

# Interim Diets for Specialist Predators of Hemlock Woolly Adelgids

Allen Cohen C<sup>1\*</sup> and Carole Cheah ASJ<sup>2</sup><sup>1</sup>Department of Entomology, Insect Rearing Education and Research, North Carolina State University, USA<sup>2</sup>The Connecticut Agricultural Experiment Station, Windsor, CT, USA

## Abstract

We provide a rationale for diets that temporarily support populations of insects but not sustained rearing of target insects. We call such sustaining media, "interim diets". We present formulation and performance details of such a diet, which sustains specialist adult predators of hemlock woolly adelgids (HWA) for several months. The diet base is ground, freeze-dried, cooked chicken breast, heat-treated chicken egg yolk with several nutritional and functional supplements. This diet has been tested in our laboratories for five years and has been validated in several mass-rearing laboratories where HWA predators are routinely produced. Although the current diet does not support complete development of HWA predators from egg to adult, it is useful as an economical and convenient means of keeping predators alive and healthy during periods when natural prey are not available. Unlike generalist predators, HWA predators would not accept factitious hosts such as scale, aphids, lepidopteran eggs or other foods that might have sustained them during "feeding droughts". The predators studied here were beetles, *Sasajiscymnus tsugae* (Coccinellidae) and *Laricobius nigrinus* (Derodontidae). We experimented with several diet-presentation systems designed to fulfill the feeding requirements of the beetles and to preserve the diets by preventing desiccation and deterioration. We developed several forms of a hen's egg-based diet and a diet-presentation system that included alginate gels and slurry diets that were made from adhering liquid materials to a solid/capture medium (freeze dried, powdered chicken breast). Some diets and diet-presentation systems induced strong feeding responses and allowed adult predators to stay alive and active for several months and to return to egg production days after being returned to HWA. The paper describes a stable, palatable diet and diet-presentation techniques that allow researchers and mass-rearing facilities to sustain healthy populations of predators during regular periods of prey scarcity.

**Keywords:** Predators; Prey; Insect; Hemlock woolly adelgids; *Sasajiscymnus tsugae*; *Laricobius nigrinus*

## Introduction

### Interim vs. continuous development diets

Generally, the goal of researchers in developing artificial diets is to provide a medium that is fully capable of producing continuous generations of the target insect [1]. Such diets may be used for research purposes or for production of insects to be used for programs that may include sterile insect techniques, production of predators or parasitoids for biological control, insects to be used as foods for other organisms, and many other uses [1,2]. The standards for judging success (listed below) include the economics, practicality, and sustainability of diet usage, but generally, the incipient diets are intended to allow production of continuous generations of the target insect. Examples of such continuous development diets are ones used in the screwworm [3,4] and fruit fly facilities [5,6] for sterile insect techniques (SIT) and for biological control of pests [7,8]. These authors describe mass-rearing programs where the target insects have been produced for hundreds of continuous generations and where addition of field-collected materials is necessitated only by the objective of increasing genetic diversity of colonized populations, rather than the need to re-establish colonies because of diet inadequacies [9,10].

### Interim diets for predators of Hemlock woolly adelgids

Hemlock woolly adelgid Annand (*Adelges tsugae*, Hemiptera: Adelgidae) or HWA is an exotic pest that is devastating eastern hemlock, *Tsuga canadensis* Carriere and Carolina hemlock, *Tsuga caroliniana* Engelm through much of their range in the Eastern US. The pest originated from Japan [11] and has challenged forest researchers and managers with its rapid spread and devastating destructive potential. Although several means of managing HWA have been attempted, biological control is currently the most widely-used

means of management of these devastating pests. Predators, which are HWA specialists, are the most emphasized biological control agents, and beetles that specialize on HWA as prey have been widely released for HWA management in infested states. While efficacy of the HWA biological control program is still being assessed, two factors that emerge consistently are that 1) the numbers of predators that are available for release are too low, and 2) the expense per predator is too high, sometimes reaching costs of \$2-3 per individual *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae), the only introduced HWA predator that is commercially available (Jayme L. Boniewicz, pers. comm., <http://www.tree-savers.com>). These factors lead to the obvious need for a less expensive and more reliable means of predator production/mass-rearing system.

Currently, biological control programs for two widely-used predators (*S. tsugae* and *Laricobius nigrinus* Fender, Coleoptera: Derodontidae) use HWA as the sole food source [12]. These adelgids are collected on host plants *T. canadensis* and the removal of branches with HWA is a costly process in terms of labor, travel, and destruction of large portions of the trees that we are trying to conserve. Clearly, this manner of providing food for predators imposes severe limitations to the scale of production that is possible. Further complicating factors are that winter mortality of HWA can be very high, reducing food

**\*Corresponding author:** Allen Cohen, Department of Entomology, Insect Rearing Education and Research, North Carolina State University, USA, Tel: 919-513-0576; E-mail: [accohen@ncsu.edu](mailto:accohen@ncsu.edu)

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supplies, and the complexity of the HWA's life cycle (Figure 1) imposes further limitations on how many predatory beetles can be produced and even more limitation on the quality of the predators. Therefore, any reduction in the need for living adelgids and the dependency on extensive field collections of prey would be a tremendous advantage to HWA biological control programs. Any artificial diet that would reduce mortality of the predatory beetles would be a welcome addition to our rearing technology.

The term, "artificial nutrition" implies either 1) insects that are not natural hosts to the predators, known also as *factitious* hosts or 2) artificial diet that is composed of non-insect derived materials. Several species of predators (such as pirate bugs and lacewings) and parasitoids (such as *Trichogramma*) have been reared successfully on factitious hosts such as the eggs of various grain moths (the Mediterranean grain moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) and the Angoumois grain moth *Sitotroga cerealella* (Oliv) (Lepidoptera: Gelechiidae). The advantages of using factitious hosts are that they are more conveniently available and generally less costly than natural hosts [1], but the disadvantages are that they are not always the most nutritious prey, and they are more expensive than artificial diets, in general, with *Ephestia* eggs costing above \$1000/kg compared to a conventionally-used artificial diet that costs about \$5.00/kg [13,14].

