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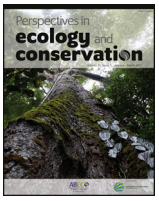

Research Letters

Can you count on counting? Retrieving reliable data from non-lethal monitoring of micro-snails

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ABSTRACT

Using non-lethal methods is especially important for the monitoring and conservation planning of endangered species and their habitats. The goal of this study is to check whether the current method of monitoring endangered micro-snail populations by soil sampling, which involves killing the snails and altering their microhabitat, could be replaced by a non-lethal method. The invasive and time-consuming soil sampling and analyzing is compared with searching the individuals by eye. Research was conducted in moist sedges meadows and seasonally inundated wetlands in western Poland and focused on two species of micro-snails differing in biology, i.e. *Vertigo moulinsiana* (a climbing species) and *Vertigo angustior* (a litter-dwelling species). Both vertiginid species are listed in the Annex II of the EU Habitat Directive and included in the IUCN Red List of Threatened Species. The results showed a significant correlation between the total number of individuals searched by eye and collected from soil samples of *V. angustior* and *V. moulinsiana*. Results of this study indicate that monitoring micro-snails by searching individuals by eye is feasible and may reduce further pressure of killing individuals of endangered species. Limitations of this method should however be taken into account – especially its inaccuracy in estimating the absolute abundance of the studied species.

Introduction

Studies on invertebrate biology and ecology often involve killing animals studied, and the rationale and ethical questions behind this practice raise vigorous discussion in literature (e.g. Horvath et al., 2013; Minteer et al., 2014). Killing animals is justified for some purposes, e.g. for describing new species for which holotypes are needed – although, even in this case, this need is questioned by some (e.g. Donegan, 2008; Minteer et al., 2014). Still, lethal research methods are used as a standard in studies where collecting individuals for documentation is not crucial, such as gathering data on invertebrate abundance, biodiversity or community structure (Lecq et al., 2015). Often, such studies are connected with killing hundreds or even thousands of animals in a single sample, including representatives of a number of non-target species (e.g. Tepedino et al., 2015). Such a practice is especially controversial and inconsistent for species that are threatened by extinction. Moreover, the data gathered this way reflect a situation that no longer exists because the animals are dead and not present in the ecosystem, and the conservation status in the studied area worsens (e.g. Jewell, 2013). Efforts are employed to develop non-lethal methods, which would provide similar accuracy to the standard-but-invasive methods (e.g. Lecq et al., 2015; Parris et al., 2010; Tepedino et al., 2015).

Invasively disturbing the endangered species and their habitats may be particularly pronounced in the case of routine monitoring carried out for the purpose of conservation planning (Jewell, 2013; Parris et al., 2010). Such activities are conducted regularly for many years at the same site, which can contribute to the depletion of studied populations and to the degradation of their habitat (Jewell, 2013). On the other hand, assessing species abundance is a crucial requirement in monitoring, providing pivotal information for making conservation decisions (Nichols and Williams, 2006; Lovett et al., 2007; Parris et al., 2010). Knowledge on patterns and trends in population numbering is essential for interpretation of monitoring results as well as planning conservation schemes (Lovett et al., 2007; Jewell, 2013). Moreover, monitoring invertebrate species, especially the smallest ones, using such methods requires considerable effort in sample processing (e.g. Cardoso et al., 2011;

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Książkiewicz and Gołdyn, 2015). These obstacles contribute to the dearth of focus on invertebrates in conservation studies and policies worldwide (e.g. Cardoso et al., 2011; Zamin et al., 2010).

