**Multi-generational impacts of the psyllid *Arytinnis hakani* (Hemiptera: Psyllidae) on
growth and survival of the invasive weed *Genista monspessulana***

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Abstract

Pre-release efficacy assessments of biological control agents can identify species with the most potential to impact the target weed. Experiments typically occur within a single generation of the agent, however, and strong impacts on target weeds may take longer to emerge. This study examined the effects of the prospective agent *Arytinnis hakani* (Hemiptera: Psyllidae) on the invasive weed French broom, *Genista monspessulana*, over three generations of the psyllid (~150 days) under quarantine conditions. All of the psyllid-free control plants survived to the end of the experiment, whereas 23 out of 28 (82.1%) of the plants infested with psyllids died. Only three plants with psyllids died during the first psyllid generation; plant mortality rate peaked at the transition between the second and third psyllid generations. Plant growth was also reduced on plants with psyllids. Impacts on proportional increases in plant height and total stem length were evident by the end of the first psyllid generation, but effects on proportional change in leaf number were not apparent until the end of the third psyllid generation. By the end of the experiment, average proportional increases in height, total stem length and leaf number were 65.7%, 60.7% and 53.2% lower, respectively, on surviving psyllid-infested plants than controls. Final above-ground biomass of surviving psyllid-infested plants was 66.4% lower on average compared to the psyllid-free control plants. The results of this study highlight the need for pre-release efficacy assessments that allow effects on target weeds to develop over time.

Keywords

agent efficacy; *Arytinnis*; French broom; *Genista*; Psyllidae; weed control

1. Introduction

Biological control is often the most sustainable and cost-effective long-term strategy for managing invasive weeds of natural areas (Van Driesche et al., 2010). By introducing host-specific natural enemies from the invader's native range, classical biological control can reduce populations of invasive pests to non-damaging levels (Crutwell McFadyen, 1998; McEvoy, 2002; Hoddle, 2004; Clewley et al., 2012; Van Wilgen et al., 2013). The release of biological control agents can have unintended ecological consequences, however (Louda et al., 2003; Pearson and Callaway, 2005; Paynter et al., 2010), and releases of ineffective agents should be avoided (McEvoy and Coombs, 1999; Strong and Pemberton, 2001; Sheppard, 2003). Many agents have established and reached high numbers without contributing to weed control (McClay and Balciunas, 2005; Bownes et al., 2010; Wang et al., 2011).

Pre-release assessments can help prioritize prospective agents with the most potential for controlling the target weed (Sheppard, 2003; van Klinken and Raghu, 2006; Conrad and Dhileepan, 2007; Müller-Schärer and Schaffner, 2008). A single effective agent may be sufficient for control (Denoth et al., 2002). Most quantitative pre-release efficacy studies have occurred under field conditions in the native range (McClay and Balciunas, 2005), where the weed may be limited by processes such as competition that may not occur in the introduced range (Morin et al., 2009), and where the prospective agent may be affected by other herbivores and its own natural enemies (Balciunas and Smith, 2006). Furthermore, this approach is not always feasible for political or logistic reasons (Balciunas and Smith, 2006). Although artificial conditions in quarantine may not be directly applicable to the field, conducting pre-release efficacy studies under strict containment conditions is often the only available option. Nonetheless, few studies have measured the impacts of prospective weed biological control

agents in quarantine (but see Balciunas and Smith, 2006; Goolsby et al., 2009; Grevstad et al. 2013).

One challenge that is particularly difficult to meet under quarantine conditions is ensuring that experiments are conducted over sufficiently long time periods for effects on target weeds to fully emerge. Experiments typically occur within a single generation of the agent, and evaluating damage to weeds over short time spans may underestimate the agent's potential (McClay and Balciunas, 2005; Gerber et al., 2008). Impacts may accumulate over several generations of the agent. For example, strong effects of the psyllid *Boreioglycaspis melaleucae* on growth of melaleuca plants did not emerge until psyllids had been on plants for four months, or two psyllid generations (Franks et al., 2006).

