



# Long-term captive-rearing affects oviposition behavior and nymphal survival of a weed biological control agent

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## HIGHLIGHTS

- Leaf toughness affects nymphal survival of long-term laboratory-reared *A. itadori*.
- Oviposition behavior of *A. itadori* is affected by long-term laboratory rearing.
- Time in laboratory rearing affects *A. itadori* performance on field *F. japonica*.
- New collections of *A. itadori* may be better able to establish in the field.

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## ABSTRACT

Adaptation to artificial rearing conditions is a well-documented phenomenon in insects and has been shown to reduce the efficacy of biological control agents. The knotweed psyllid, *Aphalara itadori* Shinji (Hemiptera: Psyllidae), is a biological control agent for *Fallopia japonica* (Hout.) Ronse Decr. (Caryophyllales: Polygonaceae) that has been released in North America and Europe, however, to date successful establishment of the insect over multiple field seasons has not been observed. We conducted a series of experiments to explore the possibility that long-term rearing in laboratory conditions has impaired the psyllid's ability to survive in the field. We used light intensity to manipulate leaf toughness in *F. japonica*, and used the resulting plants in laboratory bioassays to assess the effects of foliage toughness on the survival of the psyllids. We conducted laboratory-choice tests to compare the effects of leaf age on oviposition behavior by *A. itadori*. Finally, we conducted a field experiment, comparing the effects of foliage age on adult oviposition and nymphal survival. All experiments were conducted using a line of psyllids that have been reared in captivity since their collection from Japan, in 2004 (K1 psyllids). The final two experiments also utilized a line of psyllids collected from the same location more recently in 2019 (K2 psyllids). We observed a reduction in K1 *A. itadori* nymphal survival on tough *F. japonica* plants compared to soft plants. *Aphalara itadori* from the K1 line laid significantly more eggs on young versus old leaves, and survived best on young *F. japonica* regrowth in the field. Conversely, K2 *A. itadori* showed no oviposition preference between young and old leaves, and no preference between cut and uncut *F. japonica* foliage in the field. Our results indicate that recently collected psyllids (K2) may be better able to establish on field-conditioned knotweed in North America than older colonies and highlights the possible effects of long-term rearing on biological control agent performance.

## 1. Introduction

Adaptation to artificial rearing conditions is a well-documented phenomenon in insects (Hoffmann and Ross, 2018), and has even been shown to reduce the efficacy of biological control agents upon release (Bertin et al., 2017). The controlled conditions of the laboratory

relax selection pressures associated with variable field environments (Hoffmann et al., 2001). For example, the greenhouse-grown plants on which captive insects often feed are likely younger and possess fewer chemical and physical defenses than those found in the field (Karban et al., 1997; Bernays and Graham, 1998). Long-term rearing on such plants may reduce the ability of captive insect populations to utilize host

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plants of varying quality (Bravo and Zucoloto, 1998). We conducted a series of experiments to examine the effects of foliage quality (age and toughness) on the survival and oviposition behavior of the psyllid *Aphalara itadori* Shinji (Hemiptera: Psyllidae), a biological control agent for Japanese knotweed, *Fallopia japonica* (Houtt.) Ronse Decr. (Caryophyllales: Polygonaceae) in North America. All experiments were conducted using a population of psyllids that had been reared in captivity since their collection from Japan in 2004. Where possible, experiments were repeated with a new line of psyllids, collected in 2019, to highlight the effects of long-term captive rearing on psyllid behavior and performance.

Japanese knotweed is one of a complex of knotweed species that have become invasive in Europe and North America (Barney, 2006). The knotweed complex also includes giant knotweed, *F. sachalinensis* (F. Schmidt) Ronse Decraene, and Bohemian knotweed, *F. × bohemica* (Chrtk & Chrtková) J. P. Bailey, the latter which is a hybrid of Japanese and giant knotweed. Knotweed species are prevalent in at least 41 states in the United States (U.S.) and in nine Canadian provinces (Bourchier et al., 2013; USDA NRCS 2011), and they are projected to continue to expand their range. Analyses of temperature and precipitation thresholds indicate that 53% of the land in southern Ontario is suitable for Japanese knotweed growth (Bourchier and Van Hezewijk, 2010; Gaskin et al., 2014). Japanese knotweed is a herbaceous perennial that forms extremely dense monoclonal stands (Dommanget et al., 2019), reaching heights of up to 3 m (Bailey et al., 2009). The species reduces native plant diversity through intensive light competition (Maurel et al., 2010), heavy leaf litter accumulation (Aguilera et al., 2010), and the production of allelopathic chemicals (Dommanget et al., 2014). The weed is particularly prevalent on riverbanks, where the robust but fairly non-fibrous root system allows for extensive soil erosion, which affects stream nutrients (Urgenson et al., 2009), and negatively affects aquatic fauna (Beerling and Dawah, 1993; Maerz et al., 2005; Gerber et al., 2008; McIver and Grevstad, 2010). Additionally, the extremely vigorous root system enables knotweed species to grow through almost any substrate, including concrete, causing damage to infrastructure and in some cases affecting property values (Wilde, 2019). Traditional methods for controlling knotweed species, such as herbicide sprays and mechanical removal, have achieved limited success, not only because they are unmanageable at the scale of the problem, but because the storage of photoassimilates in underground rhizomes allows for rapid regrowth of the weed (Price et al., 2002; Shaw et al., 2009; Aguilera et al., 2010). Chemical control is also not desirable in many infestations due to their proximity to rivers and streams. Classical biological control is a crucial tool for controlling invasive knotweed populations sustainably, and at relatively low cost.

