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Research Article

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Quantifying the impact of *Psylliodes chrysocephala* injury on the productivity of oilseed rape

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Abstract

BACKGROUND: Current European Union and United Kingdom legislation prohibits the use of neonicotinoid insecticidal seed treatments in oilseed rape (OSR, *Brassica napus*). This ban, and the reduction in efficacy of pyrethroid insecticide sprays due to resistance, has exacerbated pest pressure from the cabbage stem flea beetle (*Psylliodes chrysocephala*) in winter OSR. We quantified the direct impact of *P. chrysocephala* injury on the productivity of OSR. Leaf area was removed from young plants to simulate differing intensities of adult feeding injury alone or in combination with varying larval infestation levels.

RESULTS: OSR can compensate for up to 90% leaf area loss at early growth stages, with no meaningful effect on yield. Significant impacts were observed with high infestations of more than five larvae per plant; plants were shorter, produced fewer flowers and pods, with fewer seeds per pod which had lower oil content and higher glucosinolate content. Such effects were not recorded when five larvae or fewer were present.

CONCLUSION: These data confirm the yield-limiting potential of the larval stages of *P. chrysocephala* but suggest that the current action thresholds which trigger insecticide application for both adult and larval stages (25% leaf area loss and five larvae/plant, respectively) are potentially too low as they are below the physiological injury level where plants can fully compensate for damage. Further research in field conditions is needed to define physiological thresholds more accurately as disparity may result in insecticide applications that are unnecessary to protect yield and may in turn exacerbate the development and spread of insecticide resistance in *P. chrysocephala*.

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

Keywords: thresholds; cabbage stem flea beetle; economic injury level; compensation; tolerance; integrated pest management; rapeseed

1 INTRODUCTION

Oilseed rape (OSR, *Brassica napus* L.) is the second most widely produced vegetable oil crop globally[1] and the primary oilseed crop in Europe,[2] where it forms an important component of agricultural rotations with cereals.[3] *Psylliodes chrysocephala* L. (cabbage stem flea beetle) is one of the most economically important autumn pests of winter OSR in the United Kingdom (UK) and coastal areas of Europe[4] and causes direct injury, which can be damaging in two distinct ways. First, adult beetles feed on the cotyledons and leaves of plants, causing characteristic "shot-holing" damage that can threaten crop establishment. Second, the stem-boring larvae, which feed within the leaf petioles and stem, weaken the plant and increase its susceptibility to frost damage and secondary infections, for example stem canker *Plenodomus* (*syn. Leptosphaeria*) *maculans*.[5]

Since the early 1990s, *P. chrysocephala* has typically been controlled in OSR through use of neonicotinoid insecticide seed treatments; these act systemically and give good protection during early growth to allow crop establishment.[6] However, there has been growing global concern about the impact of these seed treatments on non-target species, particularly pollinators.[7,8] In 2013, the European Union (EU) imposed restrictions on their use, banning neonicotinoid seed treatments on crops used by bees, including OSR,[9] and in 2018 extended to a blanket ban on their use outdoors.[10] There have also been calls to review their

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use in the United States, Canada, Australia, New Zealand[11,12] and some parts of Africa.[13] In the absence of the neonicotinoid seed treatments, OSR growers are increasingly relying on the use of pyrethroid insecticide sprays.[14] This overdependence on a single insecticide group has, in effect, stalled any insecticide resistance management strategy and exacerbated the development and spread of resistance to pyrethroid insecticides, a phenomenon now being reported in *P. chrysocephala* across the EU.[14–21] The ban on the use of neonicotinoid seed treatments in OSR originally raised concerns that injury from *P. chrysocephala* would lead to reductions in the amount of OSR grown across Europe.[22] These concerns have been realized;[23] in the UK, for example the area of winter OSR grown has reduced from 621 000 ha in 2014 before the ban[24] to 337 000 ha in 2020.[25] The reluctance of farmers to grow OSR has been attributed largely to *P. chrysocephala* and has resulted in the loss of OSR from many farms' cropping rotations.[26,27]

With only one synthetic insecticide control option available, it is crucial that applications of pyrethroids, and any future control products,[28] are minimized to reduce exposure of the beetle and selection pressure for resistance. Understanding what level of injury the crop can compensate for physiologically before yield loss is crucial to achieving this and ensuring that insecticides are used only when necessary. Decisions on the application of a pesticide are often based on an economic action threshold, that is, the abundance of a pest or extent of plant damage above which yield deficit is greater than the cost of management implementation.[29] The economic action thresholds available for *P. chrysocephala* in Europe vary between countries, with the UK having the highest thresholds before action is advised.[4] UK growers are advised to apply insecticide (i) against adult damage at the first sign of attack when the risk is high during emergence of cotyledons or when >25% of leaf area has been lost between the cotyledons unfolding and the two-leaf stage or when 50% of the leaf area has been lost at the three- to four-leaf stage,[30,31] or (ii) against larvae, when the mean number exceeds five larvae per plant.[30]

The UK threshold of five larvae per plant is derived from a study by Purvis[32] in which the efficacy of organophosphates to control *P. chrysocephala* was tested. However, as noted by the author, the yield responses recorded at a mean of five larvae/plant may have been due, or partially due, to the benefits from insecticidal action on reducing co-occurring virus vectors (aphids). There is little empirical evidence on the physiological threshold for leaf loss in OSR and the need exists for further research on the direct effects of *P. chrysocephala* adult feeding on OSR yield.[33,34]

OSR has been shown to have a high capacity to compensate for leaf area loss or defoliation, given time.[35–37] However, work on the effects of defoliation has largely focused on production of glucosinolates as a response to injury[38,39] or has considered only early growth stage tolerance to injury, assessed by the rate of biomass accumulation.[35,36,40,41] Most studies have not evaluated the long-term compensation capacity of the crop, and in particular the effects on OSR yield, although it has been shown that spring OSR, which has a significantly shorter growing season than winter OSR, can tolerate high levels of defoliation (from grazing by sheep or mowing) with negligible impacts on yield if the injury occurs prior to stem elongation.[42,43] To understand the true impact of *P. chrysocephala* injury to OSR and relate the data to action thresholds it is crucial to take plants to maturity and assess effects on yield (seed quantity and quality).

