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Towards a standardized experimental protocol to investigate interactions between weed seeds and ground beetles (Carabidae, Coleoptera)

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Abstract

Seed bank control is the mainspring of agricultural management practices. Using weeds seeds natural predators as a weed control strategy can be a valuable alternative to herbicide use. Among natural predators, ground beetles (Coleoptera, family Carabidae) have been claimed to be one of the most important invertebrate seeds predators in agroecosystems. However, information on the trophic relationship among these two large communities is fragmentary. Laboratory experiments have been conducted using a wide variety of protocols on a few ground beetle and weed seed species. To better characterize trophic relationship among these communities, a standardized and simple protocol is required. We propose a protocol to quantitatively assess weed seed consumption by ground beetles. Importantly, we explore parameters that should be optimized and fixed since they are not targeted, such as fasting time, test duration and substrate type. We also investigate whether, and under which conditions, tests may be replicated with the same individuals. Tests were carried out on five ground beetle species and eight weed seed species, though in the majority of tests Viola arvensis was used. Using climatic chambers, parameters such as temperature, humidity and photoperiod were fixed at 25 °C, 70% and 17-h/7-h day/night, respectively. Fasting time prior testing and test duration were fixed to 3 days and 24 h, respectively, to avoid mortality of ground beetle species that do not rely on seeds as their main food. Also, a recovery period of 24 h between successive tests is required when multiple tests are conducted on the same individuals. Among the substrate tested, we recommend to use sand as it appeared the most convenient substrate to conduct a large amount of replicated experiments.

Keywords Carabids · Insect feeding ecology · Laboratory experiments · Standardized conditions · Replicating tests · Insect diet

Introduction

A major target of public policies is to reduce negative effects of intensive agriculture, particularly regarding pesticide use (Geiger et al. 2010). Decreasing reliance on pesticides while maintaining income or yield (or both), obviously requires alternative and innovative cropping systems (Rusch et al. 2010; Pretty et al. 2006). Under the ecological intensification paradigm, equivalent or higher yield levels are expected through explicit biodiversity management, by enhancing ecosystem services (Bommarco et al. 2013). Biological control agents for pest regulation could help reduce pesticide use in agricultural systems (Wilby and Thomas 2002), especially herbicides that are massively sprayed against weeds (Petit et al. 2010; Gaba et al. 2014, 2015). Indeed, weeds regulation by their vertebrates and invertebrates predators has attracted a lot of attention (Heggenstaller et al. 2006;

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Menalled et al. 2007; Powolny 2012). While many granivorous species have been identified, quantifying their precise impact on seed populations has, however, remained challenging. For instance, ground beetles (Carabidae) have been repeatedly claimed to be one of the most important invertebrate seeds predators in agroecosystems (Honek et al. 2003; Lundgren et al. 2013; O'Rourke et al. 2006). Many studies have focused on their seed consumption rates in the field on their potential as biological control agents of the weed seed bank (Westerman et al. 2003; Honek et al. 2003, 2007; Honek and Martinkova 2005; Menalled et al. 2007; Gaines and Gratton 2010). While there is some empirical support for the latter (Thiele 1977; Holland and Luff 2000; Bohan et al. 2011), we still lack information on the trophic relationship between ground beetle and weed seed communities, such as the number and identity of ground beetle species feeding on seeds (Kulkarni et al. 2015).

Laboratory experiments are a useful tool to explore weed seed consumption by ground beetles. Seed consumption experiments have been conducted using a wide variety of conditions, ranging from seeds set up in white plasticine tested over 5 consecutive days (Petit et al. 2014; Honek et al. 2003) to cafeteria tests with 28 different real seed species with trays replacement every 24 h (Table 1). Since weed seeds and ground beetles are two large communities, a standardized and simple protocol, allowing rapid collection of information on dietary preferences and the experimental parameters which could influence weed seed consumption by ground beetles, is required (Kulkarni et al. 2015). This would allow to determine whether a particular ground beetle species consumes a particular weed seed species and to quantify this consumption. First, such protocol should be performed under standardized housing conditions: temperature and humidity during tests should be controlled to reduce environmental variation. The substrate should be easy to use and to standardize to conduct multiple and simultaneous experiments. Importantly, it should not contain any food or seeds and should allow fast counting of seed consumption. Individual level of satiation should also be standardized: a fasting period must therefore be applied (Lövei et al. 1990; Sunderland et al. 1987) and individuals should be maintained and tested individually to prevent cannibalism. In addition, if individual variation is a target parameter to be quantified, individual beetles must be tested several times according to optimal conditions. To that aim, lag time and rearing conditions between successive tests should be optimized in order to avoid carrying out tests on individuals with variable condition along successive tests and decreasing survival probability. Similarly, the seed coat imbibition (moistened) is a factor which may impact seed predation by ground beetles (Law and Gallagher 2014). Many of these parameters remain to be determined and optimized experimentally.

In the present study, our aim was to develop such a standardized protocol allowing investigation of seed consumption of ground beetles. We conducted tests to optimize the following major parameters: test duration of fasting, substrate (i.e. the matter on which seeds are provided), concealment of seeds and some weed seed physical properties (species and moisture). Furthermore, we investigated the level of intra-individual variation and in which way it was achievable to use the same ground beetle individual in successive tests while maintaining the set of initial conditions (fasting, body mass).

