

## COMMENTARY

# Importance of study design and robust analyses in ecology – what is the evidence for silica–vole interactions?

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‘What causes small rodent population cycles’ is a long-standing question in population ecology (Krebs 2013). Silica is one of the principal defences of grasses against herbivores and heavy grazing induces high levels of it in many grass species (McNaughton *et al.* 1985; Vicari & Bazely 1993; Massey, Ennos & Hartley 2007). High intake of silica has been linked to intestinal epithelium damage and lower growth rate of vole individuals (Massey & Hartley 2006; Wiczorek *et al.* 2015a). The latter is an important driver of the timing of vole maturation, which in turn modifies population reproductive output and thus population growth rate (Ergon, Lambin & Stenseth 2001; Krebs 2013). Hence, Massey & Hartley (2006) and Massey *et al.* (2008) proposed that vole grazing at high population densities induces grasses to produce silica-based defences, with a sufficiently long time-lag to lead to a negative delayed density-dependent population trajectory. Such a long time-lag of induction has been demonstrated by Reynolds *et al.* (2012), with other papers having addressed specific aspects of this hypothesis (see recent review by Hartley & DeGabriel 2016). Because the hypothesis that silica affects vole population dynamics is based on logical ecological arguments, experiments aiming at testing these relationships have the potential to really contribute to elucidate a central ecological question.

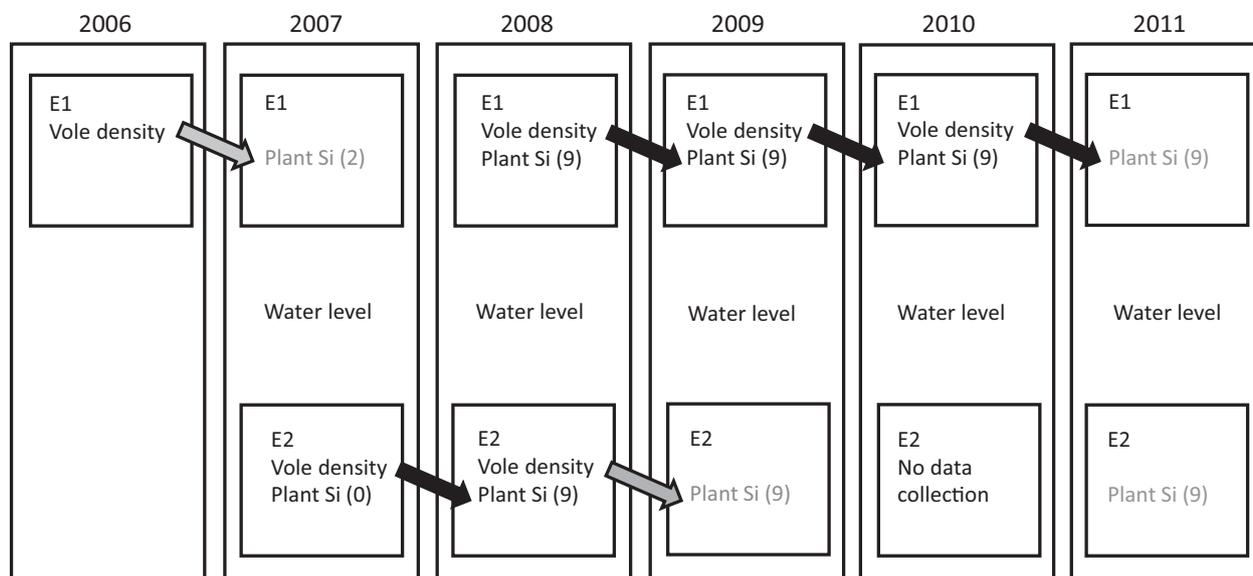
Recently, Wiczorek *et al.* (2015b) performed a field experiment to assess the link between plant silica-based defences and vole population cycles. One of their conclusions is that vole density affects plant silicon (Si) concentration. This conclusion, however, is not supported because of two fundamental problems related to their study design: (i) the analyses suffer from pseudoreplication (Hurlbert 1984) and (ii) the two predictors of plant Si concentration, vole density and water level, were strongly correlated and therefore their effects were confounded. Indeed, our reanalysis of data published by Wiczorek *et al.* (2015b) led to different results than those presented in the original study. Furthermore, the authors concluded that winter survival of voles decreased with increasing body mass, without any influence of plant Si concentration on survival. Nevertheless, our reanalysis of the survival