Data on larval infestation of OSR in the UK have shown an increasing trend in occurrence and abundance of *P. chrysocephala* larvae following the ban on neonicotinoid seed treatments in 2013.[44] This increasing abundance is of concern and requires more research to understand the carrying capacity of OSR for *P. chrysocephala* larvae before there is a loss in yield. In recent years a wide range of integrated pest management strategies have been explored for reducing the risk posed by *P. chrysocephala*,[4,28,41] but further work is required to understand the direct impacts of the beetle on OSR growth and yield to properly understand risk and to use efficiently any control methods commercialized in the future.

Here, we aimed to assess the physiological tolerance of OSR plants to leaf area loss caused by *P. chrysocephala* adult feeding and feeding by larval stages, both alone and in combination, to determine the levels of adult feeding damage and larval infestation load that impact yield. We hypothesized that high levels of adult feeding would affect plant productivity, particularly when the plant is also infested with larvae, but that plants would be able to effectively compensate for low levels of feeding damage. Using potted OSR plants in outdoor cages, we simulated *P. chrysocephala* adult feeding damage by removing controlled amounts of leaf area, and artificially infested OSR plants with varying numbers of larvae to quantify their separate and combined effects on OSR phenotype, floral rewards and yield.

2 MATERIALS AND METHODS

This study comprised two experiments using potted OSR plants in outdoor pest-excluding mesh cages (semifield conditions) to quantify the direct impact of injury caused by *P. chrysocephala* on the productivity of OSR. In Experiment 1 (2017 harvest), the leaves of the OSR plants were manually injured to varying degrees using a hole punch to simulate leaf area loss caused by adult feeding in a consistent manner. In Experiment 2 (2018 harvest), the same method was used to simulate adult feeding at varying degrees and plants were then inoculated with *P. chrysocephala* larvae to test the effects of low, medium and high larval infestation and interaction with leaf area loss. In Experiment 2, measurements of plant phenotype were used to test whether leaf area loss and/or larval infestation impacts plant biomass (height), floral abundance or floral resource quantity. In both experiments the plants were grown to maturity to assess the impact of treatments on final yield.

2.1 Experiment 1: Leaf area injury and growth stage

For Experiment 1 (2017 harvest) winter OSR (cv. DK Imperial) seeds were sown at weekly intervals in individual plant plugs (19 × 19 mm, 30 mm depth) and kept in an unlit, unheated glasshouse until germination occurred. The plants were then transplanted to 18-cm diameter pots (13th February 2017), ensuring all plants had equal amounts of compost (Rothamsted standard mix, Petersfield Products, Leicester, UK, which comprises 75% peat, 12% sterilized loam, 10% lime-free 5 mm grit and 3% vermiculite). The compost was fertilized with 16-9-12 NPK + 2MgO with added trace elements: Bo, Mo, Cu, Mn Zn, and Fe at 3.5 g/m^3 (Osmocote Exact Mini 3–4, Scotts International, ICL, Treviso, Italy). They were then placed in an outdoor net cage (4 × 4 × 2 m, with a mesh gauge of 2 mm) to exclude pests and pollinators.[45]

Four levels of simulated leaf injury were applied to the plants (0%, 25%, 50% or 90% leaf area loss) at two early growth stages (GSs) according to the BBCH scale[46]: cotyledons expanded

(GS10) and the first true leaf extended (GS11), giving eight treatments in total. These levels were selected to include the UK threshold of 25% leaf area damaged threshold for plants with two or fewer leaves.[30] The injury was done 25 days after sowing using a leather hole punch (3 mm diameter) to remove a controlled amount of leaf area (estimated by eye using a defined scoring system[47]). Multiple holes were made at random, starting from the leaf/cotyledon tip and working towards the petiole, selecting either the leaf edge or an area between the edge and the midrib, until separated holes were no longer possible. This simulated *P. chrysocephala* adult feeding, that is, shot-hole damage, as opposed to removal of a more contiguous area. It has been shown in spring OSR that defoliation by cutting a contiguous area resulted in less biomass re-growth and fewer pods than plants with multiple holes of the same area.[48] This allowed damage to be as biologically relevant as possible while ensuring standardization between replicates and treatments.

Following plant injury, two grids of 100 plants were set out. Each grid comprised 10 randomized blocks (grid columns) of 10 plants (Fig. S1). Each block contained two replicates of the two control treatments (GS10 or GS11 with 0% damage) and one replicate of each of the other three leaf area loss treatments in combination with the two GS treatments. Overall, the control treatments were therefore replicated 40 times and all other treatments 20 times. The randomized design was generated using Genstat for Windows 20th edition.[49] Plants were evenly spaced within each grid over an area of 2 m^2, thus simulating a density of 50 plants/m^2, common for OSR crops,[50] with a 1-m gap between the two grids. The plants were placed on a metal mesh stand, supported ~10 cm from the ground to facilitate slug control; slug pellets were spread on the ground underneath the mesh to prevent slug damage. Plants were hand watered using a watering lance until mid-May 2017, when automatic drip irrigation was set up; this delivered a standardized volume of water to just beneath the soil surface at regular intervals each day. Plants were maintained until harvest.

2.2 Experiment 2: Leaf injury and larval infestation

For Experiment 2 (2018 harvest), winter OSR (cv. DK Imperial) plants were grown as per Experiment 1 to reduce variation between years (sown on 5th October 2017). Leaf area loss treatments were combined with subsequent controlled infestation with *P. chrysocephala* larvae. Experiment 1 showed no effect of growth stage on effects of leaf area loss (see Section 2.1). Therefore, in Experiment 2 plants at the one to two true leaf stage were used (GS11-12) as these are more likely to be infested with larvae (SMC personal observation). The leaf area loss treatments were applied as for Experiment 1 but with 0%, 25%, and 90% leaf area removed (i.e., no 50% leaf area loss treatment) and were done 55 days post sowing. This was followed 48 h later by the larval infestation (see next Section 2.2.1) at zero (control), low (one larva), and medium (five larvae, current UK action threshold) levels. This sequence of simulated adult injury followed by larval infestation mimics the usual order of *P. chrysocephala* attack as it occurs in the field. In addition to this 3 × 3 factorial set of nine treatments, uninjured plants (0% leaf area loss) were also subjected to a high level of infestation (25 larvae, a field realistic high infestation typical of some areas in UK, albeit later in the season than GS 12).[44] Each of the 10 treatments was replicated 12 times, with plants arranged in two neighboring 10 × 6 grids; each grid comprised six randomized blocks (columns) of 10 plants (Fig. S2). Guard rows of additional OSR plants were grown around each grid (Fig. S2) to allow each of the experimental plants to be within a plant density of 50 plants/m^2 as per Experiment 1, and to allow comparable growth restrictions across all blocks, that is, no experimental plants were located on the open edges of the grids.