The knotweed psyllid, *A. itadori*, emerged in 2007 as the leading candidate insect for the biological control of invasive knotweeds (White, 2007), and host-range testing has shown the species to be specific to the knotweed complex (Grevstad et al., 2013). Two biotypes of the insect were initially collected from the native range, one from Kyushu in southern Japan in 2004 (hereafter referred to as K1 psyllids), which performs best on *F. japonica*, and the other from the northern Japanese island of Hokkaido in 2007 (northern biotype), which performs best on *F. sachalinensis* (Shaw et al., 2009; Grevstad et al., 2013). A second population of the southern biotype was collected in 2019 by CABI UK (hereafter referred to as K2 psyllids). The goal with recollecting the Kyushu population was to restore genetic diversity to the psyllid population being released, because the K1 line had been in rearing in the laboratory for over 15 years. It was expected that the K2 line might be better adapted to field conditions having spent less time in captivity.

Females of *A. itadori* lay approximately 600–700 eggs on plant surfaces, and the resulting nymphs pass through five instars (Shaw et al., 2009). Time from egg to adult is 33 days at 23 °C (Laznik and Trdan, 2012). Both nymphs and adults are sap feeders, and adults overwinter in the bark of neighboring trees. In Japan, the psyllids are known to use *Pinus densiflora* Zieb. & Zucc. and *Cryptomeria japonica* D. Don

(Miyatake, 2001). In the introduced range, however, they are expected to use other coniferous trees, as has been observed for other *Aphalara* species (Hodkinson, 2009). *Aphalara itadori* is multivoltine and is expected to complete 1–3 generations per year in the majority of North American sites, based on climate data and observed emergence dates in Japan (Myint et al., 2012; Fritzi Grevstad, personnel communication). The first *A. itadori* releases were conducted in the United Kingdom (UK) in 2010 (Grevstad et al., 2013), in Canada in late 2014, and in the U.S. in 2020. In Canada, releases of more than 200,000 *A. itadori* have now been conducted at sites in British Columbia, Alberta, and Ontario (Bourchier, unpublished data). Although diapausing adults, released in the fall, have been observed to overwinter successfully and to lay large numbers of eggs in spring, the resulting populations have failed to continue for longer than two generations, and no persistent population over multiple winters has been observed at any release site. Similarly, for releases of non-diapausing adults in spring or summer there has been limited survival through multiple generations, and establishment across multiple seasons has not been achieved (Bourchier, unpublished data). It appears in North America, that nymphal survival represents the biggest obstacle for the establishment of *A. itadori*, and that foliage quality may be a factor in the performance of the agent in North American field sites (Jones et al., 2020).

Numerous studies have demonstrated the role that host plant quality plays in the development and survival of herbivorous insects (Hinz and Müller-Schärer, 2000; Wheeler, 2001; Huberty and Denno, 2006) and it is well known that physical and chemical characteristics of plants such as leaf toughness, nutritional quality, and concentrations of secondary metabolites are affected by light conditions (Jansen and Stamp, 1997; Ingersoll et al., 2010; Diaz et al., 2011). We produced ‘soft’ and ‘tough’ *F. japonica* plants by growing under two different light conditions in the greenhouse. The most obvious effect of the light treatments was noticeable as leaf toughness (plants under strong light having tougher leaves than plants growing in shade). While there were probably other differences induced, these were not investigated. Having produced ‘tough’ and ‘soft’ *F. japonica* plants, we conducted bioassays to assess the effects of leaf toughness on K1 psyllid survival.

We also conducted laboratory choice tests to compare the effects of leaf age on oviposition behavior in K1 and K2 psyllids. Insects make use of a wide variety of visual, textural, and olfactory cues when selecting sites for oviposition, all with the goal of maximizing larval performance (Rauscher, 1979). We sought to understand whether oviposition behavior in *A. itadori* is likely to mitigate or exacerbate any nymphal mortality associated with foliage quality. We also sought to address the question of whether oviposition behavior in *A. itadori* has been affected by long-term captive-breeding.

Finally, we conducted a field experiment, comparing the effects of foliage age on nymphal survival in K1 and K2 psyllids. Many insects perform better on younger foliage because it tends to be soft and more nutritious (Steinbauer et al., 1998). Indeed, Jones et al. (2020) showed that late season survival of K1 *A. itadori* nymphs in an Ontario field site was improved on younger foliage resulting from mid-summer cutting treatments. Through the comparison of K1 and K2 psyllids we could not only examine whether foliage quality is a legitimate barrier to field establishment in *A. itadori*, but also determine the extent to which this barrier is a result of long-term captive-breeding of the insect.

## 2. Methods

### 2.1. Insect production

All *A. itadori* used in these experiments were reared at Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Alberta, Canada. Psyllids from the Kyushu (southern) biotype were used for all experiments that required psyllids (experiments two, three, four, and five), as these have been shown to perform better on *Fallopia japonica* (Grevstad et al., 2013). Experiments two and three utilized only

K1 psyllids, while experiments four and five utilized K1 and K2 psyllids as a comparison to assess the possible effects of long-term laboratory rearing.