data showed the effect of body mass was conditional to a non-random pattern in missing values. In the following, we demonstrate in more detail why the evidence provided in Wiczorek *et al.* (2015b) does not support their conclusions regarding silica–vole interactions in their study system.

## Study design issues

Wiczorek *et al.* (2015b) studied the relationship between voles and plant Si concentration in wetlands, a habitat where flooding dynamics may affect Si availability for plants (Struyf *et al.* 2009). We reconstructed the study design (Fig. 1) based on the text provided in the paper and its Appendices S1 and S2 (Supporting Information), including the data file provided online. This exercise was difficult because of the inconsistencies between the text and the data provided (Fig. 1), e.g. discrepancies in the identity of enclosure(s) where sampling was performed in a given year. The field experiment included two enclosures. Each year, the authors acquired measurements of the water level for the overall study area, i.e. having same value for both enclosures. They also adjusted each year the vole population densities inside the enclosures so that it corresponded to the natural density measured in a control plot in the study area. Over five consecutive years, they sampled Si concentration of sedges in November. They sampled between two and nine sedge tussocks located within the enclosures and measured Si concentration in both leaves and rhizomes (sample sizes indicated in Fig. 1). With the exception of one year, silica measures were taken from a single enclosure each year. Therefore, the authors had a dataset with six combinations of enclosure identity and year, representing the ‘experimental units’ (i.e. entities subjected to treatments) of the study design. Each experimental unit had measures of Si in sedge leaves and in rhizomes, representing the observational units (i.e. units on which the response is measured) of the study design. The authors used these data to test for the effects of vole density and water level on sedge Si concentration.

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**Fig. 1.** Spatiotemporal layout of the field sampling in Wieczorek *et al.* (2015b). Each large rectangle represents the study area at each year, illustrating that the water level was measured at the level of the study area. The two small boxes inside the rectangles represent the two enclosures (E1, upper row and E2, bottom row). For each rectangle (i.e. each year), we list in black data that were sampled based on the study design presented in the Appendix S2 of Wieczorek *et al.* (2015b), and added in grey data that were not mentioned but were also available in the dataset provided in the Appendix S3. In parentheses are the numbers of sedge tussocks sampled for silica (Si) concentration in each enclosure-year, based on data available in Appendix S3. Arrows link the predictor variable ‘Vole density year  $t - 1$ ’ to the response variable ‘Plant Si year  $t$ ’. Black arrows illustrate pairs that correspond to both the study design and the dataset presented in Appendixes S2 and S3 of Wieczorek *et al.* (2015b). Grey arrows show pairs that were available in Appendix S3 and appear to have been included in the analyses, but do not correspond to the description by Wieczorek *et al.* (2015b) in Appendix S2.