Our discussion of natural and factitious prey leads to a comparison real insect prey with artificial diets in a context of diet presentation systems along with diet composition. Cohen [1] explained that for an artificial diet and diet-presentation system to be considered *completely* successful it must meet all of the following six criteria:

1. Stimulate robust feeding
2. Support prolonged survival
3. Support growth and development
4. Support reproduction
5. Allow production of continuous generations indefinitely
6. Support high quality insects that are fully useful for their intended purpose (biological control, genetic pest management, food for other species, conservation, education, research, etc.).

Relatively few diet systems have been developed to meet all these specification, with a rough estimate of about 20 basic diets that have been shown to support about 300 species [1,2]. By "basic diets" we mean, for example, the wheat germ diet developed for pink bollworms by the Vanderzant team (reviewed in [1]) and modified hundreds of times for more than 100 other species or the "meat based" diets developed for predators by Cohen [15-17] and modified for dozens of other species. The current report presents information on the diet formulation, processing, and presentation that supports the first two criteria.

Fewer diets have been successful for predaceous lady beetles. The most notable success was a diet [18] which supported eight successive generations of *Coleomegilla maculata* De Geer. Several authors reported using factitious diets such as formulations with powder honey bee brood (reported in [2]). The most successful factitious host diet for a coccinellid in our experience was the use of pink bollworm eggs to rear multiple generations of *Serangium parcesetosum* Sicard in the USDA, APHIS Pink Bollworm Facility in Phoenix, AZ in the 1990s in a program developed by Dr. Robert T. Staten. Unfortunately, this work was not published, and several papers treating the whitefly specialist,

*S. parcesetosum*, have argued that this predator is so specialized that it cannot be reared on other than whitefly hosts.

### Specialists vs. Generalists

In most programs and research efforts to control forest pests, specialist predators or parasitoids have been used in classical biological control systems [19]. Because HWA are exotic pests, it is well-accepted that the best natural enemies to use against them are HWA specialists such as *L. nigrinus* and *S. tsugae*. In accord with the complex life cycle of HWA (Figure 2), both species of predators have complex life cycles, which track the prey's periods of dormancy or reductions or surges in nutritional availability. This "meshing" of the predators' life cycle with that of the prey indicates a high degree of specialization of the predator. A further indication of the high degree of specialization is the fact that both species of predators have been shown to be restricted either nutritionally or in terms of feeding stimulation to require HWA to complete their life cycle. This fastidious feeding response, which excludes acceptance of substitute (factitious) prey, raises the question about whether rearing *S. tsugae* and *L. nigrinus* can be possible on foods other than HWA.

### Preliminary testing: factitious hosts

We had tested factitious hosts and dozens of diets that were either selected from previously reported artificial diets or were developed de novo for the current study. Factitious hosts included eggs from the Mediterranean grain moth *E. kuehniella* (Pyralida) and the Angoumois grain moth *S. cerealella* (Gelechiidae), both species being used widely as factitious hosts for many predators and parasitoids [13].

### Preliminary testing of artificial diets

The artificial diets tested initially were meat-based diets developed for predators [13-15,17], where beef liver and ground beef were base materials as well as an "entomophage diet" [13] where meat products and chicken eggs were the base materials formulated with various supplements such as brewer's yeast, sugar, honey, vitamin mixtures, and various mineral mixtures. These diets were also supplemented with various functional ingredients [1] that enhanced texture and stability-including antimicrobial and anti-fungal compounds, humectants, and viscosity enhancers. We tested more than 100 different diets, counting major "diet types" (blood worm macerates, insect egg purees, freeze-dried and powdered insect larvae, bird's egg diets, meat diets, various plant materials, yeasts, etc.) and minor changes in these major classes (uses of various sugars such as fructose, sucrose, trehalose, glucose, and sugar alcohols; various proteins from soy, wheat germ, whey products, etc.; various preservatives such as potassium sorbate, sodium propionate, methyl paraben, etc; various vitamin mixtures such as the Vanderzant mixture, AIN, USDA pre-vitamin mix, etc.; various texturizing agents such as agar, carrageenan, alginates, locust bean gum, xanthan gum, guar gum, pectin, gellan, glycosamino glycans, and chitosan, to mention a partial list). The texturizing agents were various hydrocolloids that were added to the diet to give it a consistency ranging from a viscous liquid to a soft gel. We also attempted to use several types of known chemicals that had feeding stimulation qualities (yeast extracts, proline, betaine HCl, tryptophan, etc.) We also used several basic media components such as protein hydrolyzates (soy tryptone, beef tryptone, casein hydrolyzate, and yeast hydrolyzate). A special mention is in order for the alginate and chitosan gels. Both of these materials produced soft gels that simulated the consistency of internal structures of living prey. Alginate forms a gel without heat, if cross-linking agents, including divalent cations such as calcium, are

added to the diet formulation. Chitosan is activated to form a soft gel if it is mixed with 1-5 N acetic acid [1], which also gives a measure of antimicrobial activity. Both soft gels were very acceptable to both larvae and adult predators.

We also tested several diets reported as suitable for coccinellids, including the casein/wheat germ-based diet of Atallah and Newsom [18], the yeast and casein hydrolyzate diet of Ferran and Laforge [20], Smirnoff [21] diet with pulverized prey mixed with other ingredients such as alfalfa flour, the Smith [22] diet with minced liver and casein, and diets made of nitrogen sources such as fish meal, various yeast products, casein products, tryptones, spray-dried eggs, soy products, meat extracts: lipids from a variety of sources including lecithins from eggs or soy, sterols from various plant sources, and fats and oils from various plant and animal source. And we also tried a huge range of combinations of sugars, vitamin mixtures, and mineral mixtures along with texturizers such as agar, carrageenan, tapioca, locust bean gum, xanthan gum, and amino acids shown for other species to have feeding stimulation qualities. All of these efforts met with little or no feeding by the predaceous beetles. We also used several anti-microbial agents such as various paraben species (methyl-, ethyl-, propyl-, and butyl-paraben), potassium sorbate, and sodium propionate. We tested these materials over a period of 2-3 years, and we observed almost no acceptance until we used the chicken egg yolk-based diet in the following experiments. In these studies, we tested several dozen hypotheses about possible base materials that would be acceptable to the target predators. One series of hypotheses was based on the rationale that many insects, even some specialists, would accept non-natural insect (factitious) hosts. The second series of hypotheses was that certain foods of high nutritional value would be acceptable as substitute hosts. The results presented here treat both hypothesis testing tracks.