Materials and methods

Ground beetle species capture

Adult ground beetles were collected in an intensive crops area, the Long-Term Social-Ecological Research - Zone Atelier Plaine & Val de Sèvre (LTSER-ZAPVS), Deux-Sèvres, France, using pitfall traps placed in fields that were a priori chosen to provide high capture rates and high species diversity (oilseed rape, wheat, sunflower and maize). Beetles were captured between April and August in 2015 and 2016. The beetles were brought to the laboratory and kept individually in plastic boxes (diameter: 5.5 cm, height: 6.5 cm) at room temperature. They were fed ad libitum diet consisting of a weed seeds mixture (20 weed seeds every 2 days, see Table S1) and crushed cat crockets (Purina®, Friskies®). Before tests, ground beetles have been fed with this food mixture for at least 24 h, until numbers of individuals required for the experiment have been reached. Most tests were performed with five ground beetle species classified as granivorous or omnivorous. They represent a reasonable level of diversity of body size, and caught in sufficient numbers to allow several experiments with replicates. These five species were, from smallest to largest, *Amara similata* (Gyllenhal; 8.7 mm), *Harpalus affinis* (Schrank; 10 mm), *Poecilus cupreus* (Linnaeus; 12 mm), *Harpalus dimidiatus* (P.Rossi; 13 mm) and *Pseudoophonus rufipes* (DeGeer; 15 mm).

General test procedure

All tests were carried out with a single species of seed at a time. All seeds were provided by Herbiseed® (Twyford, the UK), and most tests were conducted with *Viola arvensis*, a common dicotyledonous annual weed found in our study site and which was already used in other study with ground beetles. *V. arvensis* was easily consumed (Boursault 2012; Alignier et al. 2008), allowing therefore comparisons between ground beetle species and experimental conditions. Moreover, this species was already used in many previous

Table 1 Examples of protocols used to test consumption of weed seeds by ground beetles under laboratory conditions

Ground beetle species	Fasting time	Condition for fasting, Temperature	Weed seed species	Substrate	Temperature (°C) during the test	Photoperiod	Humidity	Test time	Question of the study	Protocol type	References
19 granivorous species	5–8 days	5 °C in darkness	*Taraxacum* from different regions	Soil from 50 cm depth, sieved, seeds in plasticine	26	Natural photoperiod	–	4 days	Taxonomy, geographic origin and size of seeds	Preference experiments	Honek et al. (2011)
H. affinis, P. rufipes	5 days	5 °C	*Taraxacum*	Soil from 50 cm depth, sieved, seeds in plasticine	10.5, 14, 17.5, 21, 24.5 or 28	17/7 h	–	4 days	Effect of temperature on consumption	No-choice	Saska et al. (2010)
30 granivorous species	3–5 days	5–7 °C in darkness	28 species of dicotyledonous	Plasticine	25–27	14.5/9.5 h (April) 17/7 h (June) 11 h/13 h (Oct)	100%	5 days	Preference of seed according to size and taxonomy	Preference experiments	Honek et al. (2007)
3 species	48 h	5 °C	Canola seeds (rapeseed)	Steamed soil sterilized	22	16/8 h	–	72 h	Seed consumption according to burial depth	Choice according to depth	Kulkarni et al. (2015)
5 species	3–5 days	5 °C in darkness	10 species of seeds	Seeds in plasticine	25	Natural photoperiod	–	5 days	Seed preference and quantify consumption	Preference experiments	Petit et al. (2014)
8 species	5 days	5 °C in freezer	4 species	Soil from 50 cm depth, sieved and moistened + plasticine	–	–	–	4 days	Laboratory preference experiment	Preference experiments	Saska et al. (2008)
23 carabids species for no-choice, 2 for multi-choice	1–4 days	5–7 °C in the dark	2 for no-choice, 64 for preference	Moistened soil	25–27	Natural photoperiod	100%	3 days	Carabid species, condition Seeds: size and species on consumption	No-choice, preference experiments	Honek et al. (2003)
24 species	–	–	3 species + Leaves of wheat	Filter paper	–	–	–	7 days	Explore granivory for 12 genera of carabidae	Preference experiments	Goldschmidt and Toft (1997)

The literature survey conducted here is non-exhaustive

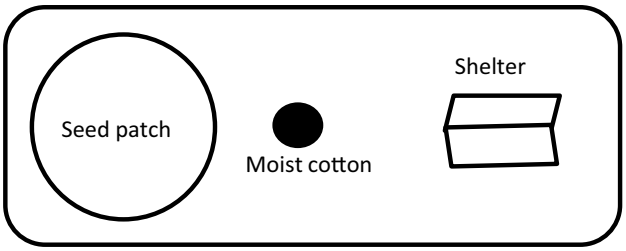

Fig. 1 Arrangement of used materials in experimental boxes (11 × 23 × 8 cm), seeds were placed in a 63.60 cm^2 arena, corresponding to 25% of the plastic box area, moist cotton and a paper shelter (5 × 4 cm)

studies and shows variable—but often high—consumption rates by ground beetles (Goldschmidt and Toft 1997; Honek et al. 2003; Saska et al. 2008; Petit et al. 2014; this study).

All tests were carried out with a single individual with a minimum of five individuals tested per experiment, which was a good compromise between time needed and statistical power. In order to control the climatic conditions and avoid any additional confounding factor, all experiments were carried out in climate chambers (Vötsch Industrietechnik®) at 70% humidity, 25 °C and 17/7-h day/night photoperiod which is optimal conditions for ground beetle feeding activity (Honek et al. 2003; Petit et al. 2014; Eskelson et al. 2011). During the experiments, we used plastic boxes (11 × 23 × 8 cm) with rounded corners and screened openings on the lid to allow good air circulation in the box (mesh 1 × 1 mm). Seeds were placed in a 63.60 cm^2 arena, corresponding to 25% of the plastic box area. Moist cotton and a paper shelter (5 × 4 cm) were placed in the plastic box (see Fig. 1). After the experiment, boxes were washed (water + liquid black soap) to remove odours of the previous ground beetles that could be detected by next ground beetles (Kielty et al. 1996). For similar reasons, substrates were used only once.