#### STUDY UNITS AND LEVEL OF REPLICATION

The first main issue with the study design is that the authors used individual plant measurements as units in the statistical analyses, whereas the predictors of their analysis varied at the higher level of the study design: spatially at the scale of enclosures (i.e. vole density) or study area (i.e. water level) and temporally between years (both predictors). This is a typical example of pseudoreplication (Hurlbert 1984), as observational units (plants) were used in statistical analyses instead of the ‘experimental units’ (enclosure-year combinations). Even though Wieczorek *et al.* (2015b) used enclosure as a random factor in the analyses, it did not fully account for the structure of the study design in this case. Having a design variable representing the experimental units as a random effect usually solves pseudoreplication issues (Zuur & Ieno 2016), but the random effect must adequately represent the study design to account for repetitions within experimental units. One way to check that this structure is accounted for is to look at the d.f. in relation to the units of the study. The d.f. at the denominator presented for the linear mixed models (Table 1 in Wieczorek *et al.* 2015b) correspond to number of plants sampled (e.g. 47 plants were sampled and the d.f. was 42 for the analysis on leaves and 41 for that on rhizomes). Thus, in spite of ‘enclosure’ being included as a random variable, plants were used as the units. In this case, however, the d.f. for testing for the effect of density and water level should have been at the enclosure-year

level, i.e. based on six units. The way to account for the replication in their experiment is to use a random effect with six levels designating the enclosure-year.

When we analysed the data using this random structure, we obtained the correct d.f. on which to measure the influence of vole density and water level (See Appendix S2, lines 127–170). For a model with vole density, water level and their interaction as predictors, the d.f. at the denominator is reduced to two (See Appendix S2, lines 134–137). Obviously, having only six units in the analysis limits strongly the complexity of the models that can be fitted, as well as the precision of the measured effects.

#### CONFOUNDED PREDICTORS

The second main issue with the study design is that it does not allow for separating the effects of the two main predictor variables of sedge Si concentration: ‘vole density in year  $t - 1$ ’ and ‘water level in year  $t$ ’. These two predictors are very strongly correlated, as evident from a visual inspection of Fig. 1 in Wieczorek *et al.* (2015b) and corroborated by a statistical analysis ( $r = 0.83$ ;  $n = 6$  enclosure-year pairs; Fig. 2a). The authors seemed to be aware of collinearity issues because they avoided including highly correlated predictors in their survival analysis (see p. 190 in Wieczorek *et al.* 2015b). For the analyses on sedge Si concentration, however, both effects and their interaction were analysed in the same models. Such correlations make it difficult to estimate separate effects of the predictors, as

**Table 1.** Reanalyses of the work of Wiczorek *et al.* (2015b). The table presents ANOVA results and parameter estimates from different mixed effect models evaluating the change in Si concentration in leaves (A) and rhizomes (B) in relation to vole density in year  $t - 1$  and water level in year  $t$ , two predictors correlated at 83%. I: Results presented in Wiczorek *et al.* (2015b). II: Reanalyses of the same model as Wiczorek *et al.* (2015b) comparing an ANOVA based on marginal sum of squares SS (a) and on sequential SS with vole density first (b) or water level first (c). For marginal SS, the effect of each predictor is evaluated after all other predictors have been accounted for, so it is the SS that would be obtained for each predictor if it was entered last in a model (also referred to as Type III SS). The sequential SS represents the incremental improvement in the error SS as each predictor is added to the model (also referred to as Type I SS). Therefore, the hypothesis tested depends on the order the predictors are specified, which is illustrated by comparing II.b (vole density first) and II.c (water level first). Parameter estimates are independent on type of SS and are therefore the same for II. a, b and c. III: Analyses presenting the influence of each predictor analysed separately, i.e. including only one predictor in the model