## 2.2. Plant production

We grew *F. japonica* from root cuttings in shade and full sun, to manipulate leaf toughness. We collected *F. japonica* roots from a site in Toronto, ON, Canada, (43.679163, -79.377795), on 08 October 2019. We chose roots of a similar thickness (1–1.5 cm diameter) and cut them into sections of around 3–4 cm in length, ensuring that each section contained at least one node. We then washed root sections, before planting them in 0.5L pots filled with Fafard Agro-Mix G2 (Sun Gro Horticulture, Agawam, MA, USA). On 22 October 2019, 80 *F. japonica* plants of similar size were selected and divided randomly between two treatments, high light and low light. High-light plants were placed on the bench of the greenhouse, underneath a grow lamp. Low-light plants were placed in the same greenhouse on a bench underneath a shade structure consisting of a wooden frame covered with black shade cloth. Temperature and light conditions for each treatment were monitored at 5-min intervals using HOBO wireless pendant data loggers (Onset, Bourne, MA, 02532).

On 27 November 2019, a single leaf from 60 experimental plants (30 high-light and 30 low-light) was subjected to mechanical testing (Zwick Universal testing machine-10KN load cell on flexure mode) to determine leaf toughness. In order to control for leaf age, the 3rd leaf down from the apical meristem was chosen in each case. Leaves were removed using a razorblade and stored in individual petri dishes with damp tissue paper before testing. In all cases, testing was carried out within 30 min of the leaf being removed from the plant. Immediately prior to testing, individual leaves were clamped between two metal plates (3 mm thick), each with an aligning circular hole (1 cm diameter). Care was taken that the hole exposed a similar location on each leaf, and that no part of the leaf midrib was exposed. The leaf apparatus was then suspended underneath the mechanical testing machine and held in place by two large weights on either side. The machine then tested the force/mm<sup>2</sup> in newtons required to puncture the leaf using a flat-ended circular rod with an area of 72 mm<sup>2</sup> (F<sub>max</sub>).

## 2.3. Experiment one: Effect of leaf toughness on *A. Itadori* establishment on potted plants

We conducted a greenhouse experiment to determine the effects of leaf toughness on psyllid establishment on potted *F. japonica* plants. On 29 January 2020, all 60 plants that were tested for leaf toughness, were moved to a small greenhouse with intermediate light conditions. Temperature and light conditions were monitored every five minutes as above. All experimental plants (30 with tough leaves and 30 with soft leaves) were enclosed in 3L breathable plastic bread-bags. Twenty *A. itadori* adults (K1) were introduced to half of the plants from each treatment (30 plants in total). Psyllids were collected into 50 ml plastic centrifuge tubes (FroggaBio, Toronto, ON, Canada) using a manual aspirator (Ward's Science, Rochester, NY, USA), and the tube containing 20 psyllids was placed at the base of each plant, inside the bread-bag. Tubes were then opened to allow psyllids access to the plants. On 5 February 2020, after a period of 7 days, the plants were moved to the laboratory, and the bread-bags were removed. Adult psyllids were removed from the plants by repeated disturbance, and the number of eggs on each plant was then estimated by scanning the upper surface of each leaf using a magnifying glass. Egg numbers were estimated to ensure the presence of similar numbers of eggs across the two treatments. Plants were then enclosed in bread bags, as above, before being returned to the intermediate light greenhouse.

On 2 March 2020, 27 days after the removal of adult nymphs, all 60 experimental plants were transported back to the laboratory. Leaves and stems of all plants were examined under a dissecting microscope, and

the numbers of surviving *A. itadori* nymphs were recorded. To determine the effects of psyllid activity on plant biomass, all above-soil parts of each plant were then collected in paper bags and placed in a drying oven (Cascade Sciences, Hillsboro, OR, USA) for one week at 60 °C. Dried plants were then weighed individually using a fine digital balance.

## 2.4. Experiment two: Effect of leaf toughness on *A. Itadori* nymphal survival on single leaves

We conducted single leaf laboratory bioassays to determine the survival rate of first instar *A. itadori* nymphs on tough and soft *F. japonica* leaves. *Fallopia japonica* plants were grown in high and low light conditions as described above. On 2 February 2020, 40 *F. japonica* leaves (20 sun and 20 shade leaves) were cut from plants using a razorblade. To control for the effects of age on leaf toughness, we used only the 3rd and 4th leaves down from the apical meristem. Experimental leaves were inserted at the petiole into a small glass vial (5 ml) containing florist foam (Oasis). Vials had been soaked in water until the florist foam was fully saturated. Leaves were held in place using parafilm, and five first instar *A. itadori* nymphs were transferred onto each leaf using a fine tipped paint brush. Vials containing leaves were glued individually to the lids of a small round plastic containers (0.5L), and the containers themselves were sealed over the leaves to prevent them from drying out. Containers were placed in a growth chamber held at 25 °C, with a 16:8 h (light/dark) diel period. On 7 February 2020, after a period of five days, leaves were removed from containers and observed under a dissecting microscope. The number of surviving nymphs on each leaf was recorded.

## 2.5. Experiment three: Effects of leaf age on *A. Itadori* oviposition behavior

We conducted laboratory oviposition choice tests to determine the effects of leaf age on oviposition behavior in both K1 and K2 psyllids. Two excised *A. itadori* leaves (one young and one old) were placed at opposite ends of an experimental arena. All experimental leaves were sourced from a naturally growing patch of *F. japonica* (Lat:43.959318, Long:-78.943353). Young leaves were newly expanded leaves immediately adjacent to the growing tip of lateral branches. Older leaves were cut from the same lateral branches, at least three leaves back from the growing tip. All leaves were searched under a dissecting microscope, prior to use in experiments, to check for herbivore damage and to ensure that no psyllids or predatory insects/mites were present. Leaf surface area was also calculated prior to experiments, to adjust egg counts and ensure that any oviposition preference observed was not simply a function of available space. Experimental arenas comprised of 15 × 20 × 30 cm plastic boxes (Sterilite, Townsend, MA, U.S.) with circular ventilation holes (5 cm diameter) cut at either end. Mosquito netting was glued over the holes to prevent insect escape. Excised leaves were inserted at the petiole into small glass vials (5 ml) containing florist foam (Oasis). Vials had been soaked in water until the florist foam was fully saturated. Leaves were then held in place using parafilm, wrapped around the stem and over the top of the vial, and the vials were secured at either end of the oviposition box (approximately 20 cm apart) using modelling clay.

