The Effects of Fluorescent Tracking Powder on Oxygen Consumption in Salamanders Using Either Cutaneous or Bimodal Respiration

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Fluorescent powder is gaining attention as an effective method for tracking terrestrial amphibian movements, particularly for species that are too small for conventional tracking equipment. The technique requires coating portions of an animal with fluorescent powder, releasing the animal, and following the trail of powder as it is progressively lost during movement. Recent studies have shown that fluorescent powder has no negative effects on survival or growth. However, a substance that coats the skin, a major respiratory organ in most amphibians, may have sublethal effects on performance and consequently behavior. We tested the effect of fluorescent powder application on the respiration of lungless Red-Backed Salamanders, *Plethodon cinereus*, and lunged terrestrial Red-Spotted Newts, *Notophthalmus viridescens*. In comparing species with contrasting skin textures and primary modes of respiration, we expected to find *P. cinereus*, the species relying solely on cutaneous respiration, more sensitive to fluorescent powder. Standard metabolic rate (SMR) and total oxygen consumption for both species were measured before and after application of the powder. We found no significant differences in respiration between control and powdered salamanders. Independent of treatment, SMR was 6–16% higher during the post-treatment trial in both species, and likewise, total oxygen consumed increased by 8–20% in *P. cinereus* and by 7–10% in *N. viridescens*. Our results, in combination with other recent work, suggest that fluorescent powder is a safe technique for tracking amphibians.

A lack of safe and reliable methods for tracking small-bodied animals has in part limited ecological studies of these organisms in natural habitats. However, fluorescent powder pigments have been used extensively for short term studies of movement and habitat use of small mammals (Lemen and Freeman, 1985; Mullican, 1988), insects (Johansson, 1959; Turchin, 1998), and reptiles (Fellers and Drost, 1989; Blankenship et al., 1990; Stark and Fox, 2000; Tuttle and Carroll, 2005). Recently, this tracking technique has been used in studies of amphibians in order to gain insight into terrestrial movements (*Pelobates fuscus*: Eggert, 2002; *Rana clamitans*: Birchfield and Deters, 2005; *Rana sphenocephala*, *Ambystoma opacum*, and *Bufo terrestris*: Graeter et al., 2008; *Notophthalmus viridescens*: Roe and Grayson, 2008; *Plethodon cinereus*: Roberts and Liebgold, 2008). Fluorescent powder applied to terrestrial amphibian skin is gradually shed as the released animal moves, creating a path that can be tracked using a portable ultraviolet or black light (Graeter and Rothermel, 2007). One major advantage of fluorescent powder tracking for amphibians is that the powder temporarily adheres to the skin without the use of an alternate attachment technique. Surgery to implant radio transmitters, as in Rittenhouse (2002), can be problematic for small amphibians and external harnesses, as in Dole (1965), can damage amphibian skin.

When evaluating the safety of a tracking technique for any organism, both the effects on survival and body condition as well as sublethal effects that impact behavior must be evaluated. A major concern associated with the use of fluorescent powder on amphibians is the potential interference of cutaneous gas exchange by the coating of the integument (Windmiller, 1996). Sublethal effects of fluorescent powder on respiration, even if temporary, could affect performance or behavior in a field study. For example, when amphibians are physiologically stressed, they often exhibit water conserving behaviors that could change microhabitat use or activity level (Pough et al., 1983; Wisely and Golightly, 2003). Several recent studies have highlighted the importance of studying the potential for methods of marking or following amphibians in the field to bias results (McCarthy and Parris, 2004; Kinkead et al., 2006; Blomquist and Hunter, 2007).

The purpose of this study was to quantify the effects of fluorescent powder application on respiration in two salamander species (*N. viridescens* and *P. cinereus*). We compared a lunged and a lungless species of salamander to determine whether effects of fluorescent powder application would vary based on reliance on the skin for respiration. We predicted that effects of fluorescent powder on respiration would be greater in the lungless *P. cinereus* compared to the lunged terrestrial stage of *N. viridescens*.