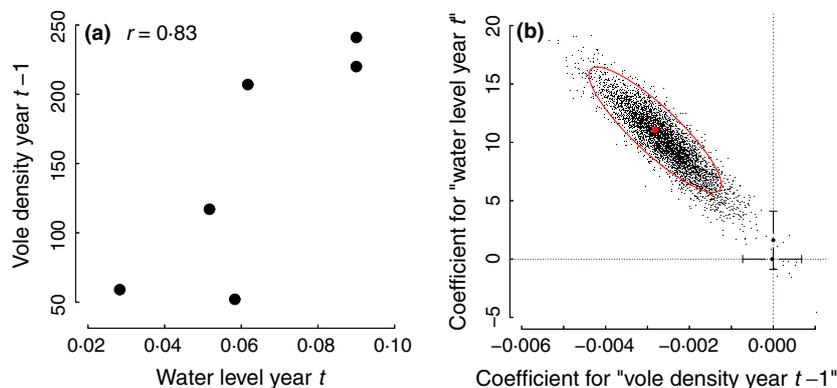
Factor	$F_{d.f.}$ value	$P$ value	Estimate $\pm$ SE
<b>A. Leaves</b>			
I. Wiczorek <i>et al.</i> (2015b)			
Vole density	$F_{1,42} = 10.6$	0.002	$0.002 \pm 0.001$
Water level	$F_{1,42} = 0.3$	0.61	$-1.29 \pm 2.54$
II. Reanalyses			
a. Marginal SS			
Vole density	$F_{1,43} = 3.7$	0.06	$0.0026 \pm 0.0013$
Water level	$F_{1,43} = 0.5$	0.5	$3.33 \pm 4.82$
b. Sequential SS - vole density first			
Vole density	$F_{1,43} = 36.5$	0.0001	
Water level	$F_{1,43} = 0.5$	0.5	
c. Sequential SS - water level first			
Water level	$F_{1,43} = 33.3$	0.0001	
Vole density	$F_{1,43} = 3.7$	0.06	
III. Analysis of single effect separately			
a. Vole density	$F_{1,44} = 36.2$	0.0001	$0.0034 \pm 0.0006$
b. Water level	$F_{1,44} = 33.6$	0.0001	$12.07 \pm 2.08$
<b>B. Rhizomes</b>			
I. Wiczorek <i>et al.</i> (2015b)			
Vole density	$F_{1,41} = 20.4$	0.0001	$-0.01 \pm 0.02$
Water level	$F_{1,41} = 5.0$	0.03	$6.94 \pm 3.10$
Density $\times$ Water level	$F_{1,41} = 10.7$	0.002	$0.08 \pm 0.03$
II. Reanalyses			
a. Marginal SS			
Vole density	$F_{1,42} = 0.16$	0.7	$-0.0008 \pm 0.0019$
Water level	$F_{1,42} = 16.5$	0.0002	$14.07 \pm 3.46$
Density $\times$ Water level	$F_{1,42} = 1.2$	0.3	$-0.027 \pm 0.024$
b. Sequential SS - vole density first			
Vole density	$F_{1,42} = 0.01$	0.9	
Water level	$F_{1,42} = 25.7$	0.0001	
Density $\times$ Water level	$F_{1,42} = 1.2$	0.3	
c. Sequential SS - water level first			
Water level	$F_{1,42} = 6.5$	0.01	
Vole density	$F_{1,42} = 19.1$	0.0001	
Density $\times$ Water level	$F_{1,42} = 1.2$	0.3	
III. Analysis of single effect separately			
a. Vole density	$F_{1,44} = 0.006$	0.9	$-0.00003 \pm 0.00035$
b. Water level	$F_{1,44} = 1.7$	0.2	$1.62 \pm 1.25$

is illustrated by the very strong correlation among parameter estimates of vole density and water level effects (Fig. 2b).

If water level was known to be a factor affecting plant Si concentration before the experiment, then the study should have been designed to be able to separate this effect from that of vole density. If not, we are left with *a posteriori* solutions to collinearity, which includes, for example choosing the variable of interest that makes more sense ecologically, combining meaningful variables, interpreting the effect of correlated variables together and removing collinearity prior to analysis (Dormann *et al.* 2013;

Ray-Mukherjee *et al.* 2014). In this case, these solutions would not have helped the authors because none of the solutions allows them to test the hypothesis their study is addressing. Moreover, in this case, the correlation cannot have arisen due to a causal effect, as vole density can obviously not influence the water level the following year.

To assess the consequence of the correlated predictors, we reanalysed the models presented in Table 1 of Wiczorek *et al.* (2015b) using the data provided in Appendix S3. For reproducing their results and enabling a comparison with them, we analysed the data with a similar random structure as in the original publication, i.e.