After each experiment, the number of remaining seeds was counted; missing seeds and those of which > 50% was consumed were considered eaten (Honek et al. 2005). In most tests, seed density was fixed to 50 (see below). This number was set to match the average seed density estimated empirically in our study site (2000 seeds per m^2, Powolny 2012).

Assessing standardized experimental conditions

Optimal fasting duration

A total of 25 individuals from five ground beetle species (five per species) were used for this experiment. They have been fed in laboratory in order to level out the presumably high variability in regard to feeding status of each individual. Then, they were exposed to 5 days of fasting period, placed singly in plastic pots (diameter: 5.5 cm, height: 6.5 cm) with only a moist cotton. Each day, beetles were put into a new plastic pot. On this occasion, we recorded individual's body mass and counted the number of faeces to monitor their body condition during the fasting period and the time needed to empty their guts.

Testing the influence of substrate

We selected four substrate types in order to investigate to which extent they could impact seed consumption rates, depending on ground beetle species. Seeds directly offered on paper were used as an extreme situation in which seeds were fully visible and very easy to handle. Three other substrates were tested: sand (granulometry: 300 µm, colour: chocolate; Le marchand de sable, Challans 85), short synthetic grass (height: 2.8 mm, saint-maclou.com) and long synthetic grass (density: 12 stitches/cm^2, height: 8 mm, saint-maclou.com). Each type of substrate was tested with 50 seeds of *Viola arvensis* on five individuals of three species, *Amara similata*, *Poecilus cupreus* and *Harpalus dimidiatus*. In the case of sand, seeds were dispersed in the arena and then seeds and the whole bottom of the experimental box were covered with sand. In the case of synthetic grass, seeds were scattered in the arena, and usually landed at the base of the plastic grass stitches.

Condition for using the same individual in several test to measure intra-individual variation

Our next aim was to conceive a protocol able to replicate the simple seed consumption test several times using the same individual, maintaining an identical set of initial conditions of body mass for each trial round. The aim here was to keep individuals alive, and feed them enough between successive tests such that their body condition and motivation to feed on seeds remained stable. We thus compared two protocols with two species (*Harpalus dimidiatus* and *Harpalus distinguendus*) and compared their mortality rates. A total of 24 individuals per species were tested with the first protocol and 32 with the second protocol. For both species, before each test, a fasting period of 72 h was imposed as described previously. Protocols then differed in test duration (48 h for the first protocol and 24 h for the second protocol). In order to maintain their body condition, ground beetles were fed during 24 h after the test in the second protocol only, with the same mixed diet as when housed after capture. To summarize, protocol 1 consisted in 72/48-h fasting/test, while protocol 2 consisted in 72/24/24-h fasting/test/feeding. Ground beetles were always kept in climatic chambers in the same conditions during all steps of the experimentation. All individuals were weighed before and after the tests.

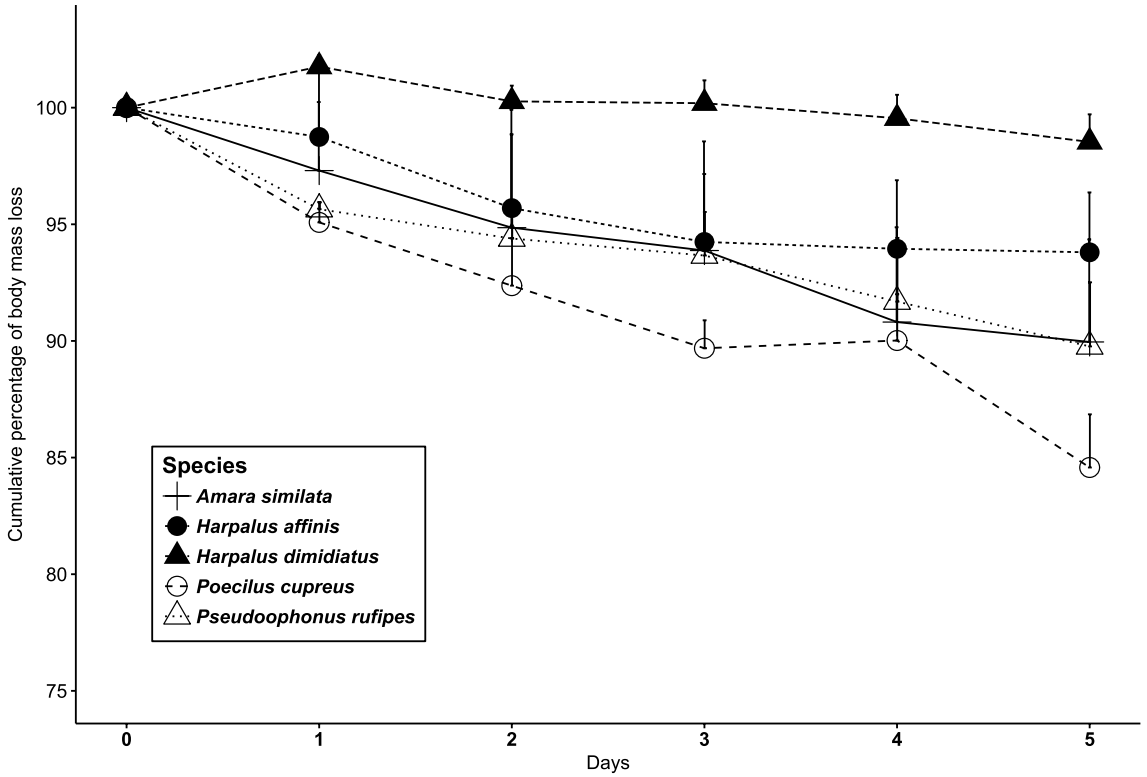

Fig. 2 Cumulative percentage of body mass loss for 5 ground beetle species (mean ± SE)