## Materials and Methods

### Insects

*Sasajiscymnus*(=*Pseudoscymnus*) *tsugae*(Coleoptera:Coccinellidae) adults used in experiments were from a colony continuously laboratory reared on HWA infesting eastern hemlock, *T. canadensis* for >15 generations at the Valley Laboratory, the Connecticut Agricultural Experiment Station in Windsor, Connecticut. The source of *S. tsugae* adults and larvae were from original field collections in 1994 and 1995 from *A. tsugae* infesting *Tsuga sieboldii* Carriere in Honshu, Japan [23-25]. *Laricobius nigrinus* (Coleoptera: Derodontidae) were reared and supplied by the Phillip Alampi Beneficial Insect Laboratory, New Jersey Department of Agriculture in 2012. The New Jersey laboratory colony of *L. nigrinus* was raised on HWA from the previous year's wild-collected beetles from Coeur d'Alene and Schweitzer, Idaho (Jenni DeSio, PABIL, personal comm).

### Experimental arenas

Large petri dishes (Nunc Labtek 150 mm diameter × 25 mm depth) were used in small scale or individual diet bioassays. If presented, the cut ends of hemlock foliage tips, infested or non-infested, were wrapped in absorbent cotton wool and kept moistened with water. For colony survival comparisons, *S. tsugae* adults were reared and maintained in 7.5 L Rubbermaid polycarbonate vented containers with HWA infested foliage kept hydrated in plastic water reservoirs. All diet testing was performed at the Connecticut Agricultural Experiment Station, Windsor, Connecticut.

## Commercial diet supplements

Bug Pro<sup>®</sup> was formerly commercially available from Gardens Alive (Lawrenceburg, IN) and the other commercial supplement tested was "Lacewing and Ladybug Food" (LLF) from Planet Natural (Bozeman, MT). The CC Diet was produced according to the formulation presented below. Gelled diets were prepared with a number of different gelling agents (see below). For other evaluations, the artificial diets were mixed with honey to form a paste in the general proportions: 30 ml of diet powder (approx 12 g) was combined with approximately 20 ml of honey in proportions that ensured that the adults would not get stuck in the paste as they fed. The mixtures were combined thoroughly with a spatula to form a smooth homogenous paste. The diet and honey paste mixtures kept at room temperatures did not develop mold; they were held in covered containers for ease of application and spread onto filter paper sections using a spatula. For presentation of diets in large insect cages, diets in honey paste were spread onto Whatman P8 filter paper (90 mm diameter) cut into quarters, each measuring around 1600 mm<sup>2</sup>, then affixed to cage sides with invisible tape.

## Diet preparation

Ingredients 1-8 (Table 1a) were mixed prior to addition to the mixture of boiling water and acetic acid. After addition of the solids plus egg yolk, the diet was mixed in a food processor (blender). More recently, we used a high speed mixer called Magic Bullet<sup>®</sup> (Homeland Housewares, Pacoima, CA). The combination of heating and low pH (from the acetic acid and betaine HCl) caused the proteins in the egg yolk to become denatured with an effect of thickening and denaturation of antinutrient proteins [1]. Various portions of the hot, viscous mixture were added to freeze-dried, cooked chicken breast in ratios of 2:1, 4:1, and 10:1, with 4:1 (4 parts hot liquid:1 part dried chicken breast) being the most suitable combination in terms of ease of mixing and handling for the freeze-drying step that follows. Mixed liquid and dried chicken slurry was placed in a freeze drying flask, frozen at -20°C, then freeze-dried. Freeze-dried diet was ground in a food processor (Cuisinart<sup>®</sup> Smart Stick Chopper).

As a means of increasing repeatability and consistency of diets, using a conventional brass wire soil sieve, we measured particle size in the powdered chicken breast and in the final version of the diet after freeze drying and grinding. The particle size distribution of the freeze dried chicken (by weight) was >2000 μM=3%, 600-2000 μM=65%, 425-600 μM=12%, and 106-425 μM=21%. After grinding the freeze dried complete recipe had size distribution of particles of >2000 μM=3%, 600-2000 μM=78%, 425-600 μM=18%, and 106-425 μM=2%. It should be noted that the particles in the combined (completed diet) were cohesive and rather sticky due to the high phospholipid content contributed by the egg yolk. This cohesiveness resulted in a tendency

Component	Amount (g)
1. Egg yolks	50
2. Fructose	0.5
3. Brewer's yeast	1
4. Vitamins (Vanderzant)	0.1
5. Betaine HCl	0.5
6. Potassium sorbate	0.1
7. Sodium propionate	0.1
8. Locust bean gum	1
9. Acetic acid (5%)	2
10. Boiling water	120

**Table 1a:** Components in the FDFE3 Diet. All components except for fresh eggs and chicken breast were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

for diet particles to clump, increasing the difficulty of measuring particle size with conventional soil sieves.