**Fig. 2.** Correlation between the predictor variables used by Wieczorek *et al.* (2015b). (a) Correlation between 'water level year  $t$ ' and 'vole density year  $t - 1$ '. (b) Correlation between parameter estimates for a model including the effects of both 'water level year  $t$ ' and 'vole density year  $t - 1$ ' on plant Si concentration (exemplified for rhizomes, a similar plot for leaves can be produced using the Supplementary R-script of the Appendix S2). Points represent possible coefficient combinations, based on a Bayesian MCMCglmm model with vole density and water level as additive fixed effects and enclosure as a random variable using 50 000 iterations. The 95% confidence ellipse for these points is depicted in red. Points and whiskers represent coefficient estimates with their 95% confidence intervals for each predictor variable alone, based on linear mixed effect models (model with one predictor variable and enclosure as a random variable).

including enclosure only. The authors did not mention if they used marginal or sequential sum of squares for computing the test statistics, but we could not reproduce the results with either type of sum of squares (compare Table 1 sections I and II). Our results differed from those of Wieczorek *et al.* (2015b), the differences ranging from small details like a difference of 1 d.f., to major changes in the significance of some predictors and the direction and strength of some estimates (compare sections AI with AII and BI with BII in Table 1). Thus, our analysis of the data provided online led to different conclusions than those presented in Wieczorek *et al.* (2015b).

The analyses presented in Table 1 clearly demonstrate that evidence towards each predictor is dependent on the method chosen, and the inconsistency among results stresses the risk of reaching spurious conclusions. From the model on Si concentration in leaves (Table 1A), the authors claim that vole density affects Si concentration in sedge leaves, but water level does not. This conclusion on water level effects is not supported because the effect of this predictor is similar to the effect of voles when the predictors are analysed independently (Table 1A.IIIa). We can also see that the order of the predictors will be decisive if a sequential sum of squares is used: there is stronger evidence for an effect of vole density when it is included first, and a stronger evidence towards an effect of water level when it is included first (Table 1A.II). We expect this result because the two variables are strongly correlated (Fig. 2a) and therefore should not be analysed in the same model. From the separate analyses of each variable, we can only conclude that Si concentration in leaves likely relates to the combined influence of both vole density and water level, but the contribution of each variable remains impossible to separate.

For the analysis of Si concentration in rhizomes (Table 1B), including both variables affects the evidence for supporting the predictors (Table 1B.II). Including both

**Table 2.** Sample size and January survival of voles in three body mass (BM) categories, for individuals that do (Si measured) or do not (Si NA) have data for Si concentration in faeces. In each cell, the ratio corresponds to the number of voles that survived on the number of voles for which Si was measured (first row) or not measured (second row), followed by the survival percentage. Only data from 2008 are included since other years had very low survival

BM (g)	19–24.4	24.5–28.1	28.2–62
Si measured	23/32: 72%	22/32: 69%	27/48: 56%
Si NA	8/42: 19%	4/37: 11%	4/23: 17%

predictors resulted in supporting evidence for one (Table 1B.IIa or 1B.IIb) or both predictors (Table 1B.IIc), whereas there is actually no evidence for either predictor (Table 1B.III). This is in sharp contrast with the conclusion of the authors that there was evidence of an effect of both variables and their interaction on sedge rhizome Si concentration (Table 1B.I).