Testing the influence of seed species and its moisture

Since most tests were performed with *Viola arvensis*, we checked whether the weed species identity may affect results obtained. We therefore tested seven additional weed seed species, selected according to two traits known to vary much between weed seed species: seed mass and oil content (Bretagnolle et al. 2015), and in order to have two weed seed species for each combination of these two traits (high and low; low and high; low and low; high and high). In addition, they have been shown to be important traits for dietary preferences in granivorous animals (Gaba et al. 2014). The seven additional species were *Lolium multiflorum*, *Fallopia convolvulus*, *Geranium dissectum*, *Fumaria officinalis* (the four heaviest), *Chenopodium album*, *Poa annua* and *Papaver rhoeas* (the four lightest, including *Viola arvensis*; see Table S1). Consumption tests were performed with four ground beetle species: *Harpalus dimidiatus*, *Harpalus affinis*, *Poecilus cupreus* and *Pseudoophonus rufipes* following the general protocol described above. We offered 50 seeds of each seven weed seed species in the no-choice experiments.

We further compared seed consumption rates according to seed coat imbibition, therefore between non-moistened and moistened seeds using only *Viola arvensis*. We tested twenty individuals for two ground beetle species (20 *Harpalus dimidiatus* and 20 *Poecilus cupreus*). We placed batches of 50 seeds in humidified cotton for 24 h before test. Seeds were placed in the 63.60 cm^2 arena. We chose sand and paper as substrates for comparison. After standardized satiation level and the 72-h fasting period, we placed ground beetles individually for 24 h in the plastic boxes for the experimentation. There were four combinations (moistened seeds on paper; moistened seeds under sand; non-moistened seeds on paper; non-moistened seeds under sand), for each one 5 individuals have been tested.

Statistical analyses

Correlation analysis between faeces produced and beetles body mass was analysed with Spearman's rank test. Body mass losses and number of faeces have been compared between ground beetle species with an ANOVA, with body mass percentage losses and number of faeces as the responses variables and species identity of ground beetle as factor. We then explored the effect of substrate (factor) and ground beetle species (factor) on the total seed consumed (response variable) using generalized linear model (GLM)

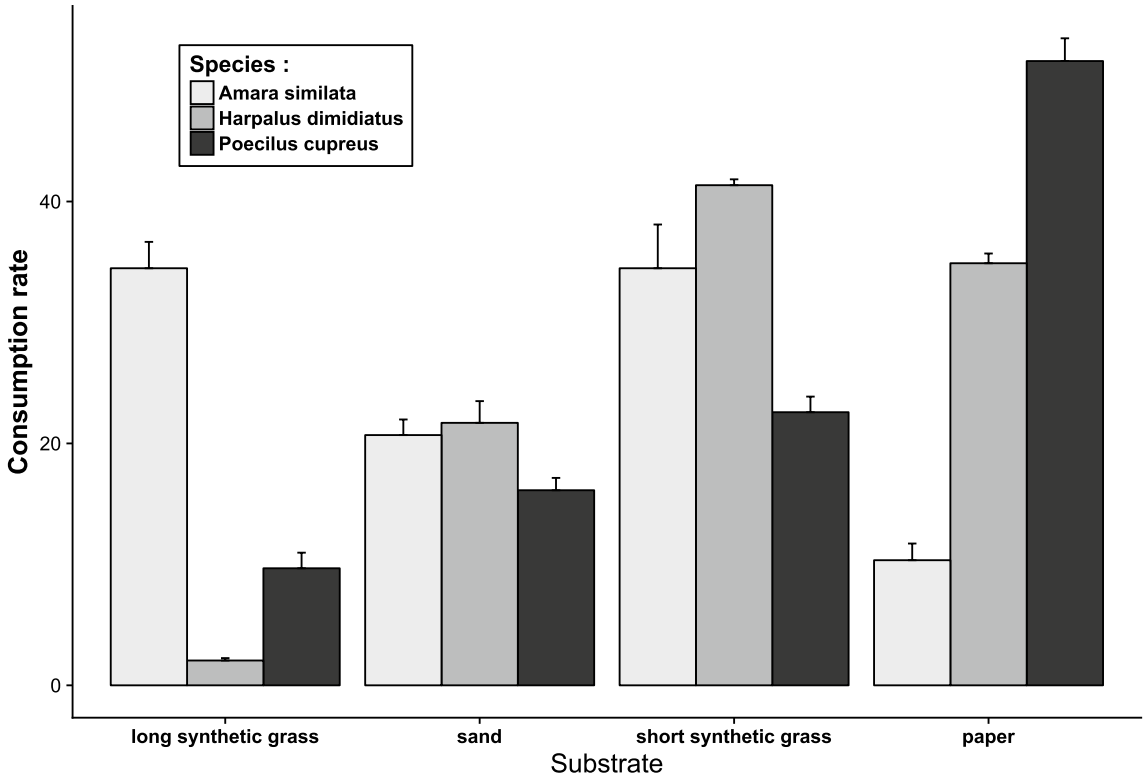

Fig. 3 Consumption rates of *Viola arvensis* seeds (% out of $N = 50$ seeds) ± SE, according to substrate type of three species of ground beetles: *Amara similata, Poecilus cupreus* and *Harpalus dimidiatus*. Seed consumption rate was affected by ground beetle species ($p < 0.0001$) and by substrate ($p = 0.001$)