This study examines the multi-generational effects of the phloem-feeding psyllid *Arytinnis hakani* (Loginova) on its host plant, the invasive weed French broom, *Genista monspessulana* (L.) L.A.S. Johnson (Fabaceae). In the United States, French broom is widespread in California and southern Oregon, and is also invasive in Australia, New Zealand and Chile (Parsons and Cuthbertson, 2001). French broom is a nitrogen-fixing perennial shrub native to the Mediterranean region that grows up to 3-5 m tall, with a life-span of 10-15 years. The plant was introduced into California as an ornamental, and now occupies at least 40,000 hectares in the state (Bossard, 2000). It colonizes both disturbed and undisturbed habitats, including grasslands, mountain slopes, riverbanks, and open forest (Sheppard, 2000). Mature plants can produce large seed banks that can lie dormant for at least five years (Parsons and Cuthbertson, 2001).

Chemical and mechanical controls for French broom are typically labor-intensive and expensive, and must be repeated for several years to combat the long-lived seed banks that

accumulate in the soil (Parsons and Cuthbertson, 2001; Sheppard and Henry, 2012). Even when controlled by these methods, the ability of *G. monspessulana* to increase soil nitrogen may favor colonization by exotic plants and impede the regeneration of slower-growing native plants (Haubensak et al., 2004). Repeated burns may help reduce soil nitrogen levels (Haubensak et al., 2004), but the fire frequency required for control may not be feasible in all locations. Thus, classical biological control appears to be the only viable long term option for controlling French broom (Sheppard and Henry, 2012), although no host-specific herbivores of French broom are known to occur in North America (Bossard, 2000).

The psyllid *A. hakani* is a prospective biological control agent of French broom in the United States (Sheppard and Thomann, 2004; Cook and Smith, 2014). The psyllid has a relatively wide native geographic distribution, occurring from Portugal, Spain, southern France to Italy in Europe, and from Morocco to Algeria in North Africa (Hodkinson and Hollis, 1987; Burckhardt, 1989). It is likely to complete 3-4 generations per year in France on average (A. Sheppard, personal communication). Nymphs feed on the phloem in the leaves and stems, and pass through five instars. In its native range *A. hakani* has been reported only on French broom (Sheppard and Thomann, 2004), although it may be capable of developing to adulthood on certain species of *Lupinus* (Sheppard and Henry, 2012). It was under consideration for release into Australia but was found in the field before a release permit could be issued (Sheppard and Henry, 2012). It has since been redistributed to several Australian states, where it is killing plants in many locations (Sheppard et al., 2014). Effects on plant survival began to appear one to two years after the psyllid was redistributed to new areas (Sullivan, 2013).

Quarantine greenhouse impact studies conducted over one generation of *A. hakani* have yielded mixed results. Reddy et al. (2015) reported no negative effects of *A. hakani* on growth or

survival of French broom (weak effects on one growth parameter notwithstanding), and Cook and Smith (2014) found that *A. hakani* reduced growth of French broom seedlings but had no significant effects on plant survival. Stronger effects may emerge over longer time periods. To examine this possibility, we conducted an experiment under quarantine conditions to examine impacts on growth and survival of French broom over several generation of *A. hakani*.

2. Material and Methods

2.1. Study Materials

A colony of *A. hakani* was maintained at a quarantine laboratory at the USDA-ARS facility in Albany, California, USA as previously described (Cook and Smith, 2014). Psyllids were reared in cages on potted French broom plants. The colony was kept in a rearing room under fluorescent plant-growth lighting with a photoperiod of 12:12(L:D) under ambient temperature (18 - 27 °C) and humidity (RH: 25 - 70%).

French broom plants were collected for the experiment from Marin County, California, in October and November, 2014 and were transplanted into 20-cm high × 16-cm diameter pots with their original soil. Plants were <1 year old and were 21.88 ± 0.60 cm tall (mean $\pm$ SE; range 15-34 cm) at the start of the experiment, and consisted of one main stem with no branches. Plants were collected at least 10 days before being used in experiments, and any plants showing signs of transplant stress were not used. Plants were examined under a microscope and any arthropods found were removed.