### Survival analysis issues

Wieczorek *et al.* (2015b) studied vole survival over winter in the same field experiment as described above. They captured voles three times during winter, equipped them with individual identification chips, and then followed their survival through the winter. During three winters (2008/2009 to 2010/2011), they collected faeces from each individual at the first capture to determine faecal Si concentration. They analysed the effects of body mass, faecal Si concentration and sex on vole winter survival. The main conclusion by Wieczorek *et al.* (2015b) was (point 5 in Summary) 'Smaller voles had lower mortality during early winter than large voles', with no effect of faecal Si on vole mortality. However, our reanalysis of the data showed that the effect of mass depended on whether or not faecal Si was

included in the model (Survival  $\sim$  Mass + Sex + Year + Si: effect of mass:  $-5.6$  (SE = 1.8,  $n = 273$ ) vs. Survival  $\sim$  Mass + Sex + Year: effect of mass:  $-1.8$  (SE = 1.4,  $n = 383$ ), models for January survival). The difference between the two estimates for mass was due to a strongly non-random pattern in missing values for faecal Si with mass and mortality (Table 2). The pattern of decreasing survival with increasing mass was seen in voles for which faecal Si was measured, but was not observed in voles for which faecal Si was not measured. When faecal Si was included in the model, the individuals with missing values were excluded, leading to the conclusion that heavier individuals had lower survival. Without further information, the evidence for a strong effect of body mass on vole survival seems weak. We want to stress, however, that the direction of the effect (large individuals having bred during the previous summer are more likely to die during the winter) is biologically plausible. Indeed, Zub *et al.* (2014) observed a similar effect of body weight on winter survival in the same study population.

### The way forward

To conclude, the data collected by Wiczorek *et al.* (2015b) do not provide reliable evidence on whether or not vole grazing and/or water levels affected the concentration of Si in sedges. Their study design did not allow for distinguishing the effects of vole density and water level because the two were almost completely confounded. Our reanalyses of each effect separately clearly demonstrated that there was no evidence for an effect of vole density on Si concentration in rhizomes, and that both vole density and water level were related to Si concentration in leaves, but it was impossible to conclude which was the determining factor due to their strong correlation. Furthermore, the study design had only six units, limiting the potential for statistical inference. Finally, our re-analysis on the survival data showed that the effect of body mass on survival was the result of missing values in one covariate and that this effect was much weaker when using the whole survival dataset.

To date, most research on the interaction between small rodent population dynamics and plant silica defences comes from laboratory conditions and local-scale field experiments (e.g. Massey & Hartley 2006; Huitu *et al.* 2014; see also Hartley & DeGabriel 2016 and references therein). Based on these studies, plant silica defences appear to have the capacity to reduce vole population growth in a negative density-dependent manner, a necessary premise for generating population cycles. Yet, to what extent plant silica defences are linked to population cycles of natural vole populations is little known and appears to differ among the few studied ecosystems (Massey *et al.* 2008; Soininen *et al.* 2013; Hartley & DeGabriel 2016). Thus, unravelling this context dependency using field experiments that combine plant Si with other factors, as attempted by Wiczorek *et al.* (2015b), is exactly what is needed. That being said, to test the hypothesis that silica-

based defences drive vole dynamics, future studies will require either natural variation in different combinations of vole density and other environmental factors, or appropriate experimental manipulations separating the different effects.

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### Data accessibility

The data used in this manuscript are published by Wiczorek *et al.* (2015b), supporting information; Appendices S3 and S6. We have added a column 'Year' in the file S3, based on Fig. 1 by Wiczorek *et al.* (2015b). This modified data file (FESoininenSA1.txt) and the R-script we used (FESoininenSA2.R) are uploaded online as supporting information.