### Rationale for diet components in CC diet

The nutritional value of egg yolk in insect diets is discussed extensively by Cohen [1]. Briefly, it contains all of the essential amino acids, lipids, and minerals known to be required by insects. Also, the cholesterol is present in egg yolk as part of a lipoprotein complex, making it more stable and biologically available than in other types of sterol presentation. Egg yolks also contain a rich complement of B vitamins and other undefined components that are potentially feeding stimuli and cryptic nutrients. An important part of the value of egg yolk is in the rich complement of polar lipids such as phosphatidylcholine (lecithin), which is an excellent emulsifier, which serves in this diet to keep polar and non-polar substances from separating. Brewer's yeast is also broadly nutritional with respect to proteins and vitamins with traces of other nutrients, many of which are not fully defined. It also serves broadly as a feeding stimulant that has been used in hundreds of insect diets reported to be successful. Early tests of diets with different types of sugar indicated that fructose was acceptable to *S. tsugae*. It has a higher sweetness value than does sucrose or glucose [1], and on a per weight basis, it offers a higher osmolarity (or lower water activity) than does sucrose, making it a slightly but significantly better humectant than sucrose. We included the Vanderzant vitamin mixture as an insurance factor to make sure that there was enough of the broad base of vitamins known to be essential or beneficial to most insects. Although we have not yet fully vetted the betaine HCl, we included it in an effort to present feeding stimuli from an array of compounds shown to have such phagostimulant qualities [26,27].

### Approaches to diet development: derivation from existing diets, heuristic methodology, and eclectic approaches

This work was performed over a 10-year period during which we used diets derived from other published sources (derived-diets) and we made modifications that were prompted by careful observation of responses to changes based on several rationales. We now understand this procedure of observation/rationale-based changes to be a heuristic method of diet development [1]. Cohen [1] discussed the heuristic and eclectic approaches to diet development and adoption of diets derived from reports of successful diets for insects with similar habits. The outcomes of these extensive efforts failed to allow us to formulate and present diets that support continuous and robust development and reproduction. The methods used and their relative degrees of successful outcome are treated further in the Discussion.

#### Step by Step Process of CC Diet Synthesis:

1. Cook chicken breasts by immersing 500 g chicken in 1,500-2,000 g of boiling water for about 40 minutes where water is at a full boil.
2. Shred cooked chicken breasts in food processor, then place in freeze dry flasks at <-40°C at least for 20 hours.
3. Freeze dry ground chicken breasts completely.
4. Place freeze dried chicken in a cutting mill or food processor that is capable of forming a fine powder from breast meat.
5. Store powder with packets of oxygen absorbent and moisture absorbent in sealed containers in a freezer until needed for further diet preparation.
6. Make liquid phase of diet by combining these components (from Table 1b): betaine HCl, potassium sorbate, sodium propionate,

locust bean gum, acetic acid, and water. Make sure that all the locust bean gum is dissolved before proceeding to the next steps. Warming the solution and stirring with a hand-held mixer with French whip attachments help the mixing greatly.

7. Mix the egg yolk, fructose, brewer's yeast, and Vanderzant vitamin mixture thoroughly and allow the mixture to come to room temperature.
8. Bring water and other materials for Step 6 to a boil.
9. Rapidly add the egg mixture from Step 7 to the boiling water mixture, and stir with French whip type mixer until the mixture begins to increase in viscosity. The mixture is ideally allowed to reach a temperature of 85-95°C so that the eggs do not become solid. This temperature serves to pasteurize the mixture.
10. Add the liquid mixture while it is warm to the powdered chicken breast at a ratio of 2 parts (by weight) chicken breast powder to 8 parts (by weight) of the liquid.
11. Mix thoroughly, and use a spatula or plastic spoon to place diet slurry into a freeze dry flask.
12. Freeze for 20 hours at <-40°C.
13. Place flask on freeze drier, and complete the freeze drying process.
14. Mixture can now be milled to a course powder and stored (with oxygen absorbent and moisture absorbent in a sealed container, preferably in a freezer for long-term storage.

### Gelling agents

Formulation of 1% Gelcarin 812 (FMC Biopolymer, New Jersey) solution involved mixing 1 g of Gelcarin with 100 ml of warm, freshly boiled water. The mixture was then stirred for 15 minutes on a hot plate to dissolve the Gelcarin. For 1% sodium alginate solution, 1 g of sodium alginate was mixed with 100 ml of freshly distilled water. For testing gelled diets, 2 g of diet powder was mixed with 5 ml of freshly boiled water and then added to 18 ml of 1% gelling agent and mixed thoroughly with a commercial drink frother for 5-8 minutes. Gelled diets were refrigerated after preparation. All gelling agents and antimicrobial agents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified. Other gelling agents tested in combination with CC Diet included Protanal (FMC Biopolymer, Philadelphia, PA), Protanal and gellan (Sigma), sodium alginate, alginate+sodium propionate, sodium alginate + chitosan (approximately 0.2 g to 2.5 ml gelling agent). Gelled CC Diets were then applied to Parafilm strips (10 × 20 mm) and then dipped in 10% calcium lactate solution for 20 seconds to set up the gels and then rinsed in distilled water. These gelled CC Diets were made every 2 weeks, kept refrigerated and refreshed every 6-7 days for *S. tsugae* consumption during 41 day diet-only experiments.

### Palatability and preference experiments with *Sasajiscymnus tsugae*

**Gelled CC diets:** Choice experiments determined the palatability of CC Diet and a bee pollen diet gelled in 1% Gelcarin at 22°C. Adult *S. tsugae* were colony adults aged 4.5 mo, isolated from foliage into vials and given water only overnight. Males and females were separately presented with diets in large petri-dishes, each containing two tips of eastern hemlock (10-12 cm length) infested with first and second instar (N1-N2) HWA nymphs. Each replicate consisted of either 5 females or 5 males, with five replicates of each. Gelled diets were added in batches with a spatula to the surface of the hemlock tips (5 batches per tip of

each diet) and the adults introduced with a paintbrush into the center of the petri-dishes. Observations were made every 15-20 minutes and the location and feeding behavior of adults in each replicate recorded for 7 hours over 2 days (maximum shelf life before advanced desiccation of gelled diets).

In another choice experiment, CC Diet and a bee pollen diet were gelled in 1% Gelcarin and then presented on infested hemlock tips with HWA third instar nymphs (N3)-adults to 100 *S. tsugae* adults (aged 6 mo) in large petri-dishes. Ten replicates of 10 adults of mixed gender were observed for 7 hours a day for 2 days. Observations were made every 15-20 minutes and the location and feeding behavior of adults in each replicate recorded.