Molluscan biodiversity is considered the most threatened by extinction according to the IUCN Red List (Lydeard et al., 2004; Régnier et al., 2009, 2015). Low motility of molluscs, especially terrestrial gastropods prevents them from escaping disturbances (e.g. Wiktor and Riedel, 2002) which makes them good bioindicators of local habitat quality and conditions (Gerlach et al., 2013). Studies on ecology and distributions of terrestrial molluscs usually involve collecting litter and soil samples and processing them in the laboratory (e.g. Sulikowska-Drozd and Horsák, 2007; Książkiewicz et al., 2013). The method gives precise results on the species abundance per unit area. However, it involves killing snails present in the sample and usually causes significant disturbances within their habitat, frequently located within protected areas. Other methods of assessing the abundance of terrestrial molluscs, which are meant to be more effective, are based on washing collected samples and separating shells from other material (e.g. Williamson, 1959; Horsák, 2003). Such methods are also relatively time-consuming, do not eliminate the aspect of killing the snails and significantly interfere with their habitat. Some researches recommend the cardboard sheets for collecting snails (Boag, 1982; Hawkins et al., 1998). This method however, is technically unwieldy, gives questionable results (e.g. the sheet can be moved by a wind) and it is recommended rather for qualitative research of slugs (Coppolino, 2010).

European conservationists focused their attention especially on one family of molluscs, namely Veritiginidae (Gastropoda: Pulmonata). Within vertiginids, two of the globally threatened species are *Vertigo moulinsiana* (Dupuy, 1849) and *Vertigo angustior* Jeffreys, 1830 (Pokryszko, 2003). These two species are hygrophilous terrestrial gastropods that usually occur in unshaded sedge meadows or seasonally inundated wetlands (e.g. Pokryszko, 1990; Cameron et al., 2003; Killeen, 2003). *V. angustior* grows to a maximum of 1.9 mm in height, and *V. moulinsiana*, reaches a maximum of 2.7 mm in height (Pokryszko, 1990). *V. moulinsiana* is usually found in *Glyceria maxima* swamps, sedge marshes or fens (Pokryszko, 1990; Killeen, 2003). Habitats of this species are characterized by high water levels – the highest population densities are reached when water levels fluctuate from 0 to 0.6 m (Killeen, 2003). *V. moulinsiana* lives over a large vertical range at different times of the year (Killeen, 2003) and tends to climb on monocots (*G. maxima*, sedges, reed) up to 2 m above ground level in late summer, especially when the water levels are high (e.g. Killeen, 2003; Tattersfield and McInnes, 2003). The climbing behavior of *V. moulinsiana* has been used in developing a monitoring methodology, which is currently based on searching snails by eye and counting individuals adhered to plants (e.g. Moorkens and Killeen, 2011; Lipińska et al., 2012). On the other hand, *V. angustior* is a litter-dweller (occasionally this species is found 10–15 cm up the stems of plants; e.g. Cameron, 2003; Moorkens and Killeen, 2011) and does not tolerate inundation (e.g. Cameron, 2003; Moorkens and Gaynor, 2003). Thus, it is usually found in moderately moist microhabitats (in western Poland *V. angustior* usually occurs in sedge meadows; Książkiewicz, 2010), although some parts of its habitat may be inundated (e.g. Jankowiak and Bernard, 2013; Książkiewicz et al., 2013; Książkiewicz and Gołdyn, 2015). For *V. angustior*, monitoring is at least partially based on collecting samples of leaf litter and processing them in the lab – a time-consuming procedure, lethal for the snails and most of the other invertebrates collected with the sample (e.g. Moorkens and Killeen, 2011; Książkiewicz et al., 2012).

Both species are listed in the annex II of the EU Habitat Directive which imposes an obligation to state members of the EU to monitor populations of these animals. The fulfillment of this obligation is however impeded due to at least two reasons. First, the size of these species makes them difficult to detect on their wetland localities (Książkiewicz and Gołdyn, 2015). Second, their habitats may be difficult to investigate due to inundation.

This study focuses on developing a universal method for monitoring micro-snails using the *V. moulinsiana* and *V. angustior* – two hygrophilous species differing in biology. We check if the currently utilized non-lethal method of searching by eye and counting specimens of *V. moulinsiana* in the field is efficient and reliable when compared to results obtained from soil samples. We also check if the time-consuming, invasive and lethal method of sampling for *V. angustior* may be replaced by a visual assessment of the population, analogous to the one already used for *V. moulinsiana*.