with Poisson error and the individual ground beetle identity as a random effect. Comparison of body mass between sessions according to species in both protocols was performed using Kruskal–Wallis rank-sum test. To compare weed seed consumption according to ground beetle species and weed seed species, we used an ANOVA with consumption as the response variable and species of ground beetles and of weed seed as factors. To separate significant results, a post hoc Tukey's HSD test was realized for pairwise comparison. To compare the effect of seed imbibition and seed visibility (sand or paper) on the total seed consumed, we used a Wilcoxon rank-sum test. All data analyses were performed using R software, version 3.1.3 (R Core Team 2016).

Results

Assessing standardized experimental conditions

After 3 days of fasting, no further faeces were found for *A. similata* and *H. dimidiatus* individuals. Faeces production completely stopped after 4 days for *H. affinis* and *P. cupreus* while there were still faeces after 5 days in a single species, *P. rufipes*. The number of faeces produce by each species was not significantly different (Anova, Df = 4; $F = 1.372$; $p = 0.279$). All species but *H. dimidiatus* lost body mass from the very first day (Fig. 2). After four days, species *P. rufipes*, *H. affinis*, *A. similata* and *P. cupreus* lost 6.5%, 6.4%, 10%, 10.3%, respectively. Surprisingly, the body mass remained stable for *H. dimidiatus* even after 4 days of fasting (body mass loss of 0.6%). Note that no correlation was found between the number of faeces produced and ground beetles body mass (Spearman's rank test; $S = 1768.596$; $p = 0.1192$). Regarding substrate type, we found that seed consumption rate was affected by ground beetle species ($F_{2,\ 3} = 36.6$; $p < 0.0001$) and by substrate ($F_{3,\ N=4} = 5.76$; $p = 0.001$; see Fig. 3). Among ground beetles tested, regardless the substrates, *H. dimidiatus* ate significantly more seeds (GLM; estimate = 2.46; $p < 0.0001$) than *A. similata* and *P. cupreus*. The long synthetic grass modality significantly and negatively impacted seed consumption (GLM estimate = − 2.07; $p < 0.0001$), we

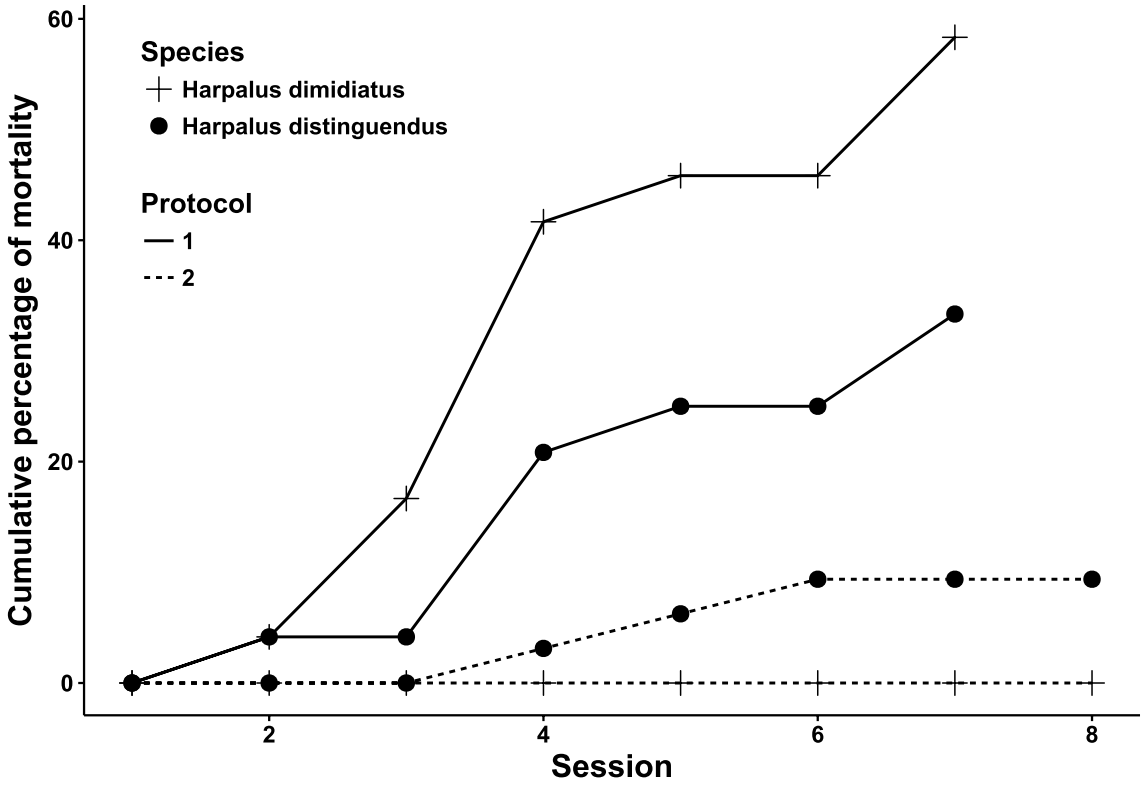

Fig. 4 Cumulative percentage of adult mortality of *Harpalus dimidiatus* and *Harpalus distinguendus* for two different experimental conditions over a period of 2 months. One session was for Protocol 1: fasting period: 72 h + test: 48 h, Protocol 2: fasting period: 72 h + test: 24 h + feeding period: 24 h

observed same results with sand (GLM; estimate = − 0.61; $p < 0.0001$).