2.2.1 *Inoculation of plants with P. chrysocephala larvae*

Larvae of *P. chrysocephala* were obtained from an untreated crop of OSR (cv. Campus) on Rothamsted Farm, Harpenden, UK in December 2017. Live larvae were carefully extracted from the plants by cutting open the stems and petioles using a scalpel under a light microscope and removing the larvae with a fine hair paint brush. Newly emerged (small) second-instar larvae (determined using the key by Ebbe-Nyman)[51] were transferred to Petri dishes lined with damp filter paper and kept refrigerated (5 °C) prior to plant infestation. Second-instar larvae were used because they were more robust (less prone to desiccation) than first instars and had a higher infestation success rate than either first or third instars (unpublished preliminary data); they were also caught most frequently in pitfall traps set in OSR crops (DJC personal observation), suggesting that they actively move between plants in the field.

Plants were infested with varying numbers of larvae to create three contrasting larval infestation levels: "low," "medium," and "high" by introducing 1, 5, or 25 larvae per plant, respectively. This was done by carefully placing larvae on the soil at the base of the stem (hypocotyl), leaving them to locate and enter the plant naturally. To make the rate of infestation comparable between treatments, and more realistic of field conditions, the larvae were introduced over a 9-day period with 20% of the total number of larvae added every other day (1–9 December 2017). This allowed time for the collection of larvae and ensured larvae were added to experimental plants within 24 h of being collected. Horticultural fleece was placed over the plants during the infestation period as freezing conditions were forecast. No larval mortality was observed on the plants' exterior or on the soil surface when plants were checked 24 h later.

2.2.2 *Confirmation of larval infestation rates and development to adult stage*

To estimate the proportion of larvae that entered the plants successfully, destructive sampling was performed on two randomly selected blocks (totaling 20 plants) from grid 2 (Fig. S2) in early spring (103 days after introduction, 21–23 March 2018; GS14). Plants were taken to the laboratory and dissected under a binocular microscope; petioles and stems were sliced using a scalpel and the larvae were counted. Plants in the remaining four blocks in grid 2 were left to flower and were removed before pod ripening (GS77-83) to assess larval survival and development to adulthood; the plants were dissected (154 days after infestation, 21–22 May 2018) as described above and the number of larvae found in the stems was recorded. As some larvae would have matured and left the plant to pupate in the soil, the plant pot (with the compost) was bagged to capture emerging adults after pupation. Bagged plants were observed every 2–3 days for 5 weeks until 27 June; any adult *P. chrysocephala* captured in the bags following emergence were counted and removed.

2.2.3 *Flower sampling and nectar and pollen measurements*

In Experiment 2, six flowers were removed from each plant for analysis of nectar (three flowers) and pollen (three flowers). All six flowers were taken from the main raceme and were of the same approximate age (i.e., between flower numbers 20 and 35 counting from the first flower opened), to ensure robust comparison between flowers and plants. Flowers produced early were

chosen for analysis to minimize effects on yield; removal of later-produced OSR flowers has been shown to affect yield more than removal of earlier produced flowers.[52] Whole flowers were cut at the base of pedicle with sharp scissors to minimize plant injury and to leave a clean, uniform wound. This was done 24 h after first opening to allow nectar secretion to occur and the pollen to dehisce,[53] and between 11:00 and 13:00 h to allow for any daily fluctuation of these resources.[52]

Nectar was extracted by inserting a micropipette (10 µL; Drummond, Broomall, PA, USA) into a single nectary and the percentage of sugar in the nectar was measured using a handheld refractometer (0–50% and 40–85%; Bellingham and Stanley Ltd, Tunbridge Wells, UK). Due to constraints during flowering, it was not possible to accurately record nectar volume and as such the values obtained are relative measures of sugar concentration, expressed as a relative treatment effect.

Flowers collected to measure pollen were stored immediately in 99.9% ethanol. Quantification of the number of pollen grains per milliliter was based on methods adapted from Hicks *et al.*[54]; anthers were removed from the flower using scissors and placed in an Eppendorf tube, and pollen extracted by sonication and vortex spinning samples. Once the pollen was in suspension, the anthers were removed, and the tubes were centrifuged to form a pollen pellet. The pellets were oven dried at 60 °C overnight and then re-suspended in ethanol (60–120 µL), vortex spun and sonicated to evenly distribute the pollen grains. A 20-µL subsample was then transferred to a hemocytometer and the grains counted under a light microscope. The counts were converted to an estimated number of pollen grains per millilitre using the equation:

$$\text{pollen grains per millilitre} = \frac{((\text{pollen count}/\text{cell number}) \times \text{cell volume}) \times \text{dilution factor}}{}$$

2.3 Yield measurements

In both experiments, all plants that survived were hand-harvested when pods were brown and dry to the touch (GS 89). Experiments 1 and 2 were harvested on 28 August 2017 and 12 July 2018, respectively, giving a total growing season, from sowing to harvest, of 211 days in Experiment 1 and 280 days in Experiment 2. Plant height (to the nearest 5 cm) and the number of pods (pod set) on the primary raceme, and the total number of pods and blind stalks (podless stalks that failed to set pods)[55–57] were recorded for each plant. Pods from the main raceme were collected and stored in paper bags in dry conditions. By adding the total number of pods and blind stalks, an estimated total flower number per plant was calculated.

From each plant, 10 pods were randomly selected from the main raceme and split to extract the seeds. Seed quality was assessed using a Near Infra-Red Analyser (Perten DA 7250 NIR Analyser, Hägersten, Sweden) which uses industry standard models derive measurements of the moisture, percentage oil content and glucosinolate concentrations from NIRS data. The same seeds were then processed through an electronic seed counter (Elmor Applied Electronics, C3 Counter, Schwyz, Switzerland) linked to a balance to determine the number of seeds and automatically calculate the thousand grain weight (TGW).