Material and methods

Research was conducted in western Poland in July and August 2013. The average annual precipitation in the study area is about 550 mm (Richling and Ostaszewska, 2006). The total rainfall in the region for the study period was about 85 mm which was considerably lower than the mean for the same period during the last five years (157.2 mm). Mean temperature oscillated around 21 °C (the mean for the last five years was 19.3). As part of a national monitoring program, snails were surveyed in nine sites located in six areas differing in protection regime–seven sites hosted *V. moulinsiana* and seven were inhabited by *V. angustior* (Table S1 and Fig. S1 in Supplementary material). Sampled sites were usually marshy, moist sedge meadows or reed beds. Most of them were moderately or severely disturbed due to lowering water levels and eutrophication resulting from human activities and natural processes.

We applied the same sampling procedure for *V. angustior* and *V. moulinsiana*. The study was conducted by two experienced malacologists (i.e. the authors of this manuscript), working in parallel on each site. Each of the malacologists independently designated one or two plots (depending on homogeneity of the habitat) per each species in each of the studied sites. As recommended in the literature (Moorkens and Killeen, 2011; Książkiewicz et al., 2012; Lipińska et al., 2012), the plots were selected to best fit the requirements of the studied species (Cameron, 2003; Killeen, 2003; Jankowiak and Bernard, 2013; Książkiewicz et al., 2013) since the snails are usually aggregated in a particular type of microhabitat (Cameron and Pokryszko, 2005). The plots chosen for both species were not inundated, however, microhabitats selected for *V. moulinsiana* were waterlogged or highly moist, covered with tall monocots (e.g. Killeen, 2003; Lipińska et al., 2012). In the case of *V. angustior*, we selected moist and moderately moist microhabitats (for details see Cameron et al., 2003). In each of the plots, one monitoring square of 25 cm × 25 cm (0.0625 m^2) was designated with the Økland frame (Økland, 1929) (i.e. in one site, one or two monitoring squares were designated by each malacologist, resulting in two to four squares per site for each species). In total, we collected data from 24 plots for each species.

In each square, one person spent 15 min counting the snails present in the litter (the litter was examined without removing), the top layer of soil and vegetation (stems and leaves) growing on the monitoring square. To avoid re-counting, counted individuals were removed temporarily from the square and when the counting finished – snails were returned to the sample. Each square was searched only once. After the visual examination, from the same squares where counting occurred, we removed the litter and dead vegetation, cut all vegetation to the ground level and collected soil to a depth of 2 cm (Cameron, 2003). The material from the squares was placed in separate plastic bags and transported to the laboratory. In the laboratory, each sample was dried on a paper sheet for two weeks. After this time, the material was divided into two fractions using a 0.5 mm sieve. Particles passing through the sieve were checked manually for the presence of snails under a

stereomicroscope. The retained fraction with the cut plants was carefully examined for snails using a magnifying glass. Processing of the samples was carried out by the two malacologists. For statistical analyses, we used only individuals that were alive during the sampling event; snails extracted from samples were identified and classified as alive or dead at the time of sampling based on shell wear and the presence or absence of a dried body (Cameron, 1982). Since the monitoring methods usually utilize separate indexes for the total population and juvenile abundance (or age structure), the snail samples were divided into adult and juvenile cohorts based on their shell development (e.g. Pokryszko, 1990; Lipińska et al., 2012).

To assess whether results of both methods showed similar results, the data on species numbering collected for each square using both methods was compared using Spearman rank correlation with a Monte Carlo permutation test (10,000 permutations) and a Wilcoxon signed-rank test for pairwise comparisons (we used nonparametric methods because of the lack of normality). In the calculations we considered data from each square as the basic sampling unit. Calculations were performed using RundomPro 3.14 software (Jadwiszczak, 2009) with $p < 0.05$ as the significance level. Due to the conservation status of the studied snail species (both species are listed in the EU Habitat Directive, see also IUCN Red List of Threatened species – Killeen et al., 2012; Moorkens et al., 2012), the number of samples has been limited to the minimum necessary for this study (Zar, 2014) following recommendations for monitoring of *Vertigo* sites in Poland (Książkiewicz et al., 2012; Lipińska et al., 2012).