2.2. Experimental Procedures

The experiment was conducted in a quarantine greenhouse at the USDA-ARS facility in Albany, CA. Two treatments were established, including 25 replicates of a no-psyllid control and

28 replicates of a psyllid-addition treatment in 25 blocks (three blocks each contained two control plants and one psyllid-addition plant; all other blocks contained one control plant and one psyllid-addition plant). Plants were blocked by height on greenhouse tables before being randomly assigned to treatments in a randomized block design. Treatments did not differ initially in plant height (t -test, $t_{51} = 0.11$, $P = 0.91$) or number of leaves per plant ($t_{51} = 0.36$, $P = 0.72$). To infest psyllid-addition plants, 2 female and 1 male *A. hakani* that had emerged within the previous five days were placed on each plant, and plants were individually enclosed in small mesh cages (~45-cm tall $\times$ 20-cm diameter). Control plants were also caged but received no psyllids. Due to time constraints, infestations started on five dates (3, 5, 6, and 7 November and 8 December); at least three infested and control replicates were started on each date. After 48 hours, all adult psyllids were removed from psyllid-addition plants using an aspirator, cages were removed from plants, and any eggs in excess of 25 per plant were removed with forceps (19.46 ± 0.66 eggs remaining per plant, mean $\pm$ SE; range 8-25) to limit overcrowding and to ensure that plants would persist for multiple psyllid generations. Initial psyllid densities were similar to those used by Cook and Smith (2014), who reported negative effects on plant growth but little or no plant mortality over one psyllid generation at densities of 1-2 *A. hakani* per cm stem length. After excess egg removal, control and psyllid-addition plants were each enclosed by a ~90 cm tall $\times$ 20 cm diameter cage, which consisted of a bag of white organdy fabric enclosing the pot and four ~90-cm tall bamboo support poles that were driven into the soil around the inside perimeter of the pot. The bag was sealed at the top with a twist-tie. This size of cage was chosen to accommodate plant growth over the course of the experiment. Plants were maintained between 16 and 26° C with a 12-hour photoperiod, under a combination of natural and LED light, and were drip-irrigated for 10 minutes per day, 4 days per week.

The trial continued for three generations of psyllids (~150 days). At the end of each generation, all adult psyllids were removed from each psyllid-addition cage eight days after the first adult had appeared; this time period allowed psyllids to lay eggs (the pre-oviposition period of *A. hakani* is ~5 days at 18-27°C [Herrera et al., 2011; Smith, 2014]) but limited overcrowding. After removing adult psyllids, plant height and total stem length (height + total length of stem and branches) were measured to the nearest 0.5 cm and numbers of leaves (each having three leaflets) per plant were counted. Numbers of live and dead adult psyllids and late-instar nymphs from the previous generation (adults represented 67.9% of psyllids overall) of the previous generation were also counted; numbers of live and dead adults and nymphs were summed to estimate realized psyllid densities. Plant health was monitored every 3-4 days, and any plant mortality (inferred by total absence of live leaves) was recorded. At the end of the experiment, plants were cut off at ground level and stored at -4°C before placing them in a drying oven at 70°C for 3 days and weighing above-ground biomass (dry weight) of each plant to the nearest 0.01 g. Root biomass was not measured because most plants were root-bound at the end of the experiment.

2.3. Statistical Analyses

Effect of psyllid infestation on the survival of French broom plants was assessed using survival analysis. Kaplan-Meier survival curves and Cox's Proportional Hazard Model were used to compare survival between the two treatments, using the `survfit` and `coxphf` functions, respectively, in the survival package in R version 3.1.0 (R Development Core Team, 2014). The `coxphf` function uses Firth's penalized maximum likelihood procedure to correct for separation (no plants died in the control treatment, while many died in the psyllid-addition treatment). The effect of psyllid density (= number of psyllids [live adults + dead adults + nymphs] per plant at

the end of the second generation/total stem length at the end of the second generation) on the longevity (days survived since the psyllid infestation date) of psyllid-addition plants was assessed using regression analysis. After each psyllid generation, proportional change in height, total stem length, and numbers of leaves and branches were calculated for each plant as $(H_t - H_i)/H_i$, where H_t is height, total stem length or leaf number on sample date t and H_i is height, total stem length or leaf number at the start of experiment. The effects of adding psyllids on plant growth parameters were assessed using repeated measures analysis of variance (ANOVA), including time (psyllid generation) as a repeated factor and psyllid addition as a fixed factor (two levels: added or not), using the lmer and lmerTest functions in R. Growth parameters were (log + 1) transformed to meet assumptions of normality. Post-hoc t -tests were used to assess the effects of adding psyllids on growth parameters at the end of each psyllid generation (time $\times$ psyllid addition interaction terms were significant for all parameters). A t -test was used to assess how the presence of psyllids affected final above-ground plant biomass (although sample sizes differed between treatments, variances were equal). Trial date was initially included in models as a random factor, but was always nonsignificant and was dropped.