Fifteen *A. itadori* adults were aspirated from rearing cages into 15 ml plastic centrifuge tubes (FroggaBio, Toronto, ON, Canada). Individual psyllids were not sexed, but colony sex ratios are consistent at approximately 50:50. Tubes were then secured to the base of oviposition boxes using modelling clay. Care was taken to ensure that tubes were secured equidistant from each experimental leaf, with the tube entrance facing the side of the oviposition box, so that leaf choice was not influenced by starting position. Following placement of the vial with insects, the oviposition box lids were secured, and boxes were placed in a dark room underneath larger upturned storage boxes (IrisUSA, Surprise, AZ, U.S.) with LED light strips (NADAIR, Laval, QC, Canada) secured to the roof. This was to ensure that oviposition boxes received even lighting,

controlling for any potential directional bias. Two oviposition boxes could fit under each storage box, and the positioning of new versus old leaves was always alternated within a storage box to further control for any directional bias. Temperature and light conditions within oviposition boxes were monitored at 5-min intervals using HOBO wireless pendant data loggers (Onset, Bourne, MA, 02532).

After 48 h, leaves were removed from oviposition boxes and examined under a dissecting microscope. The number of *A. itadori* eggs on the upper and lower surface of each leaf was recorded. A total of 60 new versus old leaf oviposition trials were conducted; 30 using K1 psyllids and 30 using K2 psyllids.

#### 2.6. Experiment four: Effects of early season cutting treatments on *A. itadori* survival on *F. japonica* at an Ontario field site

We compared the performance of *A. itadori* on bagged branches of cut and uncut *F. japonica* to examine the effects of foliage age on psyllid survival and establishment. A large patch of *F. japonica* was identified at a site in river ravines system (43.679103, -79.377532) in Toronto ON Canada. On 5 June 2020, roughly half of the *F. japonica* stems in the site were cut just above soil level using pruning shears, with cut stems distributed evenly throughout the site. Cut *F. japonica* material was then removed from the site. Five weeks after the cutting treatments, on 10 July 2020, a total of 80 *F. japonica* branches were enclosed in fine mesh bags (<1mm gauge) made from mosquito netting (Fabricland, Whitby, ON, Canada). Mesh bags were secured to the plant stems using wooden clothes pins. Thirty *A. itadori* adults were then introduced into each mesh bag by inserting a 50 ml plastic centrifuge tube containing aspirated individuals. Bagged branches were divided equally between four treatments: 1) lateral branches of mature, uncut *F. japonica* stems, inoculated with K1 psyllids; 2) lateral branches of mature, uncut *F. japonica* stems, inoculated with K2 psyllids; 3) new-growth *F. japonica* sprouted from cut stems, inoculated with K1 psyllids; and 4) new-growth *F. japonica* sprouted from cut stems, inoculated with K2 psyllids.

Between 30 July and 3 August 2020, approximately three weeks after the introduction of *A. itadori*, all 80 bagged branches were harvested and returned to the laboratory at the University of Toronto for observation. Branches were removed by cutting the stem below the bag using scissors. All leaves and stems were examined closely under a dissecting microscope, and the total number of *A. itadori* eggs and nymphs on each branch was recorded.

#### 2.7. Statistical analyses

For experiment one, leaf toughness of the experimental plants was compared between light treatments using an independent samples *t*-test. The number of eggs on plants after a 1-week adult *A. itadori* exposure was compared between tough and soft plants using an independent samples *t*-test. The number of surviving nymphs at the end of the experiment was compared between treatments using a non-parametric Mann-Whitney *U* test, as data were not normally distributed even after log transformation. Above-ground dry biomass was compared between tough and soft plants, and between plants with and without psyllids, using a two-way ANOVA. For experiment two, the percentage of first instar *A. itadori* nymphs surviving the 5-day exposure was compared between tough and soft leaves using a generalized linear model. For experiment three, total oviposition was compared between young and old leaves using paired samples *t*-tests. Paired samples *t*-tests were also used to compare oviposition on upper and lower leaf surfaces. For experiment four, the effects of foliage cutting treatments and psyllid line (K1 and K2) on the numbers of eggs and surviving nymphs were analyzed using generalized linear models. The models also analyzed the interactions between cutting treatments and psyllid line. For each foliage treatment, the relationship between the number of eggs and the number of live nymphs recovered at the point of foliage collection was analyzed using linear regression. In the case of uncut foliage, the

regression intercepts were compared between psyllid lines using ANCOVA. In the case of cut foliage, the slopes of the regression lines were compared in Excel using a modified ANOVA (Arambasi and Randhawa, 2014). With this one exception, all statistical analyses were conducted using SPSS version 26 (IBM, Armonk, USA).

### 3. Results

#### 3.1. Experiment one: Effect of leaf toughness on *A. itadori* establishment on potted plants

Plants grown in high-light experienced an average light intensity of  $267.32 \pm 2.72$  lum/ft<sup>2</sup>, while plants in low-light experienced an average light intensity of  $10.35 \pm 0.13$  lum/ft<sup>2</sup>. The mean  $\pm$  SE leaf toughness (Fmax) was significantly higher in plants grown in high-light conditions ( $0.0675 \pm 0.0021$ ) compared to plants grown in the shade ( $0.0325 \pm 0.0015$ ) ( $N = 60$ ,  $F_{(1, 58)} = 1.676$ ,  $P < 0.001$ ).