### References

- Dormann, C.F., Elith, J., Bacher, S. *et al.* (2013) Collinearity: a review of methods to deal with it and a simulation study evaluating their performance. *Ecography*, **36**, 27–46.
- Ergon, T., Lambin, X. & Stenseth, N.C. (2001) Life-history traits of voles in a fluctuating population respond to the immediate environment. *Nature*, **411**, 1043–1045.
- Hartley, S.E. & DeGabriel, J.L. (2016) The ecology of herbivore-induced silica defences in grasses. *Functional Ecology*, **30**, 1311–1322.
- Huitu, O., Forbes, K.M., Helander, M., Julkunen-Tiitto, R., Lambin, X., Saikkonen, K., Stuart, P., Sulkama, S. & Hartley, S. (2014) Silicon, endophytes and secondary metabolites as grass defenses against mammalian herbivores. *Frontiers in Plant Science*, **5**, 478.
- Hurlbert, S.H. (1984) Pseudoreplication and the design of ecological field experiments. *Ecological Monographs*, **54**, 187–211.
- Krebs, C. (2013) *Population Fluctuations in Rodents*. The University Press of Chicago, Chicago, IL, USA.
- Massey, F.P., Ennos, A.R. & Hartley, S.E. (2007) Herbivore specific induction of silica-based plant defences. *Oecologia*, **152**, 677–683.
- Massey, F.P. & Hartley, S.E. (2006) Experimental demonstration of the anti-herbivore effects of silica in grasses: impacts on foliage digestibility and vole growth rates. *Proceedings of the Royal Society B*, **273**, 2299–2304.
- Massey, F.P., Smith, M.J., Lambin, X. & Hartley, S.E. (2008) Are silica defences in grasses driving vole population cycles? *Biology Letters*, **4**, 419–422.
- McNaughton, S.J., Tarrants, J.L., McNaughton, M.M. & Davis, R.D. (1985) Silica as a defense against herbivory and a growth promoter in African grasses. *Ecology*, **66**, 528–535.
- Ray-Mukherjee, J., Nimon, K., Mukherjee, S., Morris, D.W., Slotow, R. & Hamer, M. (2014) Using commonality analysis in multiple regressions: a tool to decompose regression effects in the face of multicollinearity. *Methods in Ecology and Evolution*, **5**, 320–328.
- Reynolds, J.J.H., Lambin, X., Massey, F.P., Reidinger, S., Sherratt, J.A., Smith, M.J., White, A. & Hartley, S.E. (2012) Delayed induced silica defences in grasses and their potential for destabilising herbivore population dynamics. *Oecologia*, **170**, 445–456.
- Soininen, E.M., Bråthen, K.A., Jusdado, J.G.H., Reidinger, S. & Hartley, S.E. (2013) More than herbivory: induction of silica-based defences in grasses varies with plant species, genotype and location. *Oikos*, **122**, 30–41.
- Struyf, E., Opdekamp, W., Backx, H., Jacobs, S., Conley, D.J. & Meire, P. (2009) Vegetation and proximity to the river control amorphous silica storage in a riparian wetland (Biebrza National Park, Poland). *Biogeosciences*, **6**, 623–631.
- Vicari, M. & Bazely, D.R. (1993) Do grasses fight back? The case for anti-herbivore defences. *Trends in Ecology & Evolution*, **8**, 137–141.
- Wiczorek, M., Szafranska, P.A., Labecka, A.M., Lazaro, J. & Konarzewski, M. (2015a) Effect of the abrasive properties of sedges on the intestinal absorptive surface and resting metabolic rate of root voles. *Journal of Experimental Biology*, **218**, 309–315.

- Wieczorek, M., Zub, K., Szafranska, P.A., Ksiazek, A. & Konarzewski, M. (2015b) Plant-herbivore interactions: silicon concentration in tussock sedges and population dynamics of root voles. *Functional Ecology*, **29**, 187–194.
- Zub, K., Borowski, Z., Szafranska, P.A., Wieczorek, M. & Konarzewski, M. (2014) Lower body mass and higher metabolic rate enhance winter survival in root voles, *Microtus oeconomus*. *Biological Journal of the Linnean Society*, **113**, 297–309.
- Zuur, A.F. & Ieno, E.N. (2016) A protocol for conducting and presenting results of regression-type analyses. *Methods in Ecology and Evolution*, **7**, 636–645.

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## Supporting Information

Details of electronic Supporting Information are provided below.

**Appendix S1.** (file FESoininenSA1.txt): Modified data file based on Appendix S3 of Wieczorek *et al.* (2015b), ‘year’ column added.

**Appendix S2.** (file FESoininenSA2.R): R-script for the analyses presented in this paper.