3. Results

3.1. Psyllid numbers over time

Realized psyllid numbers (sum of live and dead adults and nymphs) on psyllid-addition plants increased over the course of the experiment. Numbers of psyllids per surviving plant increased from 20.46 ± 2.06 (mean $\pm$ SE; $n = 25$) at the end of the first psyllid generation to 133.50 ± 36.42 ($n = 18$) and 165.00 ± 116.92 ($n = 5$) at the end of the second and third generations, respectively. However, psyllid densities (sum of live and dead adults and nymphs

per cm stem length) were highest in the second generation, reaching a peak of 1.19 ± 0.25 per cm stem length (Fig. 1).

3.2. Plant mortality

The plant survival curve of the psyllid-addition treatment was significantly different from the no-psyllid control ($\chi^2 = 41.41$, $P < 0.0001$). All of the control plants survived to the end of the experiment, while 23 out of 28 (82.1%) of the psyllid-addition plants died (Fig. 2). Most plant mortality occurred at the transition between the second and third psyllid generations; only three plants with psyllids died during the first psyllid generation, whereas seven and 13 plants died during the second and third generations, respectively. There was a negative relationship between psyllid densities (sum of live and dead adults and nymphs per cm stem length) at the end of the second psyllid generation and plant longevity ($R^2 = 0.57$, $P < 0.001$; Fig. 3). Four psyllid-addition plants that survived the first psyllid generation were so weakened that they failed to support a second psyllid generation (the adult psyllids that emerged on these plants at the end of the first generation appeared too weak to lay eggs). All of these four plants eventually died, three before the end of the second psyllid generation and one before the end of the third.

3.3. Plant growth

Proportional change in plant height, total stem length and numbers of leaves were affected by addition of psyllids and by the time $\times$ psyllid addition interaction (Table 1). All three growth parameters were lower in the presence of psyllids (Fig. 4). The proportional change in height was lower on psyllid-addition plants than on control plants by the end of the first psyllid generation (post-hoc t -tests, first psyllid generation: $t_{48} = 5.87$, $P < 0.0001$; second psyllid generation: $t_{41} = 5.33$, $P < 0.0001$; third psyllid generation: $t_{28} = 3.94$, $P < 0.001$). Proportional change in total stem length was also lower on psyllid-addition plants across all three psyllid

generations (first psyllid generation: $t_{48} = 2.74$, $P = 0.009$; second psyllid generation: $t_{41} = 3.63$, $P < 0.001$; third psyllid generation: $t_{28} = 4.15$, $P < 0.001$). Effects on leaf number were not apparent until the end of the third generation, however (Fig. 4; first psyllid generation: $t_{48} = 0.96$, $P = 0.34$; second psyllid generation: $t_{41} = 1.78$, $P = 0.08$; third psyllid generation: $t_{28} = 3.18$, $P = 0.002$). By the end of the experiment, proportional change in height, total stem length and leaf number were 65.7%, 60.7% and 53.2% lower on average, respectively, on psyllid-addition plants than on controls. Final above-ground plant biomass was also affected by the presence of psyllids (t -test, $t_{28} = 2.95$, $P = 0.02$), and on average was 66.4% lower on plants that received psyllids (Fig. 5).

4. Discussion

Survival and growth of young French broom plants were significantly reduced by infestation with *A. hakani* in this study. These results lend support to anecdotal reports of damage to *A. hakani*-infested French broom plants in Europe (Sheppard, 2000), and of widespread French broom mortality and defoliation in areas of Australia where *A. hakani* has reached high densities (Sheppard and Henry, 2012; Sullivan, 2013). The psyllid clearly has potential as a biological control agent for French broom in California. By reducing the survival and growth of young individual French broom plants, *A. hakani* could conceivably limit the weed's competitiveness and dispersal and thus mitigate its impacts on the native plant community.