Psyllid establishment was better on soft plants grown in low-light conditions than on tougher plants grown in high-light conditions. The presence of psyllids on plants did not significantly affect above-ground dry biomass either on tough plants or soft plants. After a 1-week exposure to *A. itadori* adults, no significant difference was observed between the mean  $\pm$  SE numbers of eggs on tough ( $57.8 \pm 6.5$ ) and soft plants ( $62.9 \pm 7.6$ ) ( $F_{(28, 1)} = 0.741$ ,  $P = 0.612$ ). Significantly more *A. itadori* nymphs were recovered from soft plants compared to tough plants ( $N = 30$ ,  $U_{(29, 1)} = 22.533$ ,  $P < 0.001$ ) (Fig. 1). The presence of psyllids, however, did not affect plant biomass ( $N = 60$ ,  $F_{(58, 1)} = 0.191$ ,  $P = 0.664$ ).

#### 3.2. Experiment two: Effects of leaf toughness on *A. itadori* nymphal survival on single leaves

First-instar nymphs of *A. itadori* survived better on leaves obtained from soft plants than on leaves obtained from tough plants. A significantly higher percentage of first instar nymphs exposed to soft leaves were recovered alive after five days (Mean 88%, Range 60–100%) compared to those exposed to tough leaves (Mean 38%, Range 0–100%) ( $N = 40$ ,  $\chi^2_{(39, 1)} = 44.17$ ,  $P < 0.001$ ) (Fig. 2).

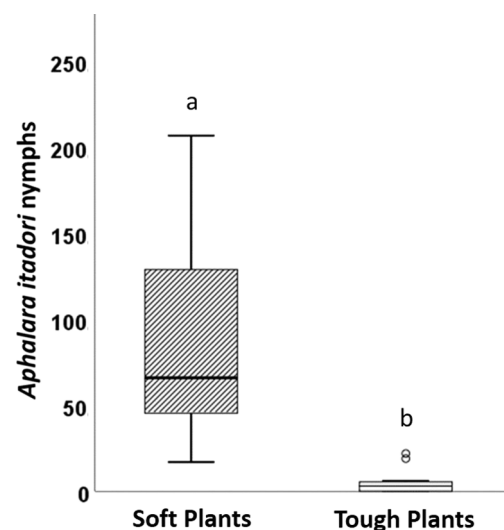


Fig. 1. Numbers of *A. itadori* nymphs recovered from soft plants ( $N = 30$ ) and tough plants ( $N = 30$ ). Nymphs were recovered 27 days after the removal of adult psyllids from plants. Box plots represent a summary of the data (minimum, first quartile, median, third quartile, and maximum). Open circles represent outliers ( $1.5 \times$  interquartile range (IQR)). Letters indicate significant differences ( $\alpha = 0.05$ ).

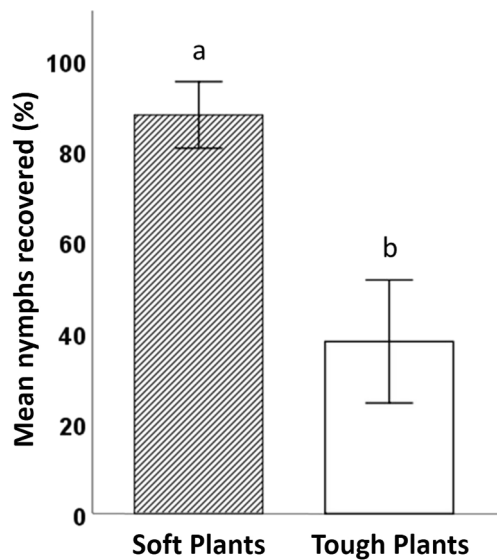


Fig. 2. Mean percentage of first instar *A. itadori* nymphs recovered from leaves of soft plants (N = 20) and tough plants (N = 20). A total of five nymphs were introduced to each leaf, and nymphs were recovered five days after their introduction. Error bars represent standard error. Letters indicate significant differences ( $\alpha = 0.05$ ).

### 3.3. Experiment three: Effects of leaf age on *A. Itadori* oviposition behavior

*Aphalara itadori* (K1) laid significantly more eggs on young leaves ( $69.4 \pm 11.06$ ) compared with old leaves ( $41.2 \pm 6.5$ ) (N = 30,  $t = 2.146$ ,  $P = 0.040$ ). In contrast, we observed no difference in oviposition by *A. itadori* (K2) between new leaves ( $53.1 \pm 6.50$ ) and older leaves ( $43.0 \pm 7.21$ ) (N = 30,  $t = 1.110$ ,  $P = 0.276$ ). There was no significant difference in leaf surface area between young and old leaves used in this experiment (N = 60,  $t = -0.657$ ,  $P = 0.513$ ); overall mean leaf size was  $81.4 \pm 1.3 \text{ cm}^2$ .

Psyllids laying eggs showed no preference between the upper and lower lamina. on either young leaves (N = 30,  $t = -0.183$ ,  $P = 0.856$  for K1 psyllids; N = 30,  $t = 0.270$ ,  $P = 0.789$  for K2 psyllids; N = 60,  $t = 0.051$ ,  $P = 0.960$  for all psyllids combined) or on old leaves (N = 30,  $t = -1.274$ ,  $P = 0.213$  for K1 psyllids; N = 30,  $t = -0.152_{(29,1)}$ ,  $P = 0.880$  for K2 psyllids; N = 60,  $t = -1.150$ ,  $P = 0.225$  for all psyllids combined).