Results

In total, on all the sampling squares we recorded 27 individuals of *V. angustior* using the searching by eye method and 128 individuals in collected samples. For *V. moulinsiana*, we noted 64 individuals when searching by eye and collected 187 individuals from samples (Tables 1 and 2).

The total number of individuals counted when searched by eye and when collected from samples was correlated for both *V. angustior* (rho = 0.687, $p < 0.001$) and *V. moulinsiana* (rho = 0.851, $p < 0.001$). Correlation was also statistically significant for juvenile and adult *V. angustior* (for juveniles: rho = 0.559, $p = 0.005$; for adults: rho = 0.612, $p = 0.001$,) and *V. moulinsiana* (for juveniles: rho = 0.741, $p < 0.001$; for adults: rho = 0.796, $p < 0.001$).

Despite the correlation, the Wilcoxon signed-rank test showed statistically significant differences for the total number of individuals and juveniles for both species when results collected using both methods were compared (*V. moulinsiana*: total number: $Z = -3.033$, $p = 0.001$; juveniles: $Z = -3.218$, $p = 0.001$; *V. angustior* total number: $Z = -2.708$, $p = 0.004$; juveniles: $Z = -2.814$, $p = 0.002$). The difference was not statistically significant for adults (*V. moulinsiana*: $Z = -0.663$, $p = 0.638$; *V. angustior*: $Z = -1.697$, $p = 0.109$).

Discussion

Our results indicate that the less time-consuming and non-lethal method provides data highly correlated with results from sample analyses for juveniles, adults, and the total number of individuals for both *V. angustior* and *V. moulinsiana*. On the other hand, differences between the number of individuals counted and collected from samples are significant for juveniles and the total number of individuals of these two species. Therefore, the non-lethal method does not provide reliable data on the absolute abundance of the species studied, but the results obtained could be effectively used to assess the conservation status of a population (see Jewell, 2013). It should be also considered that increasing the sampling area could provide more precise estimates (it is especially important in the case of smaller populations) although would not directly make the results more comparable.

V. moulinsiana, which is the largest of all *Vertigo* species in Europe and climbs tall vegetation in the summer and autumn (Pokryszko, 1990; Killeen, 2003; Moorkens and Killeen, 2011), seemed to be an easier species to count than litter-dwelling *V. angustior* (e.g. Pokryszko, 1990). For this reason, population assessment in monitoring programs is already based on counting *V. moulinsiana*. Monitoring methods for *V. angustior* either apply a time-consuming and invasive method of sample analyses or detect the presence of this species without the assessment of abundance in chosen polygons (Moorkens and Killeen, 2011; Książkiewicz et al., 2012; Lipińska et al., 2012). Thus, when both species co-occur on the same monitoring plot, both methods should be used in parallel. According to our study, these monitoring procedures may be unified and simplified. If we assume that in inundated microhabitats most of the individuals of *V. moulinsiana* indeed climb up plants (escaping from water), the method of counting them on vegetation works. However, for highly moist or marshy but not inundated microhabitats (as in this study), this method provides incomplete data since most of the individuals (especially the juveniles) stay in the litter and soil (see Table 1). Since counting only the snails on vegetation does not provide reliable data on species abundance, this method should be complemented with counting the snails remaining in the litter and the top layer of soil, as performed during our research.

Results for *V. angustior* surprisingly show that counting the litter dwelling snails may be informative and bring reliable data about the species' relative numbering (but not on their absolute abundance). The results obtained using both methods (i.e. lethal, that involves soil and litter sampling as well as non-lethal biased on visual examination) give highly correlated results and the number of adults was not significantly different when they were compared. Thus, a faster, non-lethal and less invasive counting method could be successfully used for comparing particular localities and judging general population trends.

It should be noted that the counting method may fail when the habitat is inundated as well as in dry and hot years when snails aestivate, and may be more efficient for large sized snail species. Monitoring of microsnails should be performed by a highly-qualified personnel (optimally an expert assigned permanently to a specific site), having a wide experience with monitored species and able to complete the monitoring process in the field. Snails like *Vertigo* sp. are very small and are easily overlooked by the beginners, moreover experience is needed in interpreting traits used for species determination. Identification based on photos of snails collected by nonqualified workers is definitely not recommended – the specimens are too minute to obtain a good picture in the field. Such practice was shown to be error-prone regardless of the experience of person determining the species on photos even in the case of much bigger animals (e.g. Austen et al., 2016; Ceríaco et al., 2016).

