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A protocol for increased throughput phenotyping of plant resistance to the pollen beetle

Laura Bellec,^{a,b*}  Maxime R Hervé,^a  Anne-Sophie Mercier,^a Pierre-Arthur Lenal,^b Sébastien Faure^b  and Anne-Marie Cortesero^a



Abstract

BACKGROUND: Improving crop resistance to insect herbivores is a major research objective in breeding programs. Although genomic technologies have increased the speed at which large populations can be genotyped, breeding programs still suffer from phenotyping constraints. The pollen beetle (*Brassicogethes aeneus*) is a major pest of oilseed rape for which no resistant cultivar is available to date, but previous studies have highlighted the potential of white mustard as a source of resistance and introgression of this resistance appears to be a promising strategy. Here we present a phenotyping protocol allowing mid-throughput (*i.e.*, increased throughput compared to current methods) acquisition of resistance data, which could then be used for genetic mapping of QTLs.

RESULTS: Contrasted white mustard genotypes were selected from an initial field screening and then evaluated for their resistance under controlled conditions using a standard phenotyping method on entire plants. We then upgraded this protocol for mid-throughput phenotyping, by testing two alternative methods. We found that phenotyping on detached buds did not provide the same resistance contrasts as observed with the standard protocol, in contrast to the phenotyping protocol with miniaturized plants. This protocol was then tested on a large panel composed of hundreds of plants. A significant variation in resistance among genotypes was observed, which validates the large-scale application of this new phenotyping protocol.

CONCLUSION: The combination of this mid-throughput phenotyping protocol and white mustard as a source of resistance against the pollen beetle offers a promising avenue for breeding programs aiming to improve oilseed rape resistance.

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Supporting information may be found in the online version of this article.

Keywords: mid-throughput phenotyping; quantitative traits; related species; canola; *Brassicogethes aeneus*; *Brassica napus*; *Sinapis alba*

1 INTRODUCTION

Insect pests are globally responsible for an estimated 15% of crop yield loss worldwide^{1,2} and novel management approaches are needed to reduce the environmental hazards related to the heavy reliance on synthetic insecticides.^{3,4} One alternative control strategy is breeding for plant resistance to insect pests. The development of insect-resistant cultivars requires the identification and characterization of sources of resistance as a first step. However, domesticated crops have been selected mainly for their agronomic characteristics, sometimes at the expense of defensive traits.⁵ Exploring natural variation in resistance traits among wild relatives of crop species is therefore a promising strategy to identify desirable genes,^{5,6} which could be introgressed into elite cultivars.^{7,8}

Nevertheless, introgression of valuable genes remains a challenging task since plant resistance to insects is, in most cases, a quantitative mechanism involving multiple loci.⁹ In this context, the identification of quantitative trait loci (QTLs) responsible for

the resistance requires both plant genotyping to identify the genetic bases of the traits of interest, and plant phenotyping to quantify these traits in heterogeneous plant populations. The probability to detect genotype–phenotype associations increases with the population size and the number of replicates per individual of the population, which leads to the need to genotype and phenotype hundreds to thousands of plants.^{10–13} While genomic advances have increased the speed of the identification of molecular markers of the resistance,^{14–16} the phenotyping of large numbers of plant lines remains the rate-limiting step in breeding efforts.^{11,13,17} In addition, high-throughput phenotyping also suffers from constraints related to the rearing of insects in the

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laboratory (complex or even impossible for insects that have obligatory diapause in their life cycle) or their sampling in the field (subject to regional, seasonal and year-to-year variations in insect presence), and the fact that plant-insect interactions are highly dependent on the plant's phenological stage or the insect's physiological status.^{18,19}