### 3.4. Experiment four: Effects of early season cutting treatments on *A. Itadori* survival of *F. japonica* in an Ontario field site

In terms of the number of eggs laid by *A. itadori* adults that had been released on sleeved branches of *F. japonica*, we observed a significant interaction between foliage treatment (cut/uncut) and psyllid line (K1/K2) (N = 78,  $\chi^2_{(77, 1)} = 5.025$ ,  $P = 0.025$ ). We observed no significant difference, however, in the number of eggs laid by K1 (young regrowth:  $103.3 \pm 37.1$  (Mean  $\pm$  SE), old foliage:  $44.8 \pm 14.7$ ) or K2 (young regrowth:  $49.1 \pm 12.2$ , old foliage:  $103.6 \pm 32.6$ ) psyllids on young regrowth and older uncut foliage (Fig. 3A).

The overall number of eggs laid by *A. itadori* (K1 and K2 combined) did not differ significantly between cut and uncut foliage (N = 78,  $\chi^2_{(77, 1)} = 0.006$ ,  $P = 0.936$ ). There was also no difference in the number of eggs laid, across both foliage types combined, between K1 and K2 psyllids (N = 78,  $\chi^2_{(77, 1)} = 0.008$ ,  $P = 0.928$ ) (Fig. 3A).

In terms of the numbers of live *A. itadori* nymphs recovered from *F. japonica* branches, we observed a significant interaction between foliage treatment (cut/uncut) and psyllid line (K1/K2) (N = 78,  $\chi^2_{(77, 1)} = 5.138$ ,  $P = 0.023$ ). There was no significant difference, however, in the numbers of surviving nymphs of K1 psyllids (young regrowth:  $78.89 \pm$

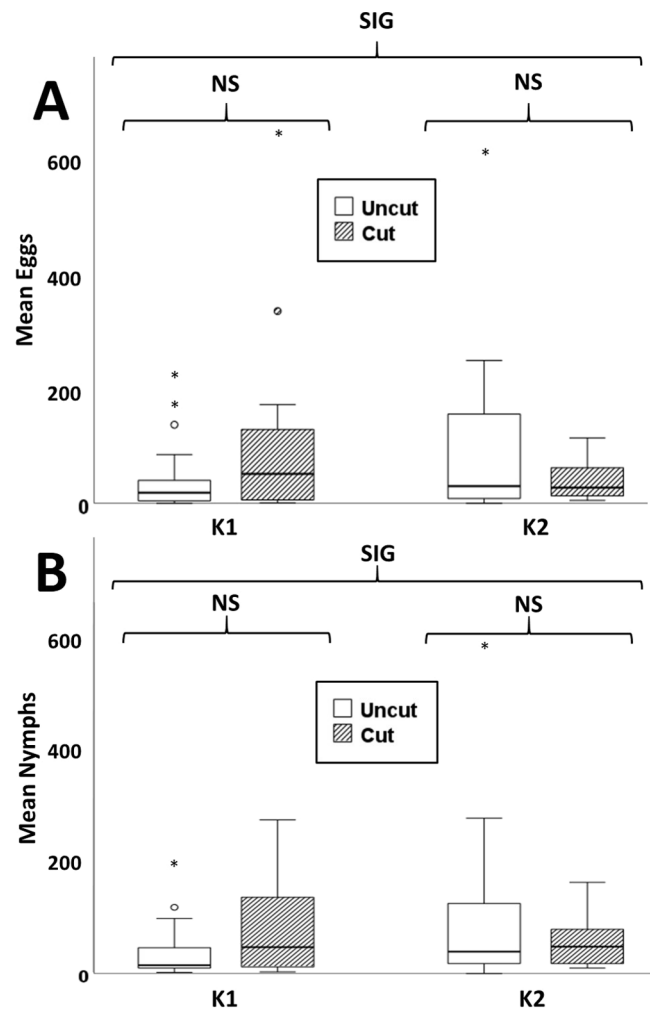


Fig. 3. Numbers of eggs (A) and numbers of live nymphs recovered (B) from sleeved branches of *Fallopia japonica* (N = 78) inoculated with 30 *A. itadori* adults. Box plots represent a summary of the data (minimum, first quartile, median, third quartile, and maximum). Open circles represent outliers ( $1.5 \times$  interquartile range (IQR)). Asterisks represent extreme outliers ( $3 \times$  interquartile range (IQR)). Brackets indicate GLM comparisons.

19.24 (Mean  $\pm$  SE), old foliage:  $36.55 \pm 9.68$ ) and K2 psyllids (young regrowth:  $56.80 \pm 9.71$ , old foliage:  $93.90 \pm 27.20$ ) between young regrowth and older foliage (Fig. 3B).

The overall number of *A. itadori* nymphs recovered (K1 and K2) did not differ significantly between cut and uncut foliage (N = 78,  $\chi^2_{(77, 1)} = 0.022$ ,  $P = 0.881$ ). We also observed no difference in the numbers of nymphs recovered, across cut and uncut foliage, between K1 and K2 psyllids (N = 78,  $\chi^2_{(77, 1)} = 1.012$ ,  $P = 0.314$ ) (Fig. 3B).