MATERIALS AND METHODS

Study species.—*Plethodon cinereus* is a member of the lungless salamander family Plethodontidae and relies entirely on cutaneous gas exchange for respiration (Feder and Burggren, 1985). Moist, permeable skin limits the activity of these salamanders to areas with suitably moist environments (Spotila, 1972; Grover, 1998). In *N. viridescens*, individuals are lunged after metamorphosis for the remainder of their terrestrial and aquatic life (Gage, 1891). In most northern parts of its range, *N. viridescens* is terrestrial through a juvenile stage (commonly called the red eft stage) and returns to an aquatic habitat to reproduce upon sexual maturity. After the breeding season, adult newts may return to the terrestrial habitat, reducing the tail fin and redeveloping granular skin that is more resistant to desiccation (Brimley, 1921; Walters and Greenwald, 1977). While the skin still has a role in gas exchange in post-metamorphic newts, Whitford and Hutchison (1965) showed that over 50% of respiration in the Roughskin Newt, *Taricha granulosa*, is pulmonary. *Notophthalmus viridescens* and *P. cinereus* were chosen for this study based on their local abundance in southwestern Virginia, their differences in skin texture and respiration mode, and their use

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in previous fluorescent powder tracking studies (Roberts and Liebgold, 2008; Roe and Grayson, 2008).

Twenty-four terrestrial adult *N. viridescens* and 24 adult *P. cinereus* were collected from Salt Pond Mountain at Mountain Lake Biological Station (Giles County, Virginia, USA, 37°22′32″N, 80°31′20″W, elevation 1160 m). *Notophthalmus viridescens* were housed individually in four-liter plastic shoeboxes with a substrate of damp leaf litter from the collection site. *Plethodon cinereus* were housed in 0.8-liter round plastic containers with a substrate of moistened paper towels. All salamanders were maintained in the laboratory at room temperature, approximately 22°C. *Notophthalmus viridescens* were fed earthworms (*Lumbricus* sp.) or crickets (*Acheta domesticus*), and *P. cinereus* were fed fruit flies (*Drosophila* sp.) twice per week until seven days prior to experiments.

Measurement of respiration.—By measuring oxygen consumption rates (O_2 ml/hr) over two consecutive 22-hour respiratory trials, we were able to quantify the total amount of oxygen consumed (O_2 ml) and estimate the standard metabolic rates (SMR) of *N. viridescens* and *P. cinereus* before and after application of fluorescent powder (i.e., a repeated measures design). Prior to the first respiratory trial, salamanders were fasted for seven days to ensure that each individual was postabsorptive. On the fifth day of the fasting period, we began a procedure to acclimate the salamanders to the respirometry chambers. Each individual was weighed to the nearest 0.01 g before being placed in a respirometry chamber (a glass culture bottle, either 100 ml for *N. viridescens* or 35 ml for *P. cinereus*). Each chamber contained a double layer of 2.5 cm × 2.5 cm paper towel moistened with 2 ml of distilled water to keep individuals hydrated during acclimation and respirometry measurements. Each chamber was sealed with a screw cap modified with compression fittings for individual air channels. Salamanders were allowed to acclimate in the chambers unattached to the respirometer in a dark environmental chamber for 24 hr at 22°C followed by 24 hr at 15°C, the temperature at which the respiratory trials were conducted.

Oxygen consumption measurements started after the acclimation and fasting period. Paper towels and water were changed before the chambers were connected to an indirect, closed circuit respirometer system (Micro Oxymax, Columbus Instruments, Columbus, OH) interfaced to a desktop computer. We checked each chamber for leaks before each trial. For each trial an empty 100 ml glass culture bottle containing a medical battery (Duracell Procell Zinc Air Medical, DA 146, 8.4 Volts) that consumes a known amount of O_2 served as a control. Prior to each trial, the instrument was calibrated using a certified gas mixture. For each salamander, O_2 (ml/hr) was measured every 66 minutes for a total of 20 measurements over a 22-hour period. Prior to measurements, every air sample was dried using a hygroscopic drier containing nafion tubing (Columbus Instruments, Columbus, OH). Carbon dioxide was also measured in each air sample and O_2 (ml/hr) adjusted accordingly by the Micro Oxymax software. All measurements were corrected for standard temperature and pressure by the software. After every eight measurements, the air in the chambers was completely refreshed. All respirometry measurements were started between 0800 and 1130 hrs.