The next experiment, conducted at 22°C, 14 L:10 D, investigated whether these same adults would oviposit in the presence of CC Diets without live HWA. Uninfested hemlock tips were presented with globules of freshly prepared CC Diet gelled in 1% sodium alginate dabbed onto the upper surface of the stems and needles. A second group of uninfested hemlock tips were prepared with batches of adelgid wool only. The HWA wool was removed with a probe from live adelgids and wrapped into small bundles and placed onto the uninfested hemlock foliage. Hemlock tips either had eight diet globules or 12 wool bundles per tip. Ten replicates of adults (10/replicate of mixed gender) were presented with one hemlock tip with CC Diet and one hemlock tip with HWA wool per large petri dish. Halfway through the experiment, a second hemlock tip with fresh gelled diet was placed in each petri dish as some mold was forming on the first diet twig. The petri dishes were lightly wrapped together with Parafilm™ strips to retain moisture for the gelled diets. At the end of one week, the adults were removed and the twigs examined for presence of *S. tsugae* eggs under a dissecting microscope.

**CC Diets in honey:** Because of problems with desiccation of gelled diets by the second day, a different formulation of artificial diets was required for the logistics of mass rearing in laboratory conditions. The initial response of *S. tsugae* to CC Diet powder mixed with honey was investigated in this second no choice experiment. Forty *S. tsugae* adults (aged 5 mo) which had been maintained on HWA at 14°C for 2.5 mo were the test subjects. The CC Diet paste (1 g of CC Diet mixed with 10 ml of honey) was applied to two filter paper sections (16 cm<sup>2</sup>), each of which was placed in the center of two large petri-dishes. Adults were divided into groups of twenty and gently introduced into the petri dishes with a fine paintbrush. Observations on the location and behavior of the adults were made every 5 minutes for 2 hours at room temperatures of around 22°C.

In a choice experiment, 50 adults (aged 4.5 mo) were presented with CC Diet+honey and HWA N1-N2 at a room temperature of 22°C. Large petri-dishes contained either 5 females (5 replicates) or 5 males (5 replicates) placed with hemlock tips infested with HWA N1-N2 on which CC Diet in honey paste was smeared lightly on the needles. Observations on the location and behavior of the adults were made every 5 minutes for 7 hours.

### Survival of *S. tsugae* on CC diets and HWA N1

Adult *S. tsugae*, Group A (8 d old, n=27; 12 ♀, 15 ♂) and Group B (42 d old, n=28; 12 ♀, 16 ♂) were set up at room temperatures of 22°C in groups of 5-8 males or females per large petri-dish with gelled CC Diet only and plastic foliage tips (Jo Ann Craft Stores) 6 cm long which simulated hemlock tips for resting. The diets were presented on Parafilm™ strips and refreshed weekly or when mold was detected.

Adults on gelled CC Diets only were individually weighed at 20 and 41 days and percent survival recorded. For comparisons, colony adults of the same age as Group B (42 d) were set up in large cages a week later, for 32 d and supplied with freshly collected HWA N1 only (Group C, n=34, 19 ♀, 15 ♂) or on HWA N1 with CC Diet and honey supplement (Group D, n=32, 13 ♀, 19 ♂). At the end of day 32, survivors were individually weighed.

Four pairs of adults that had been sustained for 30 days in large petri-dishes solely on CC Diet, gelled with 1% sodium alginate, were tested for two weeks following their subsequent return to feeding and oviposition on natural prey (HWA N2). The hemlock tips were examined for *S. tsugae* eggs under a dissecting microscope.

### Survival experiments for *S. tsugae* in large colony cages

*Sasajiscymnus tsugae*, the native Japanese coccinellid predator of HWA, is active from spring through fall in Connecticut [23,24]. Its ability to survive the summer aestivation period of HWA is unique and is attributed to its predation of the dormant settled first instar nymphs [23,24], a stage which is not nutritiously acceptable to other introduced HWA predators. However, survival of adults can be vastly enhanced if provided with artificial diet supplements in the event that fresh HWA-infested hemlock foliage is scarce or not available.

The percentage survival of *S. tsugae* adults in colony cages was compared at 14°C in a Precision 818 Illuminated Incubator (Thermo Fisher Scientific, Waltham, MA) set at 12 L:12 D when given Bug Pro® (10 cages, n=412; holding period 65-97 days; mean 72 days) or CC Diet (12 cages, n=516; holding period 70-77 days; mean 74 days) as supplements under extreme conditions of foliage deterioration and HWA depletion, where foliage was not refreshed for more than 2 months. Cages contained 30-50 adults per cage and were misted weekly with water. Both Bug Pro® and CC Diet were mixed in honey as described above and applied to filter paper sections taped to the sides of the cages at the beginning of the experiment. The diets were not in direct contact with the hemlock foliage to minimize contamination. At the end of the holding periods, numbers of live and dead adults per cage were retrieved and recorded.

Percentage survival of *S. tsugae* adults in large colony cages on HWA N1 at 14°C, 12 L:12 D, provided with CC Diet+honey supplement (25 cages, n=665 adults, holding period 64-107 days, mean 89 days) was compared to that in cages with HWA N1 without CC Diet (8 cages, n=371, holding period 66-76 days, mean 71 days). The CC Diet in honey mixture was applied on filter paper sections taped to the sides of the cages and only applied once at the start of the holding period. Cages were misted with water weekly.

Survival of colony cages provided with HWA and CC Diet and honey was also monitored at warmer room temperatures of 22°C during the fall season (60 cages, n=1283, holding period 46-82 days, mean 62 days). As before, at the end of the holding periods, numbers of live and dead adults per cage were retrieved and recorded.

### Survival experiments with *Laricobius nigrinus*

Early emergence of *L. nigrinus* adults from soil pupation during the mid-late summer is a problem for some HWA predator rearing laboratories and also in the field [28]. At this time, HWA of the new sistens generation undergoes a lengthy summer aestivation as a non-feeding settled first instar nymph, anytime from July through September in the eastern US. *Laricobius nigrinus* feeds very little, or not at all, on aestivating HWA sistens N1 during this period. Early adult

emergents suffer high mortalities under laboratory holding conditions, even when kept at artificially cool conditions and supplemented with the commercial artificial diet supplement (“Lacewing Ladybug Food” from Planet Natural, LLF). Earlier experiments (Cheah and Cohen unpublished data) with different formulations of CC Diet showed that adult *L. nigrinus* readily accepted freeze-dried powdered CC Diet mixed with honey into a paste (Figure 3) or as a gelled mixture in alginate (Figure 4). All experiments with *L. nigrinus* were carried out in a Precision 818 Illuminated Incubator set at 12°C, 14 L:10 D.