On cut foliage, the number of unhatched eggs recovered at the point of foliage collection correlated positively with the number of live nymphs recovered for both K1 ( $F_{(1, 17)} = 5.748$ ,  $R^2 = 0.264$ ,  $P = 0.029$ ) and K2 *A. itadori* ( $F_{(1, 19)} = 45.753$ ,  $R^2 = 0.718$ ,  $P < 0.001$ ) (Fig. 4A). The same pattern was noted on uncut foliage for both K1 nymphs recovered ( $F_{(1, 19)} = 20.716$ ,  $R^2 = 0.535$ ,  $P < 0.001$ ) and K2 nymphs recovered ( $F_{(1, 19)} = 8.362$ ,  $R^2 = 0.317$ ,  $P = 0.010$ ) (Fig. 4B). The positive correlation between egg numbers and nymphal recovery was stronger (steeper regression slope) for K2 than K1 psyllids on cut stems (N = 40,  $t = 2.659_{(36)}$ ,  $P = 0.0115$ ) but not for either K1 and K2 psyllids on uncut foliage where there were no significance differences in either the slopes (N = 40,  $F_{(39, 1)} = 0.002$ ,  $P = 0.967$ ) or the intercepts (N = 40,  $F = 1.323_{(39,1)}$ ,  $P = 0.258$ ) of the regressions.

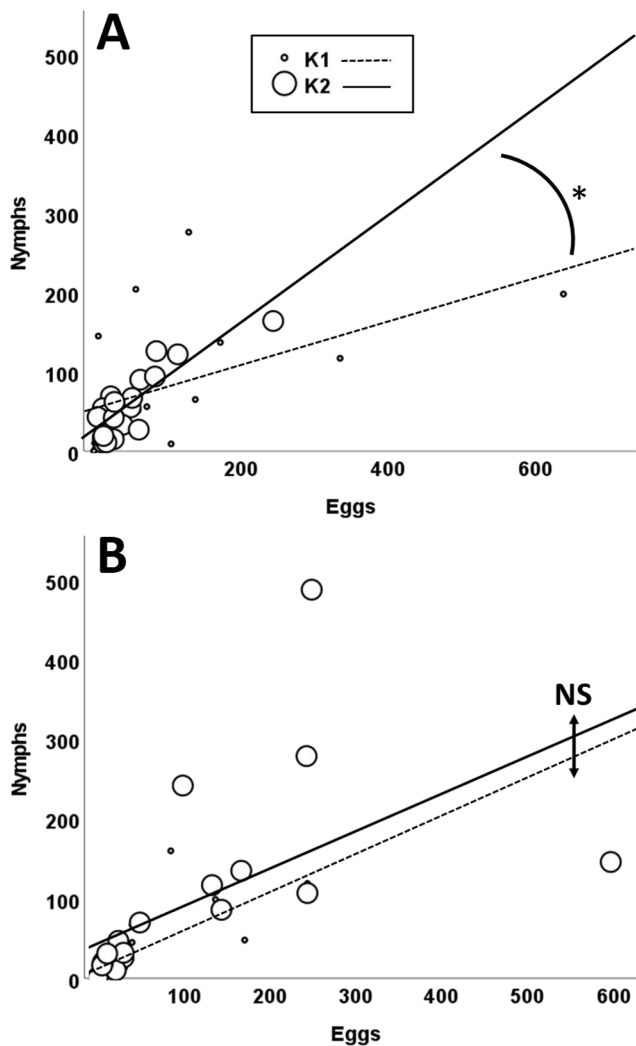


Fig. 4. A) Relationship between numbers of eggs and numbers of live nymphs recovered from cut foliage for both K1 psyllids (Nymphs =  $51.379 + 0.266 \times \text{Eggs}$ ) and K2 psyllids (Nymphs =  $23.779 + 0.673 \times \text{Eggs}$ ). Asterisk denotes significant difference between regression slopes. B) Relationship between numbers of eggs and numbers of live nymphs recovered from uncut foliage for both K1 psyllids (Nymphs =  $14.983 + 0.482 \times \text{Eggs}$ ) and K2 psyllids (Nymphs =  $45.258 + 0.470 \times \text{Eggs}$ ). NS denotes no significant difference between regression intercepts.

#### 4. Discussion

We observed a significant reduction in K1 *A. itadori* nymphal survival on tough *F. japonica* plants compared with soft plants grown in the shade. *Aphalara itadori* from the K1 line laid significantly more eggs on young versus old leaves and appeared to perform best on young *F. japonica* regrowth in the field. Conversely, *A. itadori* from the more recently collected K2 line showed no oviposition preference between young and old leaves and appeared to perform best on uncut *F. japonica* foliage in the field. These results suggest that releases of K2 *A. itadori* could result in improved agent establishment in North America and highlight the potential effects of long-term captive rearing on the performance of biological control agents.

After a 5-week exposure to the different light conditions, leaf fracture tests revealed that *F. japonica* leaves from plants in high-light conditions were significantly tougher than leaves from plants grown in low-light conditions. Our principal focus for these leaf tests was to create differences in foliage quality and explore the effects of leaf toughness on psyllid performance. Fewer *A. itadori* nymphs were recovered from

tough *F. japonica* plants compared to soft plants 27 days after the removal of adult psyllids. In addition, survival rates of early-instar nymphs were lower on the leaves removed from tough plants than those of soft plants. This increased nymphal survival on soft plants seems most likely to be the result of decreased palatability of tough leaves. Leaf toughness has often been reported to limit feeding in immature insects (e.g., Hoffman and McEvoy, 1986; Larsson and Ohmart, 1988). It should be considered, however, that several other leaf characteristics not measured here, such as water (Henriksson et al., 2003; Diaz et al., 2011), N content (Hinz and Müller-Schärer, 2000; Huberty and Denno, 2006; Diaz et al., 2011) and the production of secondary metabolites (Roberts and Paul, 2006; Ramakrishna and Ravishankar, 2011), are also known to vary with light conditions and affect herbivore performance. Future work should explore the effects of light conditions on leaf chemistry in *F. japonica*.