Strong effects on plant growth and survival did not emerge until multiple generations of the psyllid had passed. Only 3 out of 28 plants with psyllids died during the first psyllid generation (~50 days), and one growth parameter (number of leaves) did not respond until the

end of the third psyllid generation (~150 days). Cook and Smith (2014) reported similarly low plant mortality rates of seedlings over one generation of *A. hakani* feeding, with 1 out of 10 plants dying at the highest tested psyllid density of 0.7 to 1.0 nymphs per cm stem length, which was similar to psyllid densities during the first generation in the current study. In Australia, widespread mortality of French broom reportedly occurred one to two years after *A. hakani* had been redistributed to new areas (Sullivan, 2013), suggesting that accumulation of impacts of the psyllid over several successive generations was required to kill plants. Similar multi-generational effects have been observed for other psyllids. For example, the psyllid *B. melaleucae* did not impact growth of small melaleuca plants in caged field plots until four months (two psyllid generations) after addition of psyllids (Franks et al., 2006). In contrast, the univoltine psyllid *Arytainilla spartiophila* failed to reduce Scotch broom growth in a field experiment, possibly because it was actively feeding for only a short part of the year (Hogg et al., 2016). Tipping et al. (2008) reported that strong negative effects on melaleuca growth as a result of two biological control agents, including *B. melaleucae*, required five years. Cumulative impacts over multiple generations and years must accumulate for success in most biological weed control systems (McFadyen, 1998; Clewley et al., 2012). In this way, sustained attack by multivoltine agents may cause more damage to weeds than periodic feeding by univoltine agents (Wang et al., 2011). Plants may recover during lapses in feeding, and repeated bouts of feeding may be necessary for impacts on weeds to accumulate over time.

Although overall impacts on plants increased in severity over time, *A. hakani* killed plants with extraordinary speed, and it may be quite unusual in this regard. The low plant mortality that did occur during the first psyllid generation suggests that *A. hakani* may not always require multiple generations to kill French broom plants. In addition to the three psyllid-

addition plants that died in the first generation, four plants were so weakened that they did not support a second psyllid generation and eventually died. Similarly, the plant mortality that occurred at the beginning of the third psyllid generation was likely a consequence of psyllid herbivory that occurred during the second psyllid generation, when psyllid densities were highest, and plant longevity was negatively correlated with psyllid density in this generation. Psyllid densities in the second psyllid generation reached ~1.2 per cm stem length, which is somewhat lower than densities in other studies reporting strong effects of psyllids on weeds (e.g., *B. melaleucae* on *Melaleuca quinquenervia* [Franks et al., 2006] or *Aphalara itadori* on *Fallopia japonica* [Grevstad et al., 2013]). To our knowledge field densities of *A. hakani* have not been quantified in either Australia or Europe. If densities are sufficiently high, *A. hakani* may consistently kill plants over relatively short time periods.

Reddy et al. (2015) reported little or no negative effects by *A. hakani* on French broom performance after one generation despite extremely high psyllid densities, and suggested that a longer time frame may have been necessary for effects to emerge. They allowed *A. hakani* densities to increase over one generation to 1181 to 4877 psyllids per plant, far higher than in the current study in which initial egg density was controlled, or in Cook and Smith (2014). One factor that may have contributed to the differing results between studies is that Reddy et al. (2015) used plants that were initially four-fold larger (81 cm tall on average) than in the current study and over 10-fold larger than those used in Cook and Smith (2014). Young plants may show less tolerance to sap-feeding insects than older plants (Zvereva et al., 2010), and the effects of *A. hakani* may take longer to emerge or require higher densities on larger plants. However, in Australia, herbivory by *A. hakani* appears to affect both mature and immature plants (A. Sheppard, personal communication), and every French broom plant was reported dead in some

locations two years after the release of *A. hakani* (Sullivan, 2013). Quantifying the effects of *A. hakani* in relation to plant size would require further study.