In the third week of August 2012, laboratory reared first generation early emergent *L. nigrinus* adults, raised from Idaho wild collected adults, were shipped overnight to Connecticut by the Phillip Alampi Beneficial Insect Laboratory (PABIL), New Jersey Department of Agriculture. On arrival, all adults were individually weighed. Sixty adults from the Coeur d’Alene line were set aside for a replicated experiment to compare long-term survival on CC Diet or LLF, and to evaluate the potential of such supplements for reduction in emergent adult mortality.

The gender of adult *L. nigrinus* cannot be easily distinguished morphologically whilst alive so three groups comprising of 20 adults each, of unknown gender, were set up individually in large petri-dishes with high densities of aestivating HWA first instars infesting healthy eastern hemlock new growth (10-14 cm length). Infested hemlocks tips had cut ends wrapped in moistened absorbent cotton which kept the foliage fresh and adelgids alive [24]. Diet supplements were spread onto Whatman Qualitative P8 filter paper cut into strips (20 x 10 mm). Groups of replicates received either no diet supplements (controls) or CC Diet or LLF paste with honey. Individuals were provided diets on filter paper which were placed away from the infested hemlock foliage to avoid contamination in the petri dishes. Cotton wool wraps for hemlock tips and the diet strips were watered weekly with distilled water and infested foliage replaced weekly. All surviving experiment subjects were weighed individually in gelatin capsules, every 6-9 days starting on day 13 of the experiment using an Ohaus Voyager VIRR80 (Parsippany, NJ) balance. Diets were refreshed weekly for the first month then biweekly after that. Survival of individuals was monitored weekly. Individuals were tracked over 78 days while on diet supplements. Survivors were returned to hemlock foliage infested with developing HWA nymphs and CC Diets were discontinued while their weights were monitored.

In addition, another 31 *L. nigrinus* early emergent adults were maintained for 18 days in two large 7.5 L Rubbermaid polycarbonate vented containers with HWA N1 supplemented with either CC Diet (n=20) or LLF (n=11) mixed with honey, spread on filter paper attached to the sides of the containers. Survival was reduced in large containers, with only 42.1% surviving in containers with CC Diet and 36.4% in containers with LLF on day 18. Survivors (n=12) were then weighed and transferred individually to glass vials (15 x 60 mm) with absorbent cotton wool plugs, and then maintained on CC Diet+honey only. Water was supplied by moistening the cotton plugs weekly while CC Diet was presented on filter paper strips as above. Individuals were also subsequently weighed and survival recorded as above over 68 days. Survivors were returned to hemlock foliage infested with developing HWA nymphs and weights monitored for 68 days.

### Statistical analyses

The Number Cruncher Statistical Systems 2000 [29] was used for statistical analyses and graphs were prepared in Sigmaplot 2000 (Systat Software, San Jose, CA). Data on percent feeding over time, weights

of individual predators, and percentage survival from different diet treatments were initially tested for equal variances and normality of distributions to determine the appropriate t-tests for comparisons. Equal variance t-test or the Aspin-Welch unequal variance t-test were used to compare mean survival and feeding when assumptions of normality and variance were met while non-parametric Mann-Whitney U tests for difference in medians or Komolgorov-Smirnov tests for different distributions were used for comparisons when such assumptions were not met. Paired t-tests were used to compare weights of the same individual *S. tsugae* adults of different ages and gender fed on gelled CC Diets only at 20 d and at 41 d, the end of the experiments. Analysis of variance was performed to assess the effects of supplement type and time on survival and weights of *L. nigrinus* beetles.

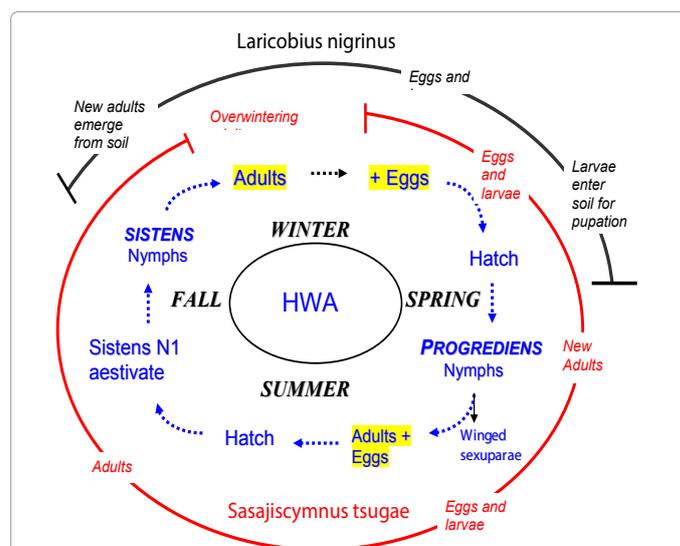
### Results

Of well-over 150 diets tested more than 25 presentation strategies, only two diets elicited sustained feeding and predator longevity. We named these diets the FDFE3 Diet with a basic formulation of chicken egg yolks supplemented with nutrients and functional materials (Table 1a) and the CC Diet (Table 1b), which is a derivation of the FDFE3 Diet where the later diet in its freshly-prepared liquid form is absorbed into powdered (coarsely ground) freeze-dried chicken breast and then freeze dried.

Over the long duration of these studies (>10 years), the negative results could be understood in the context of Figure 1, which depicts the complexity of the HWA life history and the adaptive characteristics of the predators that had to meet these complex fluctuations in the prey’s nutritional value and its biological activity [23-25,28,30,31]. For example, during the sistens immature nymph stages, the nutritional

Component	Amount (g)
FDFE3 Hot mixture	85
Freeze Dried chicken breast powder	15

**Table 1b:** Components in the CC Diet. All components except for fresh eggs and chicken breast were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Eggs and chicken breast were obtained locally from a grocery store.