2.4 Statistical analyses

All statistical analyses for both experiments were performed using Genstat for Windows 20th edition.[49]

To assess the impacts of early leaf-area loss and timing of damage (growth stage) on OSR phenotype and yield, we used multi-stratum ANOVA to analyses the data from Experiment 1 (with strata corresponding to the two whole grids, blocks within grids and pots within blocks within grids). To remove variance heterogeneity, the number of flowers were transformed to square roots; all other responses were untransformed. Eight plants died during Experiment 1 (Fig. S1) so to maintain the original balanced and orthogonal design structure, these few unrecordable values were set to missing and estimated using the method of Healy and Westmacott[58] as implemented in Genstat's ANOVA algorithm, with a corresponding loss of 1 residual df for each missing value estimated.

For Experiment 2, only five plants died, but due to several plants not producing flowers in the correct place for measurement according to the protocol, there were more missing values than in Experiment 1 (Fig. S2); the percentage of sugar in the nectar and the number of pollen grains were therefore analyzed using a linear mixed model fitted using restricted maximum likelihood (REML) with the design strata (as described above for Experiment 1) as random effects and the missing plants removed. All other data from Experiment 2 were analyzed using multi-stratum ANOVA as described above, incorporating a variance-stabilizing transformation where appropriate (see results). A nested treatment structure was used to compare (a) the treatment with high larval infestation (25 larvae introduced) and no leaf area loss, with the remaining nine treatments combined (from hereon called the nested set), and (b) to assess the main effects of larval infestation and leaf area loss and their interaction within the nested set.

3 RESULTS

3.1 Confirmation of larval infestation rates and development to the adult stage

The plant dissections in Experiment 2 showed that *P. chrysocephala* larvae successfully entered the experimental plants although the intended infestation levels were not always achieved, especially for the high larval infestation treatment (25 larvae introduced); 33% of larvae were recovered when one larva was introduced, 47% were recovered when five larvae were introduced but only 26% larvae were recovered when 25 larvae were introduced. However, increased introduction rates generally led to increased infestation (Fig. 1(a)), with significant differences between the high larval infestation treatment (25 larvae introduced) and the low (one larva) and medium larval infestation (five larvae) treatments combined (ANOVA, $F_{1,6} = 32.58$, $P < 0.001$) and between the low and medium larval infestation treatments ($F_{1,6} = 8.54$, $P = 0.027$). No *P. chrysocephala* larvae or adults were recorded in the control treatments, therefore each treatment returned low (<1), medium (c. 2), and high (>5) larval infestation (Fig. 1(a)). A similar trend was recorded for adults collected later, from the larval development assessment in summer (GS 77-83). Evidence of *P. chrysocephala* larval feeding activity (external scaring on leaf petioles and stem, and internal feeding tunnels) was recorded from a total of 19 out of 28 plants sampled from treatments with introduced larvae (no larvae were found at this assessment), but only eight plants produced adults, with the highest capture being from the treatment with 25 larvae introduced (Fig. 1(b)). Despite the lower infestation levels actually achieved, we will continue to refer to the treatments by the number of larvae introduced.

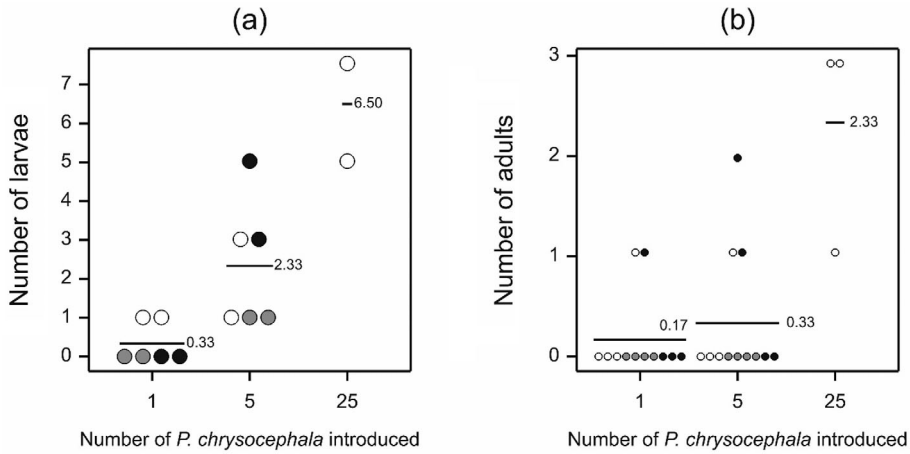

Figure 1. Dot histograms of (a) the number of *Psylliodes chrysocephala* larvae recovered *Via* plant dissection from oilseed rape plants following artificial infestation (103 days post introduction) and (b) adults emerging having successfully completed development (154 days post introduction). Different numbers of larvae were introduced, aiming for a low (1), medium (5), or high (25) infestation. Horizontal lines are located at the mean and values are given numerically alongside. Plants were also exposed to manual leaf area loss simulating *P. chrysocephala* adult feeding damage: shaded circles represent 0% white, 25% gray, and 90% black leaf area loss treatments.

3.2 Plant mortality

A total of eight plants died in Experiment 1 (leaf area loss): five controls and three from the 90% leaf area loss treatment when applied to plants at the cotyledon stage (GS10) (Fig. S1). In Experiment 2 (leaf area loss × larval infestation) a total of five plants died (Fig. S2). There was no clear trend with treatment for losses in either experiment. All other plants survived and produced harvestable pods.

3.3 Flower production

In Experiment 1 (analysis on square root scale), the total number of flowers produced (range 0–542, overall square root scale mean 9.51) was not affected by the level of leaf area loss or by growth stage and there was no interaction between these factors ($F_{3,172} = 1.26$, $P = 0.291$, $F_{1,172} = 1.51$, $P = 0.221$ and $F_{3,172} = 2.24$, $P = 0.085$, respectively) (Table S1). In Experiment 2 (analysis on the $\log_{10}$ scale), flower production was similar ($F_{2,40} = 0.43$, $P = 0.652$) for the 0-larvae introduced control, low and medium larval infestation level treatments (one and five larvae introduced, respectively) when averaged across leaf area loss treatments (log scale means of 1.614, 1.591, and 1.639, respectively, $n = 18$, SED = 0.0515). Flower production was higher among the nested set (log scale mean 1.615, $n = 54$) than for the high infestation treatment (25 larvae introduced; log scale mean 1.379, $n = 6$, SED = 0.0665, Fig. 2(a)) (Table S2). There was no effect of leaf area loss ($F_{2,40} = 2.20$, $P = 0.124$) and no interaction between leaf area loss and larval infestation level ($F_{4,40} = 1.91$, $P = 0.128$).

