected to an exhaustive exercise protocol. Nonexhausted fish (control) were used as baseline for calculating anaerobic energy expenditure (AEE). AEE was calculated as in McDonald et al. (1998). There were no significant differences between bold and shy individuals in any of the above measures.

do not support this conclusion for bluegill sunfish, but rather indicate that there is a direct link between individual boldness and the biochemical processes governing aerobic locomotor performance.

Greater aerobic capacity in bold sunfish might have been a direct consequence of higher routine activity levels in bold individuals. Although we did not measure routine activity in the current study, previous studies on bluegill sunfish have shown that bold individuals stray further from shelter (Wilson et al., 2011) and are more active than shy individuals (Wilson & Godin, 2009, 2010). Physiological changes in response to exercise have been noted in several fish species (Davison, 1997). Exercise training in fishes has been shown to increase locomotor performance by increasing the percentage contribution of red muscle to overall muscle mass (Meyer-Rochow & Ingram, 1993; Sanger, 1992; Young & Cech, 1993), improving cardiovascular performance (Davie, Wells, & Tetens, 1986; Farrell, Johansen, & Suarez, 1991; Hinterleitner, Huber, Lackner, & Wieser, 1992; Sanger, 1992) and enhancing metabolic function (Farrell et al., 1991; Hinterleitner et al., 1992). Furthermore, the act of exercise training itself may alter personality traits in individuals. For example, mosquitofish, *Gambusia holbrooki*, that were forced to swim continuously for 28 days showed an increase in both boldness and aggression that was reversed after 21 days of inactivity (Sinclair, Souza, Ward, & Seebacher, 2014). There are other possible explanations for the correlation between boldness and aerobic capacity, including the presence of genetically linked differences in aerobic metabolism, and the possibility that bold individuals might compensate for riskier behaviours by voluntarily attaining a higher level of athletic capacity (Halsey, 2016). Therefore, further research into the

proximate cause(s) of boldness-related differences in aerobic capacity is needed.

RMR did not differ between bold and shy individuals in our study, suggesting that juvenile bluegill sunfish conform to neither the performance or allocation models of energy metabolism proposed by Careau et al. (2008). The conceptual framework outlined by Biro and Stamps (2010) links energy metabolism to personality and life-history traits through the hypothesis that behavioural traits that require greater energy will also require a 'larger metabolic engine', with higher energy costs for maintenance (i.e. the performance model of metabolism; Careau et al., 2008). Our finding that bold juvenile sunfish had greater MMR suggests that boldness is indeed associated with a 'larger metabolic engine'; therefore, it is surprising that we did not also see a corresponding difference in RMR between bold and shy fish. To date, the majority of studies comparing behaviour and metabolism have supported the performance model (Biro & Stamps, 2010); however, failure to observe a significant relationship between personality and RMR (here we use RMR synonymously with standard metabolic rate) has not been uncommon, particularly in fishes (Farwell & McLaughlin, 2009; Killen et al., 2014; Myles-Gonzalez, Burness, Yavno, Rooke, & Fox, 2015; Vaatainen, 2013). Performance and allocation processes may occur simultaneously, cancelling each other out so that no phenotypic correlation is detectable (Careau et al., 2014). We, therefore, offer two possible explanations for the lack of difference in RMR between bold and shy juvenile sunfish. First, a positive relationship between RMR and boldness in our juvenile fish may have been masked by a proportionally large allocation of energy towards somatic growth. Second, failure to observe a relationship between RMR and boldness in the current and other fish studies could be due to comparatively low metabolic cost of maintaining tissues in ectotherms, relative to endotherms, which have as much as seven-fold greater basal energy demand (Frappell & Butler, 2004; Hulbert & Else, 2004).

Anaerobic metabolism has received comparatively less attention in the personality literature than aerobic metabolism (Killen et al., 2014; Metcalfe, Van Leeuwen, & Killen, 2016). Nevertheless, our observation that there was no correlation between boldness and anaerobic capacity in juvenile bluegill sunfish is consistent with two previous studies that tested for correlations between willingness to take risks (i.e. boldness) and anaerobic locomotor performance. Farwell and McLaughlin (2009) observed a positive relationship between foraging activity in the field and willingness to explore a novel environment in juvenile brook trout, *Salvelinus fontinalis*, but did not find a similar relationship between activity and anaerobic swimming performance. Likewise, Le Galliard et al. (2013) found no relationship between willingness to explore a novel environment and anaerobic locomotor performance in juvenile lizards (*Z. vivipara*). However, they did observe a weak positive relationship in yearlings, which they attributed to differences in motivation rather than to differences in physiology.

Use of appropriate tests of locomotor performance is critical to assessing physiological differences in locomotor capacity. Nelson, Gotwalt, Reidy, and Webber (2002) proposed three criteria for developing useful tests of locomotor performance: (1) individual performance should be repeatable over time, (2) tests should be within the range of performance experienced by individuals, and thus have possible relevance to Darwinian fitness, and (3) the performance test should supply information relevant to the in situ biology of the animal. While we did not test the individual repeatability of our performance test, we submit that the chasing protocol, which simulated a predation event, satisfied the latter two criteria. Therefore, we hypothesize that the lack of relationship between boldness and anaerobic capacity in our study might be due to evolutionary constraints on anaerobic locomotor



performance, which is critical in predator–prey interactions. Support for this hypothesis comes from the fact that anaerobic swimming usually constitutes only a small fraction of an individual fish's behavioural repertoire, but white muscle, which is primarily responsible for anaerobic swimming, comprises the bulk of a fish's mass (Altringham & Ellerby, 1999).

In conclusion, our results provide weak support for a pace-of-life syndrome relating boldness and metabolic capacity in juvenile bluegill sunfish. As far as we are aware, ours is the first study to directly test for a pace-of-life syndrome linking boldness and metabolic limits for locomotor performance. Our observation of a greater aerobic scope for activity in bold fish than shy ones partially supports a pace-of-life syndrome relating boldness and maximum capacity for sustained locomotor activity in juvenile bluegill sunfish. However, we were unable to determine whether greater aerobic scope in bold fish represented direct coevolution of these traits, or indirect correlated phenotypic plasticity. In contrast, contrary to the pace-of-life syndrome, we observed no relationship between boldness and anaerobic capacity for locomotor activity in juvenile bluegill sunfish. Killen et al. (2014) similarly provided support for a pace-of-life syndrome linking aerobic capacity, but not anaerobic capacity, to dominance in damselfish. Combined, these results suggest that aerobic and anaerobic capacities may be subject to different selection pressures, and that physiological processes governing maximum anaerobic performance are independent from behavioural and physiological traits related to boldness and dominance. More work is needed to unravel the complexity surrounding the relationships between personality, metabolic capacity and locomotor performance, both in fishes and other taxa. Future studies should attempt to control for motivation by conducting experiments under controlled conditions that use species-specific, or even population-specific, metrics of performance based on evolutionarily relevant stimuli that minimizes opportunity for individuals to perform at submaximal levels (Careau & Garland, 2012).

## Acknowledgments

We thank K. McGrath, S. McConnachie, K. Cook and K. Stamplecoskie for their assistance in the laboratory and two anonymous referees for their constructive comments on the manuscript. This work was funded by the Canada Research Chairs program (S.J.C.) and Discovery grants from the Natural Sciences and Engineering Research Council of Canada to S.J.C. and J.-G.J.G. (no. 8074-2010). We thank the Ontario Ministry of Natural Resources and Forestry for providing scientific collection permits, and also the staff of the Queens University Biological Station for their logistical assistance.

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