In this study, we present a new phenotyping protocol to quantify plant resistance against the pollen beetle in large plant populations. The pollen beetle (*Brassicogethes aeneus* F. syn. *Meligethes aeneus*, Coleoptera: Nitidulidae) is one of the major pests of oilseed rape (OSR; *Brassica napus* L., Brassicaceae) in Europe.²⁰ Adults are generalist pollinivores that destroy flower buds just before blossoming to feed from the pollen they contain, inflicting serious yield losses.²¹ Hervé and Cortesero,²² Seimandi-Corda *et al.*²³ and Austel *et al.*²⁴ showed that although pollen beetle susceptibility varies among OSR cultivars, none of those tested could be considered resistant. In parallel, a relative species, the white mustard (*Sinapis alba* L., Brassicaceae), has shown some level of resistance to the pollen beetle in multiple studies.^{24–27} Thus, introgressing the white mustard resistance into elite OSR cultivars appears a promising strategy.¹⁸ However, we currently lack mid-throughput (*i.e.* increased throughput compared to current methods) phenotyping protocols allowing the quantification of resistance levels to the pollen beetle in large plant populations. This is due to the many constraints associated with the phenotyping of plant resistance to this insect species, such as the need for mass sampling of insects from the field since it cannot be reared and the use of crop plants at a particular and short-lived phenological stage, *i.e.* just before flowering (the 'green-yellow bud stage'), which is the most vulnerable stage to damage by this pest. This last constraint makes large field screenings practically impossible since homogeneity in plant phenology between genotypes tested is required, which is unrealistic with large plant panels. Grown stage homogeneity is important because, it is known that flowering OSR plants are more attractive than non-flowering ones for pollen beetles^{28,29} and that differences in plant height also influences plant colonization.²⁸ Field screenings, then, seem too prone to bias for large-scale phenotyping, although they might be useful to give first hints on plant genotypes worthwhile to be phenotyped rigorously under more controlled conditions.

Here, we selected contrasted white mustard genotypes from an initial field screening and then assessed their level of resistance to the pollen beetle in controlled conditions using a standard phenotyping method using 'classically potted plants' and a plastic container as a partitioning system.³⁰ We then tried to upgrade this protocol for more mid-throughput phenotyping, by testing two alternative protocols: 'miniaturized plants' with a tube as partitioning system, and detached single buds. The alternative protocol which best matched contrasts found with the standard protocol was then evaluated for its applicability on a large panel composed of hundreds of plants.

2 MATERIALS AND METHODS

2.1 Plants

All genotypes used in this study were Innolea-supplied lines initially obtained from genetic resource centers (Supporting Information, Table S1). An initial field screening was conducted in 2016 with 50 genotypes of white mustard sown in Rosenthal (Germany) to identify potential contrasts of pollen beetle feeding intensity in this species. Briefly, 100 seeds per genotype were sown in a nursery row in March 2016. Damage was visually

assessed on each plant and from these observations a unique, approximate mean number of buds damaged per genotype was estimated. Genotypes used for laboratory experiments were selected based on the initial field screening (see Supporting Information, Fig. S1) and consisted of one moderately susceptible OSR genotype (var. 'Liho',³⁰) used as a control, and three contrasted genotypes of white mustard: one susceptible genotype ('M1') and two resistant genotypes ('M2' and 'M3'). To validate the phenotyping with miniaturized plants and a tube as a partitioning system, an additional large panel of 62 genotypes was used. All plants used in the experiments were at the 'green-yellow bud stage', *i.e.*, BBCH 55–57.³¹ Experiments with classically potted plants and detached flower buds were performed in 2018, while experiments with miniaturized plants were performed in 2020.

2.1.1 Classically potted plants

Seeds were sown in individual propagation plugs (65% blond peat, 20% black peat, 15% perlite, 2% Danish clay; pH = 6.2) and placed in controlled conditions (14:10 L:D, 70% RH, 18 °C) for 2–3 weeks. After this period, plantlets were transplanted in individual 1 L pots with the same substrate and placed in controlled conditions as before until bud development (approximately 3 weeks). The whole growth process took 5–8 weeks, depending on the genotype.