Assessing conditions for using the same individual in several tests to measure intra-individual variation in seed consumption

Mortality rate increased for both species tested with increasing test duration. *H. distinguendus* and *H. dimidiatus* showed, respectively, 33% and 58% mortality rate with 48-h test duration without recovery period, compared to 9% and 0% for 24 h with recovery period (Fig. 4). These results suggest that test duration should not exceed 24 h, and that a recovery period with food items other than only seeds, even for granivorous species, is much recommended. Under these conditions, ground beetles were able to be survived up to 2 months in laboratory (before releasing them in the wild). In the other hand, body mass remained stable during both protocols (Table 2).

Comparing seed consumption according to seed species and conditions

As expected, seed consumption rates varied between both seed species (Anova; Df = 7; $F = 18.64$; $p < 0.001$) and ground beetle species (Anova; Df = 3; $F = 13.11$; $p < 0.001$) (Fig. 5). Overall, small seeds (i.e. < 0.001 g) were more consumed than large ones. Some ground beetle species, e.g. *P. cupreus*, appeared more selective than others. *P. rufipes* consumed more seeds than any other ground beetle tested (a total of 96 seeds consumed out of 400 offered), followed by *Harpalus* species (52 seeds consumed for *H. dimidiatus* and 46 for *H. affinis*), whereas the lowest consumer was *P. cupreus* (21.8 seeds consumed). For this latter ground beetle species, however, we found that the number of seeds consumed was higher when seeds were moistened (Wilcoxon Test, W = 1114.5, $p = 0.0025$; Fig. 6), and when seeds were placed on paper (thus, fully exposed) than hidden by sand (Wilcoxon Test, $W = 1012.5$; $p = 0.04$; Fig. 6). Conversely,

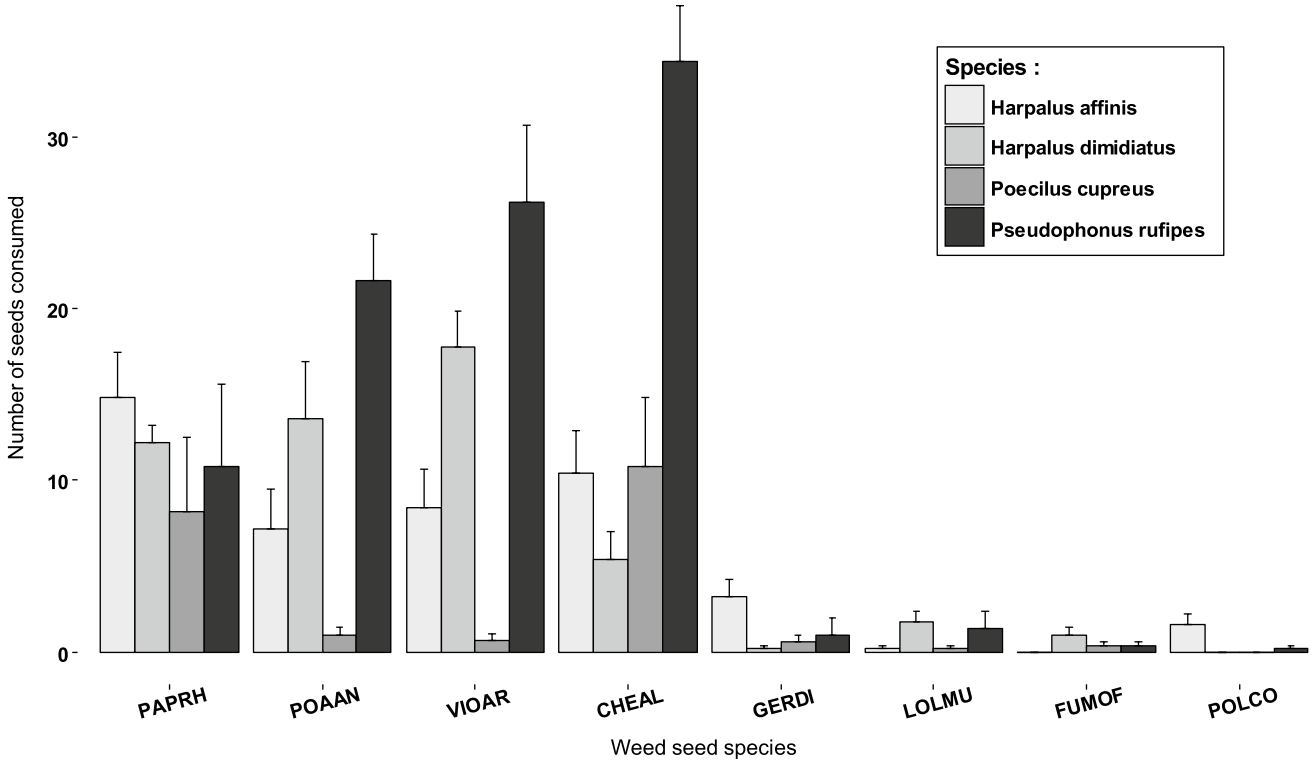

Fig. 5 Number of seeds consumed by four ground beetle species for eight weed seed species, Seeds are classified along increasing mass (see Table S1): PAPRH (*Papaver rhoeas),* POAAN *(Poa annua),* VIOAR (*Viola arvensis),* CHEAL (*Chenopodium album),* GERDI (*Geranium dissectum),* LOLMU (*Lolium multiflorum),* FUMOF (*Fumaria officinalis) and* POLCO (*Fallopia convolvulus).* Values given are mean ± SE. With Tukey's HSD post hoc test on total consumption per weed seed species, two groups were identified: group A for fourth the lightest, higher consumed than those of group B the heaviest seeds

H. dimidiatus was unaffected by seed condition (Wilcoxon Test, $W = 207$, $p = 0.86$; Fig. 6), but was affected by substrate, with higher consumption when exposed (Wilcoxon Test, $W = 302$, $p = 0.006$; Fig. 6).