Following the pre-treatment measurements, individuals were paired by mass. One individual of each pair was randomly selected to receive a powder application treatment (12 *N. viridescens* and 12 *P. cinereus*) and the other was designated as a control (12 *N. viridescens* and 12 *P. cinereus*). The pairing was done to evenly spread variation in mass between the treatments. Individuals receiving the powder treatment were removed from the chamber, grasped gently behind the head, and then dipped lightly into a shallow plastic container containing dry yellow fluorescent powder (Radiant Color, now available from DayGlo Color Corp., Cleveland, OH). Powder was applied to cover the ventral and lateral surfaces on the lower half of the body and tail while carefully avoiding the head and eyes. To control for the effects of the application procedure, control salamanders were treated in an identical manner with an empty plastic container. After treatment, both powder-treated and control salamanders were immediately returned to the chambers for the post-treatment trial. After the post-treatment trial, which was conducted identically to the pre-treatment trial described above, salamanders were removed from the chambers, rinsed with distilled water, blotted to remove excess moisture, and weighed.

Data analysis.—For each salamander, we determined total oxygen consumption and SMR. These two metabolic parameters provide different insights into the bioenergetic status of an animal and are most powerful when measured simultaneously (DuRant et al., 2007). Whereas total oxygen consumption allows estimation of the total energy expended over a prescribed time period, SMR allows estimation of energy required for basic maintenance over the same period. If total oxygen consumption changes as a result of an environmental factor such as powder application, this change could be attributable to factors other than increases in SMR. Most notably, altered activity patterns caused by distress can be detected measuring total oxygen consumption, but not necessarily by measuring SMR. Conversely, animals could compensate for an increase in SMR by decreasing activity or other energy demands, resulting in no change in total oxygen consumption (DuRant et al., 2007).

Total oxygen consumption was calculated as the total volume of O_2 (ml) consumed over the 22-hour respiratory trial. To estimate SMR, we had to reduce the influence of activity on our estimates (Janes and Chappell, 1995). We used the lowest quartile value, the boundary between the lowest two quarters of the data set, as the estimate of SMR. This method removes the measurements influenced by activity bouts, producing a reliable baseline estimate of SMR in all individuals (Hopkins et al., 2004; DuRant et al., 2007). The change in SMR and total oxygen consumption from the first and second trials was compared between control and fluorescent powder-treated individuals using a repeated measures ANCOVA for each species with mass included as a covariate (Proc Mixed, SAS, SAS Institute, Cary, NC). We specified the covariance structure as compound symmetric. Mass and SMR were log transformed to achieve normality in both statistical models for *N. viridescens*, but this adjustment was not necessary for either analysis for *P. cinereus*.

RESULTS

Application of fluorescent powder had no significant effect on total oxygen consumption or SMR in either species ($F < 0.45$, $P > 0.51$ in all cases; Figs. 1, 2). However, independent of treatment, SMR was 6–16% higher during the post-treatment trial compared to the pre-treatment trial in both species (*N. viridescens*: $F_{1,21} = 6.71$, $P = 0.02$; *P. cinereus*: $F_{1,21} = 12.58$, $P =$