2.1.2 Miniaturized plants

Plants were grown in seedling trays with 3.5 × 5 cm cells filled with fertilized substrate (Premier Tech: Horticulture – NFU 44–55). Nutrition (N-P-K: 2.5–5–2.5) was provided *via* irrigation two or three times a week, depending on the requirements of the plants, plants were maintained in controlled conditions (16:8 L:D, 70% RH, 18 °C). The whole growth process took 3–5 weeks.

2.2 Insects

Overwintered pollen beetles were collected in winter OSR crops at Betton (Brittany, France) from February to May and maintained under controlled conditions on an artificial substrate as described in Bellec *et al.*³² Insects were placed in individual Petri dishes (\varnothing = 35 mm) containing a moistened filter paper for 48 h and starved prior to all experiments. Since both females and males are pests, the sex of the individuals was not recorded in the experiments as we aimed to find sex-independent sources of resistance. Moreover, determination of the sex of pollen beetles is a laborious task that is unrealistic in large scale phenotyping. No ethical approval was required for the use of this species.

2.3 No-choice feeding experiments on whole classically potted plants

Following Hervé *et al.*,³⁰ four pollen beetles were placed on the main inflorescence of an entire plant and enclosed in a plastic container (h = 85 mm, \varnothing = 65 mm) that separated the main inflorescence from the rest of the plant. The pot was suspended by a nylon-thread fixed to the wall to avoid bending of the stem. To prevent insect escape, a piece of cotton wool was placed around the stem at the bottom of the pot (Supporting Information, Image S1.a). After 24 h, the total number of buds present on the inflorescence, as well as the number of buds damaged by feeding, were counted to calculate the proportion of buds damaged. Here and in the following experiments, preliminary tests were performed to define the number of introduced pollen beetles per plant, as well as the experiment duration, so that sufficient feeding contrasts could be observed but without reaching the total

destruction of the inflorescence (*i.e.*, saturation). Twenty-one replicates were performed per genotype.

2.4 No-choice feeding experiment on detached flower buds

One pollen beetle was placed for 24 h in a Petri dish ($\varnothing = 35$ mm) containing a moistened filter paper and a flower bud excised from a plant. All buds were of length 2–3 mm, *i.e.* the length preferred by pollen beetles on OSR plants and the mean length of white mustard flower buds, for which no specific preference according to bud size is observed.³² After the experimental period, the presence of feeding damage on the bud was recorded. Forty-six replicates were performed per genotype, with flower buds excised from at least 10 different plants per genotype.

2.5 No-choice feeding experiments on whole miniaturized plants

Four pollen beetles were placed on the main inflorescence of an entire plant totally enclosed in a plastic tube divided into two sections (a piece of cotton wool surrounding the stem at their junction was used to prevent insects moving to the part of the plant below). Plants were transplanted in the device in plastic cups of the same size as seedling tray cells used for growth. The height of the tube section containing the inflorescence was constant ($h = 100$ mm, $\varnothing = 60$ mm), while the height of the section containing the vegetative part was adjusted according to the height of the plant (Supporting Information, Image S1.b). After 48 h, the total number of buds present on the inflorescence, and the number of those damaged by feeding, were counted to compute the proportion of buds damaged. Phenotyping lasted for 3 weeks in total and was divided in two sequential sessions comprising half of the replicates for all genotypes. The phenotyping of the second session started when the first was completely over. Due to differences in development time among genotypes to reach the green-yellow bud stage, phenotyping of all the genotypes in a session lasted for maximum 1.5 weeks, *i.e.* not simultaneously for all plants. Fifteen replicates were performed to assess the phenotyping method on the four previously described genotypes and 10 replicates were performed to validate the phenotyping method on a large panel of 62 genotypes.