Discussion

Weed seed bank could be regulated by granivorous species and would be a key option for weed management in agroecosystems (Marshall et al. 2003; Fox et al. 2013). However, a recent extensive review by Kulkarni et al. (2015) concluded that studies investigating the relative role of omnivorous and granivorous ground beetle species for weed management were lacking. One of the reasons would be the lack of a standardized protocol that limits any comparative analyses. For example, the way seeds are exposed varies strongly between studies (Honek et al. 2007; Saska et al. 2008). Saska et al. (2014) showed that seed exposure strongly affects their consumption: when presented on seed cards or on filter paper, seeds were more consumed than when mounted on a tin tray. In addition, there has been a long debate on the efficiency of no-choice vs. cafeteria tests (e.g. Withers and Mansfield 2005), after which the authors finally agreed that these two approaches test different hypotheses. In a no-choice experiment, one can efficiently compare seed consumption rates between seed species and use many seeds, which is indeed needed since farmland landscapes often harbour several hundreds of weed species (Meiss et al. 2010; Gaba et al. 2010). On the other hand, cafeteria tests allow to explore weed seed preferences in a controlled seed bank, i.e. with a limited number of seed species (Petit et al. 2014; Honek et al. 2003, 2006). In the present study, we used no-choice experiments for the sake of simplicity as our overarching aim was to set up standardized experimental conditions for a simple and easy-to-replicate protocol. However, our experimental conditions can be used to conduct cafeteria tests.

Here we contribute to elaborate a standardized protocol, fixing values for parameters such as temperature, humidity and photoperiod, and studying experimentally the effect of other parameters, such as fasting period or substrate. In

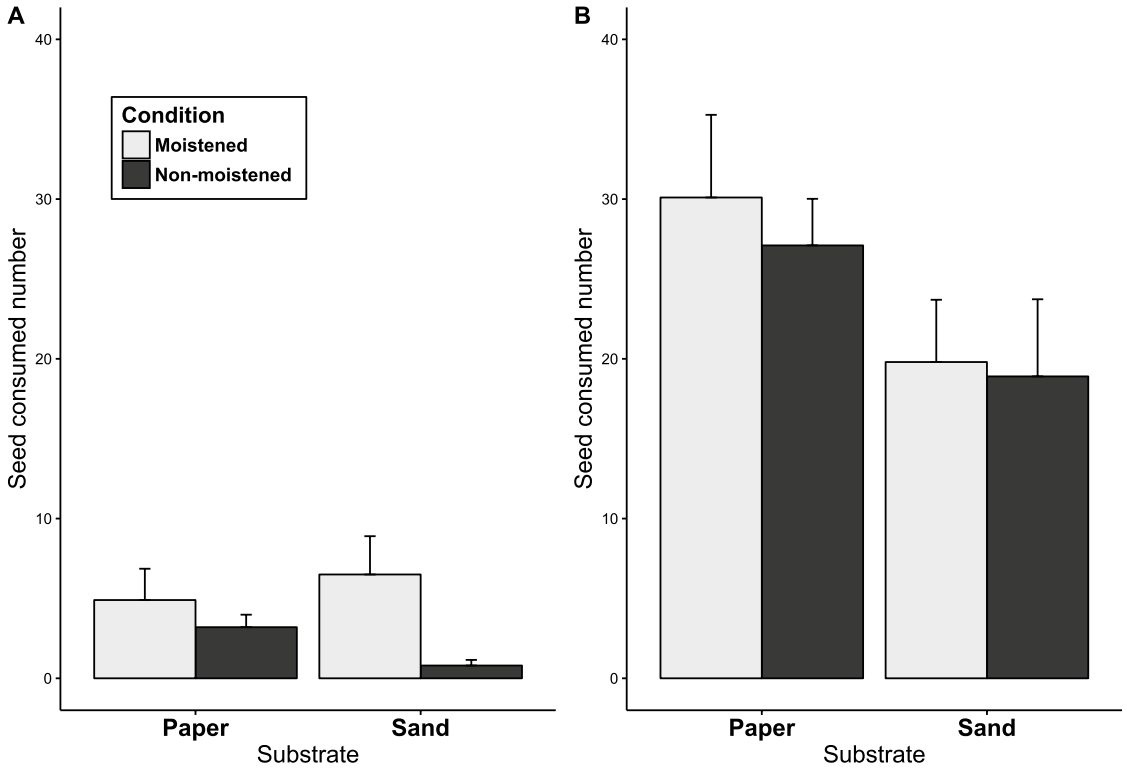

Fig. 6 Seed number of *Viola arvensis* consumed with moistened and non-moistened seeds hidden by sand or offered on paper for **a** *Poecilus cupreus;* For which *s*eed consumed increased significantly when seeds are moistened ($p=0.0025$) and when seeds are fully exposed on paper ($p=0.04$) and **b** *Harpalus dimidiatus;* For which seed condition did not impacted seed consumption ($p=0.86$), but *H.dimidiatus* seemed affected by substrate ($p=0.006$)