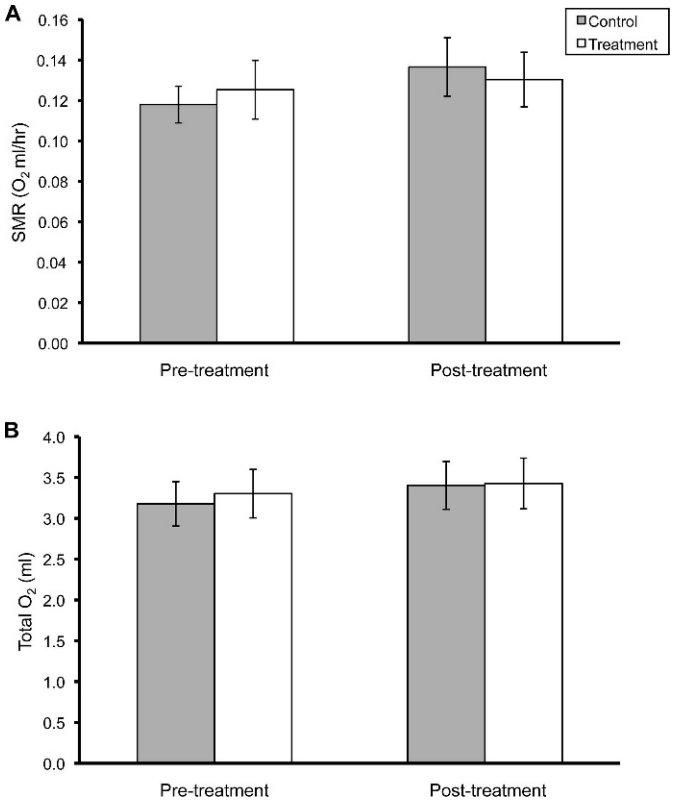

Fig. 1. Repeated measures of (A) standard metabolic rate (SMR, O_2 ml/hr) and (B) total oxygen consumption (O_2 ml) over the entire 22-hour trial in Eastern Red-Spotted Newts (*Notophthalmus viridescens*) weighing 2.36 ± 0.07 g SE. Treatment individuals received an application of fluorescent powder between the first and second trial. Control individuals were taken through the same handling procedure with no fluorescent powder applied (see methods). n = 12 treatment and 12 control individuals. Means are ± 1 SE.

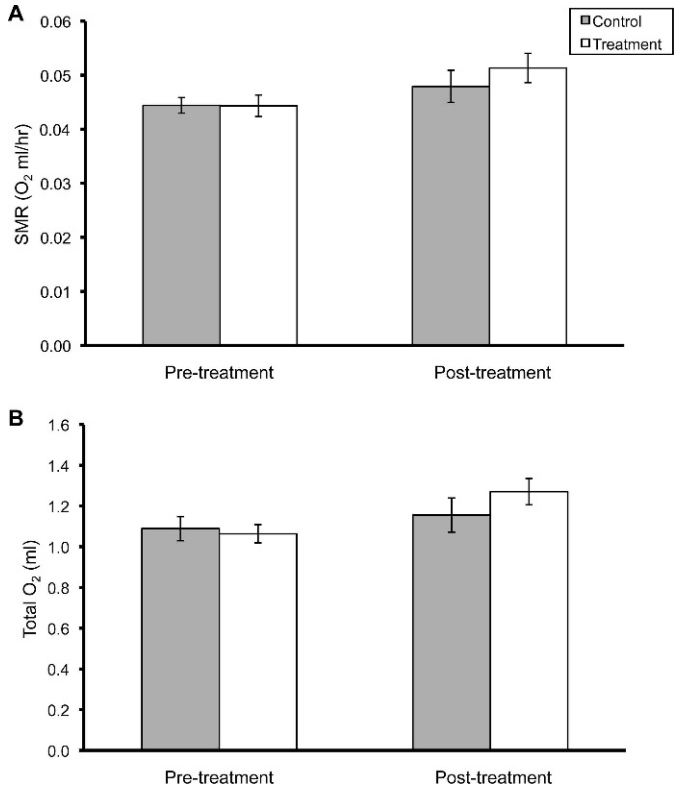

Fig. 2. Repeated measures of (A) standard metabolic rate (SMR, O_2 ml/hr) and (B) total oxygen consumption (O_2 ml) over the entire 22-hour trial in Red-Backed Salamanders (*Plethodon cinereus*) weighing 1.10 ± 0.02 g SE. Treatment individuals received an application of fluorescent powder between the first and second trial. Control individuals were taken through the same handling procedure with no fluorescent powder applied (see methods). n = 12 treatment and 12 control individuals. Means are ± 1 SE.