**Figure 1:** Life cycles of hemlock woolly adelgid (—) and its introduced predators, *Sasajiscymnus tsugae* (—) and *Laricobius nigrinus* (—) in eastern North America. Solid lines indicate relative active periods of adult and larval predator feeding on the adelgid. Note the two periods highlighted in yellow when prey biomass is high and of excellent nutritional value [25,28-30].

content per adelgid unit is inferior to the reproductively active part of the sistens stage where there is a suitable nutrient reward to meet the predators' demands, in particular for *L. nigrinus*, whose primary diet is HWA eggs, necessary for reproduction [31]. This theme is seen in several of the graphs that follow, where what we are calling low quality prey (dormant-aestivating sistens HWA N1), fail to support growth, reproduction, or even survival of *L. nigrinus* [28]. Therefore, whenever predators such as the *Laricobius* species those are in captivity become unsynchronized with their prey and they become active (breaking their diapause prematurely or remaining active for periods extended beyond the nutritional high quality periods of the HWA), the predators die, causing considerable economic losses and program disruption.

The search behavior of both *S. tsugae* and *L. nigrinus* involves the movements of these insects up and down stems until they make contact with prey (or artificial diet). Discovery of prey or an acceptable diet terminates foraging and stimulates feeding efforts (Figures 2a-2c, 3a-3d and 4a-4c). It is illustrated in Figures 3c and 3d that sometimes, even with suitable HWA prey), predators will feed on the diets that we are describing in this paper (FDFE3 and CC Diets). In light of the complete rejection of the more than 100 diets presented in our earlier studies, the acceptance of artificial diet even in the presence of their natural prey came as a surprise. The only other artificial diets that *S. tsugae* and *L. nigrinus* accepted as palatable were the commercial preparations "Lacewing and Ladybug Food (LLF)" and "BugPro®." Although our diet formulations (FDFE3 and CC Diet) and the two commercial diets were eaten robustly, comparisons of longevity and maintenance of body weight (sometimes weight gain) was found to be superior in the diets presented here. This point is explored further in the Discussion.

In Figure 3, feeding trials are illustrated where the diet was applied directly to hemlock stems. The diet here was presented in a gelled form, and this was well-accepted by larvae and adults (Figures 3a and 3b). It is also noteworthy that diet-feeding was frequently observed even in the presence of high quality natural host materials (developing or mature HWA). The alginate-gelled diet was prone to dry up or develop mold within a few days, so its palatability declined to the extent that predators would no longer accept it. Efforts to coat diet failed to achieve success at keeping the diets moist. Therefore, presentation of an uncoated gelled diet could not be used in place of prey as part of a mass-rearing system.

Although the FDFE3 and the subsequent CC Diets were developed for *S. tsugae* as the target species, other predators such as *L. nigrinus* that are HWA specialists readily accepted the diet both in its gelled form and as a honey paste (Figure 4a-4c). It is also evident from Figure 4 that all the predators tested accepted the diet both on the natural vegetation (hemlock tips) and in presentations that were free of plant materials.

### Palatability and preference experiments with *Sasajiscymnus tsugae*: Gelled CC diets

Adult and larvae of *S. tsugae* readily accept gelled CC Diets (Figures 2 and 3). For comparisons of adult feeding on HWA N1-N2 and gelled CC diets, initial feeding responses to CC Diets were low on day 1, but by the second day, adults fed preferentially on gelled CC Diet over HWA N1-N2 (Figure 5; Wilcoxon Signed Rank Test  $Z=2.52$ ,  $p<0.01$ ) and the bee pollen (BP) diet (Wilcoxon Signed Rank Test  $Z=-2.52$ ,  $p<0.01$ ). Feeding responses over the 48 h period showed that gelled CC Diet was significantly preferred over HWA N1 - N2 (Mann Whitney U test  $Z=4.65$ ,  $p<0.0001$ ), gelled BP diet (Mann Whitney U test,  $Z=-2.94$ ,  $p<0.01$ ). Gelled BP diet was also preferred over HWA N1-N2 (Mann Whitney U test  $Z=-2.88$ ,  $p<0.01$ ).

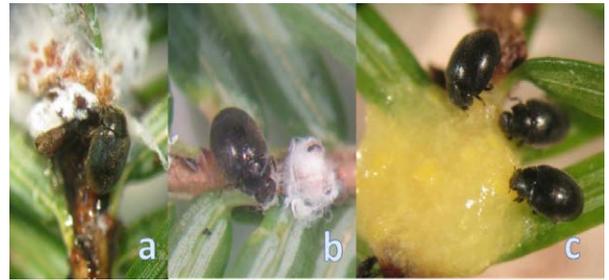


Figure 2: Establishment of normal feeding behavior as basis of comparing responses to artificial diets. *Sasajiscymnus tsugae* feeding on HWA eggs (2a), late instar HWA nymph (2b) and alginate-gelled CC Diet (2c).