2.6 Data analysis

All statistical analyses were performed with the R software v.4.0.2.³³ Only data from replicates where all pollen beetles were alive at the end of the experiment were used in analysis. The proportion of buds damaged (for experiments on entire plants) and the probability of being damaged (for experiments on detached buds) were compared among genotypes using a likelihood ratio test applied on a Generalized Linear Model (GLM, family: binomial, link: logit). Estimated Marginal Means were used since they are preferable to classical means, as they are computed independently of other explanatory variables included in the model (*e.g.* covariates, confounding or other experimental factors, random factors, *etc.*). Pairwise comparisons of Estimated Marginal Means (EMMeans) were then performed (R package 'emmeans'³⁴) with *p*-values adjusted using the False Discovery Rate correction.³⁵ Pearson's correlation tests were used to assess the relationship between results of the different phenotyping protocols.

3 RESULTS

Significant differences between genotypes in the proportion of buds damaged were observed in feeding tests on entire plants with classically potted plants ($\chi^2 = 168.59$, $df = 3$, $p < 0.001$, Fig. 1(a)). The proportion of buds damaged was the highest for the OSR treatment, followed by white mustard genotypes M1, M2 and M3, in that order. The same feeding contrast was observed with miniaturized plants ($\chi^2 = 88.38$, $df = 3$, $p < 0.001$, Fig. 1(b)), and a strong positive correlation was found between results obtained with these two phenotyping methods ($r = 0.998$, $df = 2$, $p < 0.01$, Fig. 1(d)). The feeding test with detached buds did not provide the same contrast as observed with classically potted plants. The probability of bud damage was the lowest for M2 while no significant difference was found between the bud damage of OSR treatment, M1 and M3 ($\chi^2 = 88.38$, $df = 3$, $p < 0.001$, Fig. 1(c)). Moreover, no relationship was found between feeding damage observed with classically potted plants and detached buds ($r = 0.263$, $df = 2$, $p = 0.734$, Fig. 1(e)). Based on these results, the protocol based on miniaturized plants was retained for further evaluation. When applied on a large panel of 62 white mustard genotypes with 10 replicates each (*i.e.* 620 individual plants), it allowed the discrimination of a significant variation in the proportion of buds damaged, with EMMeans proportions (+/–SE) ranging from 0.013 ± 0.006 to 0.085 ± 0.015 ($\chi^2 = 281.42$, $df = 62$, $p < 0.001$, Fig. 1(f)).

4 DISCUSSION

This study was designed to develop a phenotyping protocol allowing the quantification of plant resistance against the pollen beetle in large plant populations. Since field phenotyping of plant resistance to this insect faces several critical challenges,^{28,29} a protocol to move towards a mid-throughput phenotyping in controlled conditions, where the plant phenological stage can be homogenized, was developed. Contrasted white mustard genotypes were first selected from an initial field screening, then assessed and compared to one OSR genotype in controlled conditions using the phenotyping protocol developed by Hervé et al.³⁰ Although this protocol showed the same contrasts as observed in the field (*i.e.* the highest feeding damage on OSR, followed by white mustard genotypes M1, M2 and M3 in that order), it presents constraints in terms of time required (5–8 weeks of plant growth) and space (11 cm diameter; approximately 8 pots per m²), which limits use with large numbers of plants. To optimize this phenotyping method, plants were 'miniaturized' to speed up growth (3–5 weeks), to save space both during growth and experiments (6 cm diameter tubes; approximately 27 tubes per m²), and to ease plant manipulation. The early flowering observed with this plant growth protocol is an indicator of stress,³⁶ but although stressful conditions (such as limited space and growing substrate) can modify the plant physiology,^{37–39} and thus traits associated with resistance, the same resistance contrasts as observed in the field and the classically potted plants were found with miniaturized plants. Although experiments on miniaturized plants were not performed the same year as those on classically potted plants, results of these experiments were highly correlated. On the contrary, results from experiments performed on detached flower buds did not correlate with those obtained on classically potted plants, although these experiments were performed the same year. This excludes year-related biases in our results. It should be noted that while the phenotyping protocol with miniaturized plants was developed for white mustard and