There are several possible ways that feeding by *A. hakani* may have affected French broom plants. Sap-feeding insects can extract large amounts of nutrients such as carbohydrates from plants, and, as shown here, sustained attack over multiple generations can severely weaken or kill plants (Zvereva et al., 2010). The removal of phloem by *A. hakani* may have depleted nutrient reserves in French broom plants to the point of starvation, as surviving plants with psyllids added little or no new stem length or leaves after the second generation. Feeding damage by psyllids has also been attributed to the injection of toxic saliva into the plant tissue (Burckhardt, 1994). Still another possibility is that *A. hakani* carries a pathogen. At least four other psyllid species have been reported to vector *Candidatus* Liberibacter bacteria, including the psyllids *Diaphorina citri* Kumayama and *Trioza erytreae* Del Guercio, which vector citrus greening disease (Gottwald et al., 2007), and the potato/tomato psyllid, *Bactericera cockerelli* Sulc, which is associated with zebra chip disease in potatoes (Munyaneza, 2010). *Candidatus* Liberibacter europaeus was recently reported in the related psyllid *Arytainilla spartiophila* on Scotch broom in New Zealand, and is suspected to cause symptoms in Scotch broom such as stunted shoot growth, leaf dwarfing and leaf tip chlorosis (Thompson et al., 2013).

The results of this study, in combination with observations from Australia, strongly suggest that *A. hakani* would contribute to the control of French broom if released in California. However, effects on individual plants may not necessarily translate into decreases in weed populations (Crawley, 1989, Thomas and Reid, 2007), and we may have overestimated the effects of *A. hakani* on plant survival by using small immature plants in our experiment. Furthermore, caution should be exercised when extrapolating from controlled quarantine

conditions to the field. This study was conducted in an artificial setting that bears little resemblance to heterogeneous field conditions, and many factors could influence the performance of the psyllid if it were to be released in California. For example, the low tolerance of *A. hakani* for high temperatures may limit its success in some locations in California, although it is likely to be well-suited to cooler coastal areas (Smith, 2014), where French broom is particularly abundant. These considerations notwithstanding, pre-release assessments of impact are an important step in demonstrating the weed control potential of prospective agents, and post-release studies generally confirm predictions from pre-releases tests in the few instances where such data are available (Gerber et al., 2008). The results of this study also highlight the necessity of conducting experiments over sufficiently long time frames for effects on target weeds to fully emerge.

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472

473 **Contribution of authors**

474 BNH designed and conducted the experiment and analyzed the data with input from PJM

475 and LS. BNH, PJM and LS wrote the manuscript.

476 **Table 1.** Results of repeated measures ANOVA testing the effects of psyllid additions on plant
 477 growth parameters.

Response variable	Source of variation	df ^a	<i>F</i>	<i>P</i>
Height	psyllid addition	1, 60	48.73	<0.001
	time	2, 77	94.12	<0.001
	time × psyllid addition	2, 77	15.99	<0.001
Total stem length	psyllid addition	1, 57	29.34	<0.001
	time	2, 75	273.04	<0.001
	time × psyllid addition	2, 75	16.93	<0.001
Leaf number	psyllid addition	1, 53	8.41	0.005
	time	2, 71	219.58	<0.001
	time × psyllid addition	2, 71	6.92	0.002

478 ^aThe decimal values given by Satterthwaite's approximation for degrees of freedom were
 479 rounded to the nearest whole number.

Figure Captions

Fig. 1. Psyllid densities (sum of live and dead adults and nymphs per cm stem length) at the end of each generation. Data are means $\pm$ SE.

Fig. 2. Kaplan-Meier survival curves for French broom plants in the no-psyllid control (dashed line) and the psyllid-addition treatment (solid line).

Fig. 3. Relationship between psyllid density (sum of live and dead adults and nymphs per cm stem length) at the end of the second psyllid generation and plant longevity ($R^2 = 0.57$, $P < 0.001$).

Fig. 4. Effect of psyllid infestation on proportional change in height (a), total stem length (b) and number of leaves per plant (c) measured at the end of each of three psyllid generations in the no-psyllid control (open circles) and in the psyllid-addition treatment (closed circles). Data are means $\pm$ SE. Asterisks indicate that means for each psyllid generation are significantly different (post-hoc t -tests, $P < 0.01$ in all cases). Because of plant mortality, 25, 18 and 5 psyllid-addition plants were included in analyses from the first, second and third generations of psyllids, respectively; all 28 no-psyllid control plants survived the experiment.

Fig. 5. Difference between no-psyllid and psyllid-addition treatments in above-ground biomass (dry weight) of French broom plants at the end of the experiment.

The psyllid *Arytinnis hakani* dramatically reduced growth and survival of French broom plants

no-psyllid
control

with psyllids

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5