3.4 Floral resource measurements of nectar and pollen

The nectar sugar concentration was highly variable (range 29–75%, observed mean 65.75%, $n = 189$) with no effect of leaf area loss (Wald statistic, $\chi^2_2 = 0.88$, $P = 0.644$), larval infestation level ($\chi^2_2 = 3.03$, $P = 0.220$) or interaction ($\chi^2_4 = 7.91$, $P = 0.095$). The same was found for the numbers of pollen grains (range 53333–9 666 666, observed mean 300 081 grains/ml), with no effect of leaf area loss ($F_{2,50.7} = 0.71$, $P = 0.496$) or larval infestation ($F_{2,52.0} = 0.91$, $P = 0.410$), and no interaction ($F_{4,51.0} = 0.38$, $P = 0.821$).

3.5 Plant height

In Experiment 1, plant height was unaffected by the amount of leaf area loss ($F_{3,165} = 1.9$, $P = 0.132$) or the growth stage (GS10 or GS11) when damage was incurred ($F_{1,165} = 0.09$, $P = 0.765$), and there was no interaction between these two treatment factors ($F_{3,165} = 0.07$, $P = 0.976$, range 80–150 cm, overall mean 116.5 cm, $n = 200$; Table S1). However, in Experiment 2, plants in the high larval infestation treatment (25 larvae introduced) were significantly shorter (range 45–64 cm, mean 52.7 cm, $n = 6$; Table S2) compared with the mean (90.4 cm, range 40–125 cm, $n = 54$) of plants in the nested set of treatments ($F_{1,40} = 27.59$, $P < 0.001$, SED = 7.183; Fig. 2(b)). Otherwise, within the nested set, plant height was unaffected by lower levels of larval infestation ($F_{2,40} = 0.77$, $P = 0.472$). However, plant height was affected by injury level ($F_{2,40} = 3.40$, $P = 0.043$, average height 91.09 cm, 97.28 cm, and 82.82 cm for 0%, 25%, and 90% leaf area loss, respectively) but there was no interaction between the injury level and larval infestation ($F_{4,40} = 1.64$, $P = 0.182$).

3.6 Pod production

In Experiment 1, the total number of pods per plant (range 7–169, overall mean 53.9, $n = 200$; Table S1) was unaffected by leaf injury level ($F_{3,172} = 1.49$, $P = 0.218$) or growth stage when injury occurred ($F_{1,172} = 0.02$, $P = 0.892$), and there was no interaction ($F_{3,172} = 1.59$, $P = 0.194$). In Experiment 2, all surviving plants produced harvestable pods (range 3–51, mean 20.26 pods/plant; Table S2) but the mean total pod count for plants from the high larval infestation treatment (range 3–16, $n = 6$, mean 9.83; Table S2) was significantly lower than for the combined nested set (nested set range 8–51, $n = 54$, mean 21.42, $F_{1,40} = 13.02$, $P < 0.001$, SED = 3.211). No difference was found between the mean number of pods produced on larval infested plants among the nested set ($F_{2,40} = 1.05$, $P = 0.361$). However, the level of injury did affect pod production ($F_{2,40} = 4.81$, $P = 0.013$, a mean number of 20.87, 25.52, and 17.86 pods were produced on plants with injury levels of 0%, 25%, and 90%, respectively). There was no interaction ($F_{4,40} = 0.95$, $P = 0.447$; Fig. 3(a)).

3.7 Seed measurements

In Experiment 1 there was no effect of the level of leaf area loss ($F_{3,162} = 0.30$, $P = 0.829$) or the growth stage when the damage