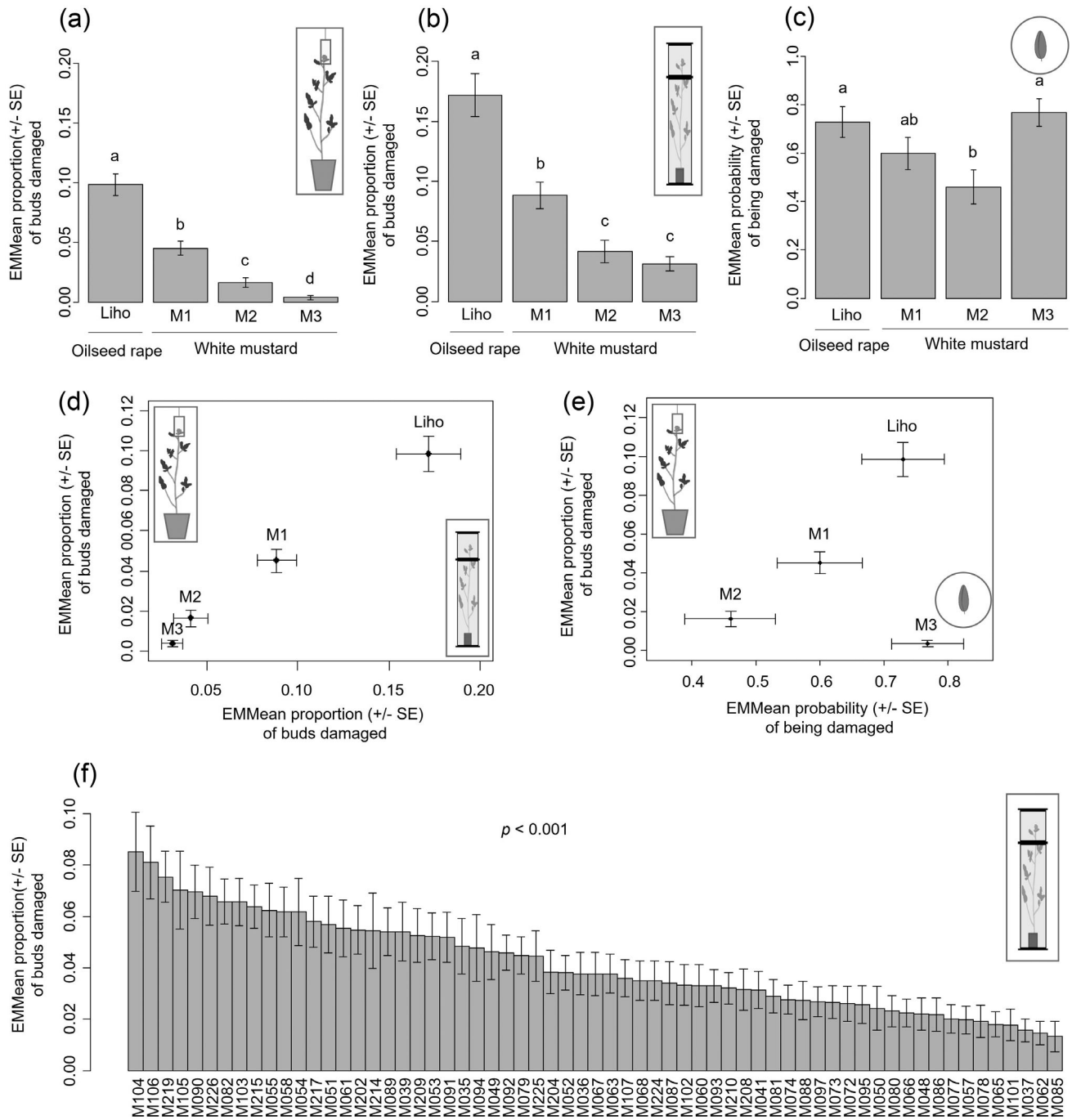


Figure 1. Pollen beetle feeding intensity on oilseed rape and white mustard genotypes in controlled conditions. (a, b, c) EMMean proportion (+/- SE) of buds damaged and EMMean probability (+/- SE) of being damaged depending on the plant genotype with (a) classically potted plants ($n = 21$ per genotype); (b) miniaturized plants ($n = 15$ per genotype); (c) detached buds ($n = 46$ per genotype). (d, e) Relationships between feeding intensity observed with classically potted plants and (d) miniaturized plants; (e) detached buds. (g) EMMean proportion (+/- SE) of buds damaged on a large panel of white mustard genotypes using miniaturized plants ($n = 10$ per genotype). Different letters indicate significant differences between genotypes.

spring oilseed rape genotypes, it is applicable to winter oilseed rape with the addition of a vernalization step prior to transplantation in the phenotyping device. Besides its advantages with the pollen beetle, this phenotyping setup with miniaturized plants could allow the phenotyping of other insects restricted to a particular organ, such as cabbage root fly larvae (*Delia radicum*) on roots, cabbage stem flea beetle (*Psylliodes chrysocephala*) adults on leaves or cabbage seed weevils (*Ceutorhynchus assimilis*) on reproductive organs.²⁰ Strategies to accelerate plant growth are

common, but are primarily based on adjustments of the photoperiod, temperature, light quality or planting density,^{40–42} rarely on space restriction for root growth.

Speed plant growth approaches are of particular interest for plants that are phenotyped at pre-flowering growth stages that are longer to reach.^{43,44} Promising results obtained on a small plant panel led to the phenotyping method being tested on a large population of miniaturized plants. Since the probability to detect associations between phenotypic and genotypic traits

increases with the population size and the number of repetitions per individual,^{10–13} this protocol was tested on a large white mustard population of 620 individual plants. We found significant variation in pollen beetle feeding damage among white mustard genotypes, which indicates the potential for large-scale application of this phenotyping protocol. Further improvements of this protocol can be envisaged. Placing plants and insects inside the setup and especially recording the proportion of buds damaged, which requires visual inspection of all individual buds, remain time-consuming and labor-intensive tasks. Phenotyping on detached buds therefore seems to be an attractive solution, however our results show that feeding contrasts observed on detached buds are not consistent with those obtained on entire plants, which supports results obtained by Hervé *et al.