In conclusion, results of our study show that the development of a universal method of monitoring micro-snails inhabiting moist and/or wet areas (litter dwelling species as well as climbing species) is possible and may be based on a simple and relatively fast method of counting individuals by eye. Moreover, it is not lethal and has a considerably lower impact on the habitat. This method can show trends in relative population numbering (but certainly not in absolute abundance) and with this respect will be characterized by similar efficiency to the time-consuming sample processing, without raising ethical questions typical for destructive methods (Parris et al., 2010; Jewell, 2013; Lecq et al., 2015). Since the method provides data on relative numbering but not on the total abundance calculated per unit area, monitoring schemes based on such a method should use new classification developed especially for

Table 1
The total number of individuals of juvenile and adult *Vertigo moulinsiana* in the studied sites. "Counted" column: counted when searched by eye (non-lethal method); "Sampled" column: extracted from samples (lethal method). The Expert column denotes which of the two malacological experts performed the count (Expert 1 or Expert 2).

Site	Plot	Adults [0.0625 m^2]		Juveniles [0.0625 m^2]		Expert
		Counted	Sampled	Counted	Sampled	
Jezioro Liptowskie	1	4	4	3	22	1
	2	1	0	7	23	2
	3	2	1	2	0	1
	4	2	6	5	13	2
Debrzynka 1	1	0	0	0	0	1
	2	0	0	0	0	2
	3	0	1	0	1	1
	4	1	3	2	6	2
Debrzynka 2	1	2	1	0	2	1
	2	3	3	8	48	2
	3	6	5	0	5	1
	4	1	1	0	4	2
Samborka 1	1	4	3	0	4	1
	2	0	0	0	0	1
	3	1	0	0	0	2
Samborka 2	1	0	0	0	0	1
	2	1	0	0	0	2
	3	0	0	0	2	1
	4	0	0	0	0	2
Jezioro Tuczno	1	0	0	0	0	1
	2	1	0	0	0	1
	3	1	1	1	3	2
Pliszka	1	1	1	1	13	1
	2	2	2	2	9	2

Table 2
Total number of individuals of juvenile and adult *Vertigo angustior* in the studied sites in particular plots. "Counted" column: counted when searched by eye (non-lethal method); "Sampled" column: extracted from samples (lethal method). The Expert column denotes which of the two malacological experts performed the count (Expert 1 or Expert 2).

Site	Plot	Adults [0.0625 m^2]		Juveniles [0.0625 m^2]		Expert
		Countied	Sampled	Counted	Sampled	
Jezioro Liptowskie	1	0	0	0	0	1
	2	0	0	0	0	2
	3	0	0	0	0	1
	4	0	0	0	0	2
Debrzynka 1	1	0	0	0	0	1
	2	0	0	0	0	2
	3	1	3	0	8	1
	4	1	3	0	4	2
Samborka 2	1	0	0	0	0	1
	2	0	0	0	0	2
	3	7	7	0	8	1
	4	0	0	0	0	2
Flinta	1	3	1	0	0	2
	2	0	1	0	0	1
	3	1	26	0	9	2
Szczyra	1	2	1	0	0	1
	2	1	1	0	0	2
Jezioro Tuczno	1	4	3	0	1	1
	2	1	1	0	0	1
	3	1	1	1	10	2
Pliszka	1	2	2	1	11	1
	2	0	3	0	9	2
	3	0	1	0	2	1
	4	0	7	1	5	2

this particular approach to population estimation (Jewell, 2013). Future studies aiming in calibrating the method as well as extrapolating it for the use in the monitoring of other species or the whole molluscan assemblages would be worth conducting.

Conflicts of interest

The authors declare no conflicts of interest.

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The authors of the manuscript declare that had appropriate permissions to conduct presented study; permissions were issued by Regional Directorates for Environmental Protection in Poland.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pecon.2017.03.005.

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