cases when individuals are caught in the field (as in our case), individuals show a strong variation in body mass, as a likely consequence of different levels of nutritional reserves at the time of capture. This inter-individual variation must be controlled by a fasting period. Our results indicate that, at 25 °C, fasting period should last at least 3 days, a shorter period than the one suggested by Sunderland et al. (1987), where Carabidae were starved (at 20 °C) for 7 days to empty the gut and induce hunger. This shorter period was expected since temperature affects metabolism, hunger, activity of insect, such as ground beetles (Chown and Nicolson 2004; Saska et al. 2009). In fact, most fasting periods recorded in the literature varied from three to 5 days (see Table 1), though shorter times were also suggested, e.g. 2 days (Fawki and Toft 2005; Lundgren and Rosentrater 2007). After 4 days, the 5 ground beetle species tested had very little food content left in their guts, and accordingly, fixing the fasting period to 3 days was a reasonable compromise to avoid carrying out experiments on individuals which were too weak and had a low survival probability. However, to be sure ground beetles are on the same level of hunger in our experimentation, it could be better to include a longer feeding period with the food mix between the catch and the beginning of the test. In our experimentation, sex of ground beetles was not considered. However, differences among sex have been previously identified (Saska et al. 2010) suggesting to take into account this parameter in future studies. The mechanisms by which seeds are detected by ground beetles are variable according to the feeding preferences of ground beetles (Wheater and Evans 1989). Most granivorous ground beetles use olfaction to detect food (Kielty et al. 1996), and moistened seeds may be more fragrant, which would facilitate their detection by ground beetles (Law and Gallagher 2014), although further research is needed to validate this hypothesis (Kulkarni et al. 2015). In our study, we found that *Poecilus cupreus*, one of the most abundant beetles in Western Europe farmland landscapes (Cléré and Bretagnolle 2001; Marrec et al. 2015), consumed more seeds when moistened and when presented on

Table 2 Results of data analysis (Kruskal–Wallis test) between body mass, session and protocols for *Harpalus dimidiatus* and *Harpalus distinguendus*

Species	Protocol	χ^2	Df	*p* value
Kruskal–Wallis				
Harpalus dimidiatus	1	4.51	6	0.61
Harpalus dimidiatus	2	1.76	7	0.97
Harpalus distinguendus	1	3.17	6	0.79
Harpalus distinguendus	2	14.42	7	0.04

a paper substrate rather than when buried in sand. But this was not true for other species such as *Harpalus dimidiatus*, suggesting possible differences across species in seed search when foraging. We chose to burying seeds in sand, to include a searching time, as it is required to investigate functional response (Holling 1966). Although there are other soil types not tested here for hiding weed seeds, such as sieved soil, sand appeared the most convenient to conduct a large amount of replicated experiments. In a field study, Alignier et al. (2008) observed a predation rate around 40% for *Viola arvensis* seeds during 1 week, this seed species was the most consumed among seven other weed seed species. As expected, we found that consumption rates of *Viola arvensis* were fairly representative of ground beetle species appetence for weed seeds, being consumed by all ground beetle species tested here (though consumption rates varied), and belonging to the seed group that attracts ground beetles. Our tests showed a strong preference for smaller seeds (see also Honek et al. 2007; Petit et al. 2014), i.e. weighing less than 0.001 g. In the field, Jonason et al. (2013) found that *Viola arvensis* was the most consumed seed. For *H. affinis*, our daily consumption rates were around 10.8 seeds/24 h/individual, a value that is close to those found in another study (11.8 seeds/day/ind: Petit et al. 2014; Boursault 2012; Honek et al. 2006).

Moreover, granivorous behaviour of some ground beetle larvae has been demonstrated (Klimes and Saska 2010), and as adult ground beetles, foraging behaviour of larvae is impacted by the differences in weed seed characteristics (Lundgren 2009). Thus, our protocol may be adapted to investigate weed seed consumption with ground beetle's larvae and compare seed characteristics which limit their consumption.

To summarize, our recommendations for a standardized protocol to test ground beetles seed consumption are the following: (i) Individuals should be stored and tested in individual container and maintained with an insert, with a mixed diet of seeds and animal food ad libitum, until the number of individuals required for the experiment have been reached. Prior to the start of experiments, individuals should be fed for a period of 24 h minimum to homogenize their level of satiation. (ii) Then, we recommend a fasting period of 3 days (72 h), in order to level the hunger between each ground beetle with moistened cotton to provide water resource. (iii) We recommend a 24-h duration of test, which appeared sufficient to identify differences among treatments or species. Weed seeds should be disposed in a patch to mimic seed spatial distribution in the field (barochory). We recommend burying seeds in sand as it appeared the most convenient substrate to conduct a large amount of replicated experiments. Indeed, sand without any seeds and other food

supply is easy to find and identification of remaining seeds is simple, in particular with small-grained sand.

In addition to our protocol and general experimental setup, we further propose some general care conditions to keep ground beetles alive between successive tests and even for extended experimental sessions of up to 2 months with the same individuals. Our results suggest to add a feeding period with food mixture (24 h). A shorter period of test (24 h vs 48 h) may also explain the lower mortality rate measured in the second protocol of replicated test. Our general housing conditions were tested on a total of 28 different ground beetle species (Deroulers and Bretagnolle 2018) using the same conditions (box size, food items, temperature and humidity), and all species accustomed to these conditions despite supposed large diet differences. For future research, this protocol type will allow to replicate tests, as an example, to compare interspecific consumption between weed seed by ground beetles, or to measure and to compare consumption with the same individual of ground beetle species with different seed densities.

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