0.002). Similarly, total oxygen consumption was 8–20% higher during the post-treatment trial for *P. cinereus* ($F_{1,21}$ = 7.76, P = 0.01). Total oxygen consumption in *N. viridescens* also increased by 7–10% in the post-treatment trial; however, this difference was not statistically significant ($F_{1,21}$ = 2.58, P = 0.12). The interaction between treatment and trial did not significantly affect total oxygen consumption or SMR in either species (in all cases, $F < 2.39$, $P > 0.14$).

Average mass loss was negligible after the two trials for each species. For *N. viridescens*, average mass prior to the start of the experiment was 2.36 ± 0.07 g (Mean ± 1 SE hereafter) compared to 2.29 ± 0.08 g after the respirometry measurements (mass range = 1.66–3.01 g). Similarly, the average mass of *P. cinereus* was 1.10 ± 0.02 g before and 1.08 ± 0.02 g after the respirometry measurements (mass range = 0.83–1.24 g). In *N. viridescens* mass significantly affected SMR ($F_{1,21}$ = 7.78, P = 0.01) and total oxygen consumption ($F_{1,21}$ = 10.07, P = 0.005), but not in *P. cinereus* for either SMR ($F_{1,21}$ = 3.07, P = 0.09) or total oxygen consumption ($F_{1,21}$ = 2.63, P = 0.12), likely due to the narrow size range available for the latter species.

DISCUSSION

This study investigated the effects of covering amphibian skin with fluorescent powder on respiration, a valid concern given the substantial respiratory role of the integument in the majority of amphibian species (Duellman and Trueb, 1986). By studying a lungless terrestrial salamander, *P. cinereus*, which relies exclusively on cutaneous respiration, we examined a species likely to be sensitive to fluorescent powder application.

In both species examined, *N. viridescens* and *P. cinereus*, repeated measures of oxygen consumption did not differ between control individuals and individuals given an application of fluorescent powder. We did find a significant effect of trial. Standard metabolic rate of both species and total oxygen consumption in *P. cinereus* were higher during the post-treatment trial compared to the pre-treatment trial. This small increase could be due to handling stress from the application procedure (Davis and Schreck, 1997). Control salamanders were handled in exactly the same manner as salamanders receiving powder. This short procedure may have elevated oxygen consumption compared to the pre-treatment trial, where all individuals were acclimated to the chambers and not handled for 48 hours before the start of respirometry measurements.

Fluorescent powder has also been shown to have no significant effects on survival and growth in controlled studies of three species of amphibians. Rittenhouse et al. (2006) showed that fluorescent powder did not affect short-term water loss or survival and mass gain over six weeks in *Ambystoma maculatum* and *Rana sylvatica*. They suggested the method seems to be similar to being covered with soil or organic debris. In addition, Roe and Grayson (2009) found

no significant effects of daily fluorescent powder application on body condition in terrestrial juvenile and adult *N. viridescens* over two weeks.

We conclude, in corroboration with previous studies, that using fluorescent powder for tracking has minimal physiological effects on amphibians. Even in what we considered to be a worst case scenario, use of the powder on a species that relies solely on cutaneous respiration, we were unable to detect any adverse effects on metabolism. In fact, our results suggest that the mild stress of being handled may actually have a greater effect on respiration than having the skin surface covered with fluorescent powder. Thus, fluorescent powder trailing appears to be a safe method for following amphibians in the field, and little or no bias in movement measures should be expected due to interference with integumental gas exchange.

As in previously published studies, we noticed that the powder did not adhere long to the skin, with salamanders ending the 22-hour respirometry trial with the majority of the powder lost. While it is possible that repeated (e.g., daily) application of the powder could eventually affect respiration, available data suggests that important characteristics, such as growth, are not modified even after weeks of use (Rittenhouse et al., 2006; Roe and Grayson, 2009). However, because the chemicals used in commercial powders can vary (Roe and Grayson, 2009), we recommend that investigators check the composition of powders before applying them to the delicate skin of amphibians.

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