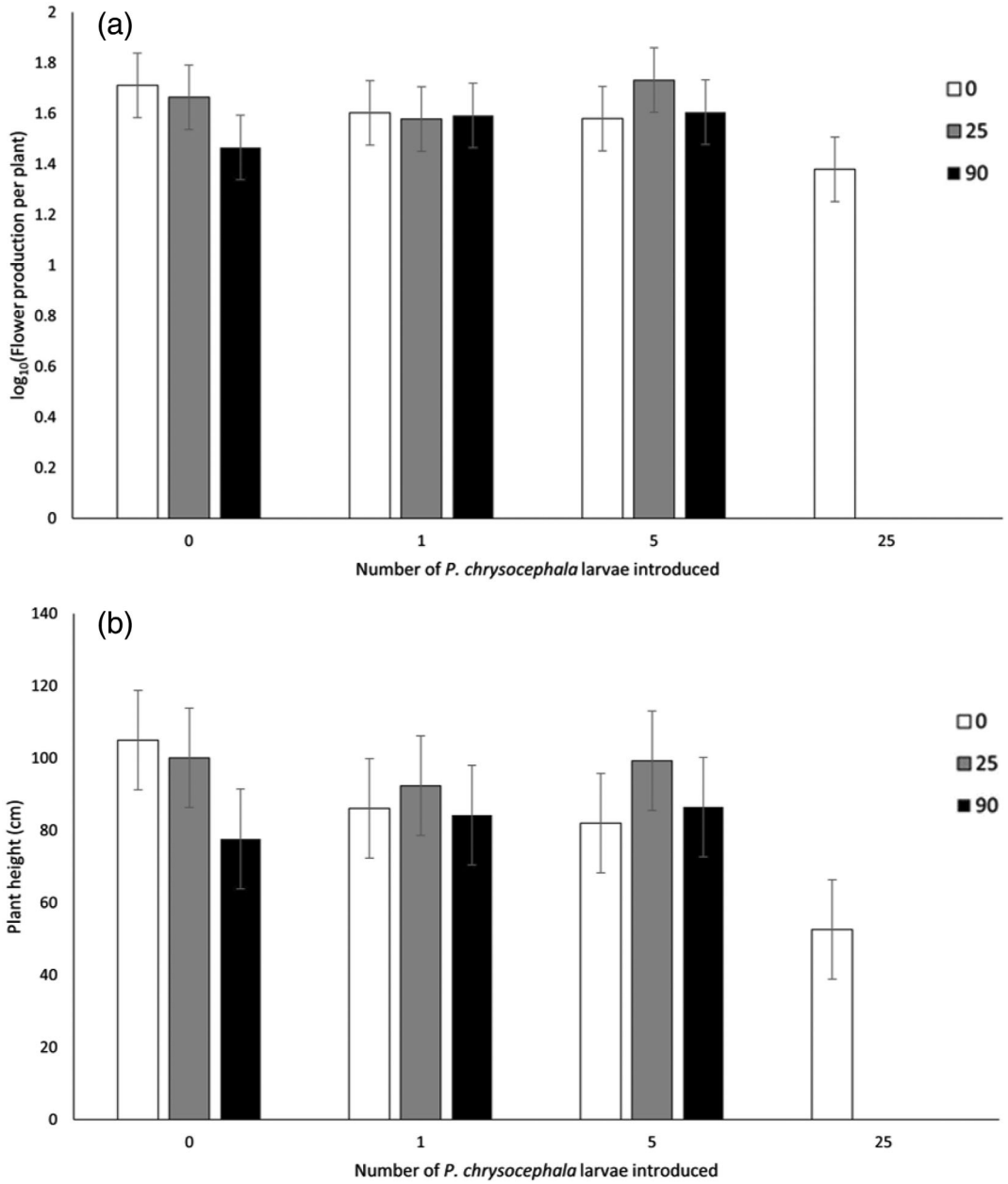

Figure 2. Oilseed rape phenotype effects of variation in leaf area loss (0%, white; 25%, gray; 90%, black) and low, medium, and high levels of *Psylliodes chrysocephala* larval infestation (following introduction of one, five, or 25 larvae/plant, respectively†): (a) Mean number of flowers produced (log_{10} scale, ±95% confidence interval) and (b) mean plant height (±95% confidence interval). Note high larval infestation (25 larvae introduced) was only applied to plants with no leaf area loss. †The actual numbers of larvae in each plant are unknown as larvae are cryptic; the mean infestation levels of a subsample are shown in Fig. 1(a).

was incurred ($F_{1,162} = 0.22$, $P = 0.642$) on the average total number of seeds from 10 pods (range 13–275, overall mean 198.7; Table S1), and there was no interaction between the two factors ($F_{3,162} = 0.12$, $P = 0.947$). However, in Experiment 2 the mean number of seeds per pod was significantly reduced for plants from the high larval infestation treatment (range 2–21, $n = 6$, mean 10.0; Table S2) compared with the mean of treatments in the nested set (range 8–25, $n = 54$, mean 18.1, $F_{1,40} = 16.70$, $P < 0.001$, SED = 2.7; Fig. 3(b)).

The thousand grain weight (TGW) of seeds was not affected by the amount of leaf area lost in Experiment 1 (range 1.529–8.947 g, mean 4.651 g, $n = 200$, $F_{3,161} = 0.78$, $P = 0.508$; Table S1) and there was no effect of growth stage ($F_{1,161} = 2.17$, $P = 0.143$) and no interaction between growth stage and leaf area loss ($F_{3,161} = 1.38$, $P = 0.251$). In Experiment 2, the TGW (log_{10} scale) was similar for seeds from plants of the high larval infestation treatment (range 1.51–3.94 g, $n = 6$, log scale mean 0.416; Table S2) compared with plants within the nested set of treatments combined (range 1.57–6.28 g, $n = 54$, overall log scale mean 0.459, $F_{1,38} = 0.65$, $P = 0.426$, SED 0.0524; Table S2). Within the nested set of treatments, there was no effect of the amount of leaf area lost ($F_{2,38} = 0.77$, $P = 0.471$) or the level of larval infestation ($F_{2,38} = 0.59$, $P = 0.557$), and there was no interaction between these two factors ($F_{4,38} = 1.01$, $P = 0.414$).

3.8 Seed quality

In Experiment 1, the oil content of the seeds (range 31.6–53.9%, overall mean 48.5%) was not affected by the leaf area loss level