*²⁸ Indeed, both the feeding contrasts between OSR and white mustard, and between white mustard genotypes, were lost. The wounding provoked by the cutting of flower buds can induce different chemical responses among genotypes that could explain such discrepancy.^{45–48}

The use of mid-throughput phenotyping protocols such as the one described here allows the acquisition of resistance data, which may then be used for genetic mapping of QTLs. Although traditional marker-assisted selection has proven successful for monogenic traits or dominant genes,^{49–51} there is still a lack of studies for multigenic traits conferring partial resistance, especially on polyploid genomes like in OSR.^{52,53} A complementary approach to marker-assisted selection could be to understand mechanisms behind resistance, in order to identify resistance biomarkers and their associated molecular markers.^{54,55} The use of molecular markers in combination with biomarkers may indeed be a successful strategy to select for complex traits. In addition to simplifying breeding for resistance by avoiding large phenotyping with insects, the identification of resistance traits would provide pest-specific responses, knowledge on relationships between resistance traits and other plant attributes, and an understanding of the context in which resistance may evolve in insects.⁵⁶ In the absence of resistance biomarkers, phenotyping protocols such as the one described here are valuable tools. Although results obtained in controlled conditions may not be fully reproducible in the field and multiple field validations are needed,^{57–60} our system opens a promising avenue for breeding programs aiming to improve OSR resistance against the pollen beetle.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

M.H., A.M.C., S.F. and L.B. conceived the ideas and designed the methodology. M.H., S.F., A.S.M., P.A.L. and L.B. collected the data. M.H. and L.B. analyzed the data. L.B. wrote the first draft of the manuscript and all authors contributed to revisions. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Figshare at <https://figshare.com/account/articles/20764747>.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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