For plants in the field, changes in leaf characteristics, and therefore herbivore performance, occur not only in response to environmental conditions, but in response to changing seasons and plant phenology (Kitajima et al., 2016; Uyi et al., 2018). During oviposition choice tests, *A. itadori* psyllids from the K1 line laid significantly more eggs on younger leaves compared with older leaves. Leaf age is known to be one of the most important factors governing oviposition choice in many insects (Steinbauer et al., 1998), likely because younger leaves generally have higher nutrient and water content and a relative lack of mechanical defenses (Larsson and Ohmart, 1988; Leather, 1994). Indeed, the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is less able to access phloem sap in fully mature citrus leaves due to a ring of sclerenchyma fibers that thicken around the phloem as the leaf develops (George et al., 2017). The effect of leaf age on oviposition behavior, however, is not consistent among psyllid species. Females of the Eucalyptus psyllid, *Ctenarytaina bipartita* Burckhardt et al. (Psylloidea: Aphalaridae), for example, settle disproportionately on younger leaves of eucalyptus (Steinbauer et al., 2016). Conversely, the oviposition choices of the potato psyllid, *Bactericera cockerelli* (Sulc) (Hemiptera: Trioziidae), are not influenced by plant or leaf age (Prager et al., 2014). Similarly, in our study, *A. itadori* females from the K2 line showed no preference between young and old leaves.

The preference of K1 *A. itadori* for soft, younger, foliage may be a result of long-term rearing under laboratory conditions, where insects are generally fed on young, unstressed, potted plants. Adaptation to artificial rearing conditions is a well-documented phenomenon in insects (Hoffmann and Ross, 2018), and has even been shown to reduce the efficacy of biological control agents upon release (Bertin et al., 2017). Laboratory-reared insects are often maintained in discrete generations which selects for individuals that develop quickly and reproduce early (Matos et al., 2000; Diamantidis et al., 2011). Additionally, the controlled conditions of the laboratory relax selection pressures associated with variable environmental conditions. As a result, tolerance of thermal extremes and water stress (Hoffmann et al., 2001) and the ability to use a variety of food sources can be reduced in captive populations (Bravo and Zucoloto, 1998). It is possible that long-term rearing of the K1 *A. itadori* under laboratory conditions (since collection of the original population in 2004) has relaxed selection for traits that enable individuals to feed on the tougher foliage often found in field plants, and that this is contributing to the failure of *A. itadori* to establish in North American field sites. The fact that *A. itadori* from the more recently collected K2 line showed no oviposition preference between young and old leaves is a positive sign that these psyllids are less adapted to the laboratory and may be better able to survive on tougher foliage found in the field.

The field experiment comparing the performance of K1 and K2 *A. itadori* on cut and uncut *F. japonica* foliage showed no direct effects of cutting treatments on nymphal survival but did reveal a significant interaction between cutting treatments and psyllid line. Linear regression analyses showed that on uncut (older) foliage, the relationship between egg numbers and surviving nymphs was similar for K1 and K2

psyllids, but with slightly higher nymphal survival rates in K2 psyllids. Similar analyses on cut (younger) foliage showed a stronger positive correlation between egg numbers and surviving nymphs for K2 psyllids than for K1 psyllids. These regression analyses suggest that K2 psyllids were more able to take advantage of the favorable conditions provided by younger foliage. It should be noted, however, that these regression analyses assume that the number of eggs at the time of foliage collection correlated strongly with earlier oviposition rates. The results of experiment five support not only the previous experiments in this study, but also a similar field study conducted by Jones et al. (2020) that showed K1 *A. itadori* nymphal survival after a mid-summer cutting treatment to be significantly higher on *F. japonica* regrowth than on older uncut foliage. The fact that Jones et al. (2020) observed a clearer effect of foliage age on K1 nymphal mortality than we observed here is likely because our field experiment was conducted earlier in the growing season than theirs, when the 'older' uncut foliage had not toughened up to the same extent. The comparison between these results underlines the effects of plant phenology on herbivore food quality, and emphasizes that, regardless of habitat preferences (both of these field experiments were conducted in similar shaded habitats), any population of *A. itadori* will need to survive on tough, late-season, *F. japonica* foliage in order to become established in North American field sites, just as they would in their native range. It should be noted that this field study was conducted at a single, semi-shaded, field site. Future work should compare both the response of *F. japonica* to cutting treatments, and effects of foliage age on *A. itadori* performance across a range of environmental conditions.

The increased survival of K1 *A. itadori* nymphs on soft leaf material in the laboratory, their preference for laying eggs on young leaves, and their diminished performance on uncut field foliage all suggest that long-term captive rearing has resulted in a laboratory-adapted population that is less able to tolerate the *F. japonica* foliage encountered in North American field sites. This conclusion is further supported by the lack of oviposition preference between young and old leaves by K2 *A. itadori*. These results suggest that the more recently collected K2 psyllids will be better able to establish in North American field sites and enhance the potential for biological control of *F. japonica*. Furthermore, our results provide documentation for biological-control scientists and practitioners of the problem of long-term rearing for the performance of biological control agents.

#### CRedit authorship contribution statement

**Ian M. Jones:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing - original draft, Writing - review & editing. **Rob S. Bouchier:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Sandy M. Smith:** Conceptualization, Funding acquisition, Project administration, Resources, Software, Supervision, Writing - review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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