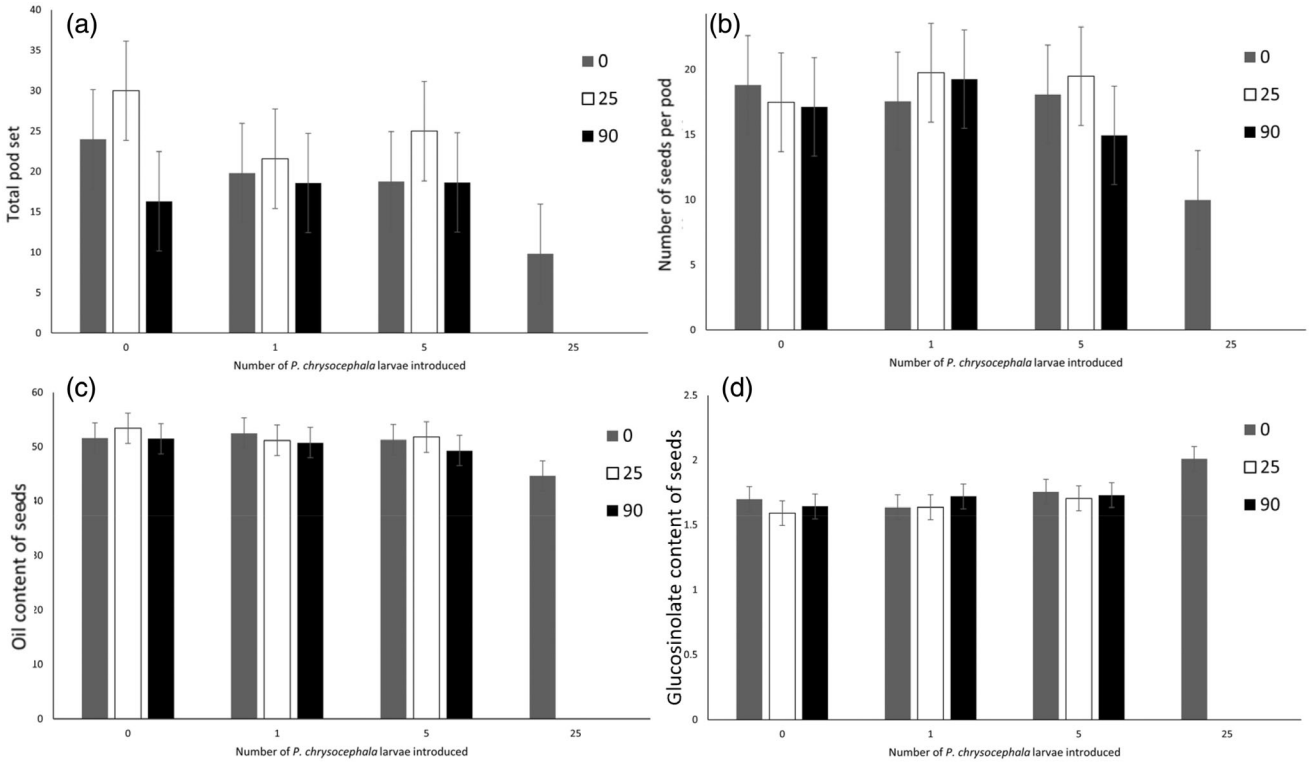

Figure 3. Oilseed rape yield effects of variation in leaf area loss (0%, white; 25%, gray; 90%, black) and low, medium, and high levels of *Psylliodes chrysocephala* larval infestation (following introduction of one, five or 25 larvae/plant, respectively*). (a) Mean number of pods per plant (± 95% confidence interval), (b) mean number of seeds per pod (±95% confidence interval), (c) mean oil content of seeds (±95% confidence interval), and (d) mean glucosinolate concentration of seeds expressed as $\log_{10}$ (μmol/g) (±95% confidence interval). Note high larval infestation (25 larvae introduced) was only applied to plants with no leaf area loss. *The actual number of larvae in each plant is unknown as larvae are cryptic; the mean infestation levels of a subsample are shown in Fig. 1(a).

($F_{3,163} = 0.48$, $P = 0.694$) or growth stage at which the injury occurred ($F_{1,163} = 0.62$, $P = 0.431$) and there was no interaction between these factors ($F_{3,163} = 0.20$, $P = 0.899$). However, in Experiment 2, there was a significant reduction in the percentage oil content of seeds from plants in the high larval infestation treatment (mean 44.6%, $n = 6$) compared with the mean of the nested set ($F_{1,40} = 21.84$, $P < 0.001$, mean 51.5%, $n = 54$, SED = 1.47%). Within the nested set there was no effect of either larval or damage level and no interaction ($F_{2,40} = 0.71$, $P = 0.496$, $F_{2,40} = 1.12$, $P = 0.335$, and $F_{4,40} = 0.40$, $P = 807$, respectively; Fig. 3(c)).

In Experiment 1 (analysis on the $\log_{10}$ scale) there was no effect of leaf area loss on the level of glucosinolates in seeds (range 21.56–320.88 μmol/g, log scale overall mean 1.680, $F_{3,163} = 0.35$, $P = 0.790$) or of growth stage ($F_{1,163} = 0.23$, $P = 0.631$) and there was no interaction ($F_{3,163} = 0.26$, $P = 0.852$). However, in Experiment 2 (analysis on the $\log_{10}$ scale), the concentrations of glucosinolates in seeds were significantly higher in the high larval infestation treatment (range 61.4–225.11 μmol/g, log scale mean 2.010) than the mean for the nested set ($F_{1,40} = 43.59$, $P < 0.001$, range 30.59–77.47 μmol/g, log scale mean 1.679, SED = 0.050). Within the nested set there was no effect of either the level of leaf area loss or larval infestation and no interaction ($F_{2,40} = 2.70$, $P = 0.079$, $F_{2,40} = 1.25$, $P = 0.297$, and $F_{4,40} = 0.69$, $P = 600$, respectively; Fig. 3(d)).

4 DISCUSSION

In the field, adult *Psylliodes chrysocephala* feeding on OSR causes shot-hole leaf area loss that can range from very small areas to most of the leaf area removed and death of the plant. Action thresholds based on the amount of leaf area loss vary between countries on the European continent, with advice to treat the crop at the growth stages relevant to this study (i.e.,cotyledon to two true leaves) when 10% of the leaf area has been lost in Denmark, Germany, Italy, Luxembourg and the Netherlands and 25% in France and the UK.[4] There is very little published information on the substantiation for these thresholds and little evidence for the effect of varying levels of leaf loss on yield. Although we found some evidence that high leaf area loss (90%) reduced plant height and the number of pods, in both our experiments no effect on yield was observed at any level of leaf removal at the two growth stages tested (cotyledon or one true leaf stage). This highlights the potential of OSR to recover from very high levels (90%) of leaf area loss at early growth stages (akin to severe adult *P. chrysocephala* feeding damage) and the ability, given good conditions and time, to grow away from leaf loss and deliver yields comparable to unaffected plants.

The lack of statistically significant effects on OSR flower production, resource quality of flowers, and yield parameters from any of the leaf area loss treatments applied at early growth stages in this study are in line with studies showing little to no yield penalty from leaf area loss at early growth in both simulated injury experiments[35,59–62] and field observations under natural pest infestations.[63] For example, Susko and Superfisky[48] reported no significant impact on reproductive biomass production following 50% simulated leaf area loss applied during early growth stages (to GS 30) of spring OSR. The level of injury they applied was simulated in a similar manner (hole punch) to our study and both studies may not be truly representative of actual pest feeding injury. It has been shown that responses to actual feeding of another Brassica

pest, the crucifer flea beetle (*Phyllotreta cruciferae*), differ according to the type of damage, with no response from simulated defoliation but a reduction in seed yield and oil content from actual feeding injury compared with simulated injury.[59] This is possibly due to the absence of biological factors such as pest saliva that impact the production of secondary chemicals, which in turn entails a metabolic cost to the plant.[64] However, a study comparing the glucosinolate content of manually damaged and *P. chrysocephala*-damaged plants showed no difference,[39] which supports the relevance of the method used in this study. Further work is clearly required to understand the level of influence direct adult feeding has on plant recovery; the lack of effect observed in our study using potted plants, grown in relatively optimal conditions, is in contrast to the many crops that are lost every autumn as a result of intense adult feeding damage, especially in suboptimal conditions. Our methods may not truly represent the impact of *P. chrysocephala* adult feeding activity in the field, where plants are exposed to cumulative feeding injury over an extended period whilst also under potentially suboptimal conditions in terms of water and nutrient status and additional pest damage from other species. Nonetheless, our results concur with field experiments that show that OSR plants can respond to early growth stage defoliation with strong biomass recovery.[35,43,62] Thus, the use of current action thresholds for adult damage, based on economic calculations, may result in the unnecessary use of insecticides whereas the use of physiological injury thresholds could lower insecticide use, thus lowering input costs, reducing selection pressures for insecticide resistance, and lowering risks to nontarget species.

This is the first study to experimentally infest OSR with field-collected *P. chrysocephala* larvae to quantify their chronic effect on crop yield. The intended infestation rates were not 100% successful, indeed reaching only 26% of the target for the high infestation level (25 larvae/plant). This target was likely too high and failure was probably due to the plants at GS 11-12 being too small and physically unable to support such high larval infestation, and possibly other factors such as interspecific competition, particularly as second-instar larvae were used. We used newly emerged (small) second-instar larvae as our preliminary experiments showed that these survived the outdoor cage conditions better than first instars, and they were the most commonly found stage in pitfall traps, indicating they are most adapted to intraplant movement (akin to the artificial infestation process). However, although we have previously recorded the presence of second-instar larvae in similarly small plants (Ortega-Ramos and Cook, unpublished data), second-instar larvae are uncommon in large numbers at the time when most OSR plants develop their first true leaves. Despite these limitations, larvae were successfully recovered later in the experiment from test plants and the capture of adult *P. chrysocephala* towards the end of the experiment demonstrates the potential of the method; while in need of optimization (e.g., comparison of pupation rates in the field compared with potted plants), the method allowed the empirical test of the impact of full larval development on OSR growth and yield. Previous studies have used laboratory reared larvae to artificially infest plants[65] and/or sampled effects on plants only a few days or weeks after infestation.[38,39,65] However, the method reported here can be used as a simple way of artificially infesting plants with *P. chrysocephala* larvae in relative abundance (low, medium, high) and could facilitate further research into the tolerance of OSR cultivars to prolonged larval feeding, the effects of various agronomic practices, and meteorological conditions such as drought as well as for testing effects of new insecticides.

In the current study, there was in general no interaction between the early leaf area loss and larval infestation, implying that the main yield limiting factor, once the crop is established, is from the developing larvae. The larval economic action threshold varies from country to country, with treated advised at only one larva/plant in the Czech Republic and Poland, two to three larvae/plant in France, and five larvae/plant in Germany and United Kingdom.[4] However, information on the development of the thresholds and effects of different numbers of larvae is sparse.[32,33,66–68] Our results show that there was no significant yield effect when plants were infested with larvae at GS11-12 at current European action thresholds of five larvae or lower. However, introducing high numbers of larvae (i.e., 25, but with a projected mean infestation rate of 6.5 larvae per plant with a range between 5 and 8), did significantly reduce plant height, flower and pod production, the number of seeds produced, and their quality (lower oil content and higher concentrations of glucosinolate), suggesting that the physiological injury level, the level of injury where a plant begins to ensure yield penalties,[69] for *P. chrysocephala* larvae is between five and 25 larvae/plant in this experimental set up. Overwintering temperatures are likely important; field studies showed that high larval infestation (six to 10/plant) followed by mild winter conditions did not inevitably lead to yield loss.[66] In Experiment 2, temperatures did fall below freezing but maximum temperatures were relatively high with overall mild conditions (Fig. S3). Further study is needed to assess the physiological threshold more precisely, especially in field conditions, and using a range of OSR cultivars (e.g., conventional *versus* hybrids) under varying management conditions. Physiological thresholds are likely to vary with agronomy and meteorological factors, facilitating future economic thresholds which also consider product cost, efficacy and application costs *versus* yield loss and its value.

This study has revealed a potentially novel effect of *P. chrysocephala* larval infestation which may impact bees and other flower visiting insects. When high levels of *P. chrysocephala* larvae (25) were introduced (subsampled mean of 6.5/plant) the plant's capacity to produce flowers was significantly reduced. This could reduce resource availability for pollinating insects, resulting in an unintended consequence of the neonicotinoid ban (designed to protect pollinators), as larval numbers in OSR have significantly increased since the ban[44] and are therefore more likely than before the ban to exceed the infestation levels, impacting flower availability. Although no effects were seen in either nectar sugar concentration or amount of pollen, these values were based on only one plant for the treatment with 25 larvae introduced (and it is unknown how many larvae this plant was infested with). Nectar volume and total sugar concentration were not recorded, and pollen was not assessed for protein or amino acid content but may have been affected by plant stress caused by *P. chrysocephala* larval infestation as these resources have been found to vary among different OSR cultivars,[52] so other flower resource effects might have been missed. For instance, if the total volumes of nectar available were substantially lower in damaged than in undamaged plants, then pollinator visitation and pollination could be reduced, with further impact on seed production and oil content.[70] This requires further investigation as OSR is an important early, mass-flowering crop that is visited by a wide range of insects, including pollinators.[71–75]

5 CONCLUSION

We detailed a method to artificially infest oilseed rape plants with varying quantities of the larval stage of *P. chrysocephala*. This could aid the development of biologically relevant action

thresholds, which would inform farmers and policy makers to better calculate the cost:benefit of insecticide application based on empirical data and not simply yield responses following application. The data reported here suggest that, under relatively optimal conditions, high levels of leaf area loss at early growth stages, that is, leaf injury as caused by adult *P. chrysocephala* feeding, does not impact the final yield and quality and low levels of *P. chrysocephala* larvae infestation (<5 larvae/plant) can also be tolerated. However, higher larval infestation (>5/plant) resulted in negative effects on plant growth, which would in the field negatively affect both crop production and the flower-visiting activities of insects such as bees.

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CONFLICT OF INTEREST

There are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are available from the corresponding authors upon reasonable request.

SUPPORTING INFORMATION

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

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