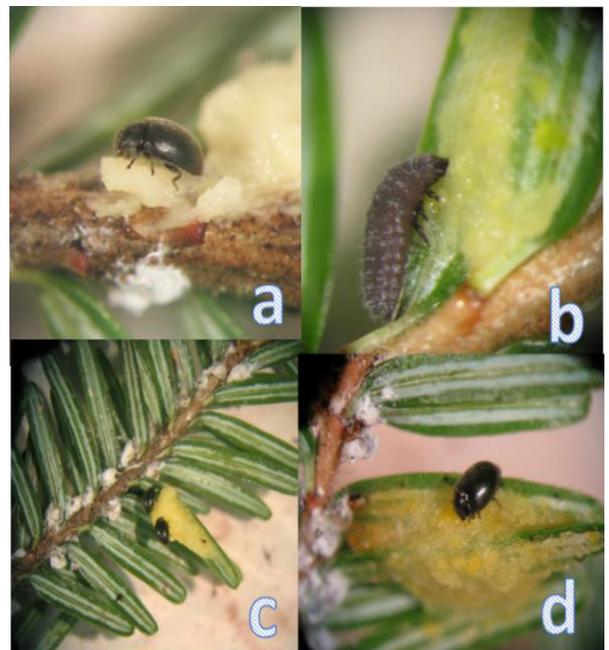
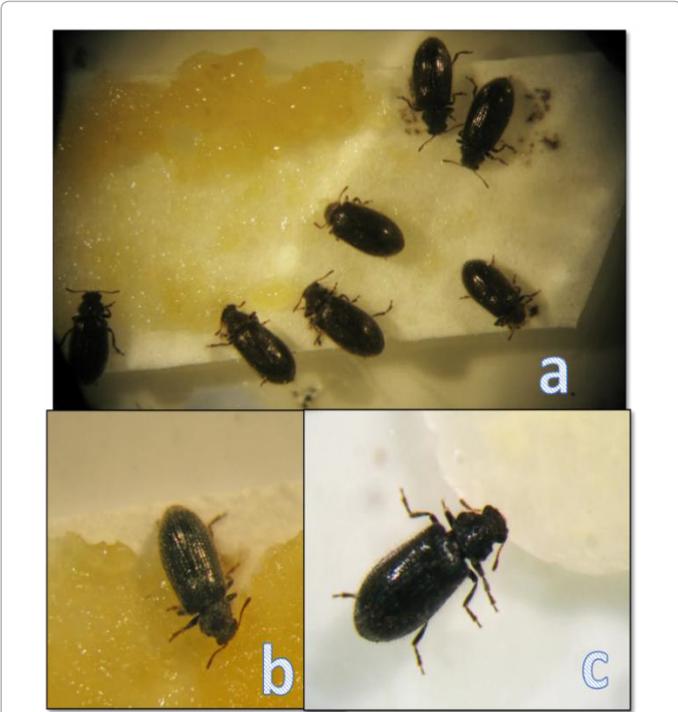


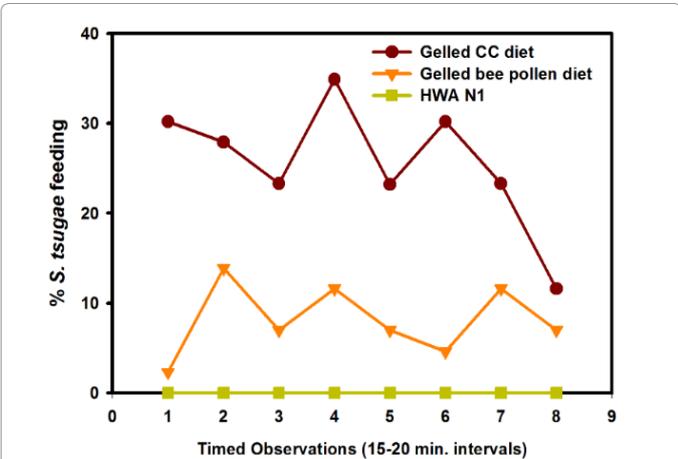
Figure 3: *Sasajiscymnus tsugae* feeding on CC diet, including an adult feeding on alginate-gelled diet (3a), a larva feeding on gelled diet (3b), adults feeding on gelled diet in the presence of healthy natural HWA prey (3c), adult feeding on 2 d old gelled diet in the presence of HWA nymphs (3d).

When provided a choice between a pollen-based diet (an 80% pollen mixture with sugar, vitamins, and an alginate gelling agent), HWA 3<sup>rd</sup> instar nymphs, and CC Diet, *S. tsugae* initially selected HWA, but with the opportunity to explore the pollen and CC Diet, the predators displayed a decline in feeding responses to HWA and increased their responses to the two artificial diets (Figure 6). The *S. tsugae* adults again preferred gelled CC Diet over gelled bee pollen diet (Mann Whitney U test  $Z=-2.55$ ,  $p<0.01$ ) and fed more on CC Diet than on HWA N3-adults on day 2 (Mann Whitney U test  $Z=-1.72$ ,  $p<0.01$ ), although initial feeding on HWA was robust on day 1 (Figure 6). But feeding levels were similar on HWA and gelled bee pollen diet on day 2 (Mann Whitney U test,  $Z=0.11$ ,  $p>0.05$ ). Switching in feeding to gelled CC Diets on day 2 could have been also due to depletion of HWA after initial day 1 feeding.

After one week of confinement with gelled CC Diet and HWA wool on uninfested hemlock tips, oviposition was recorded in 80%



**Figure 4:** *Laricobius nigrinus* consuming CC Interim Diet as a honey paste (4a and 4b) and in a cold-gelled alginate form (4c).



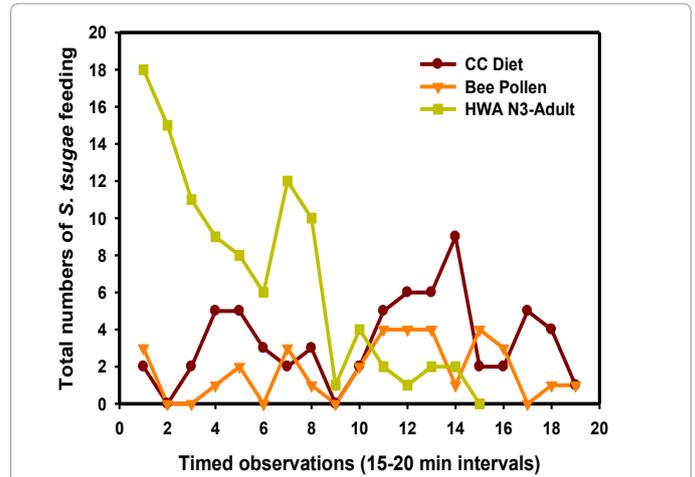
**Figure 5:** Preferential feeding by *Sasajiscymnus tsugae* adults on gelled CC Diet over gelled bee pollen diet and dormant first instar nymphs of HWA on Day 2. Observations were made every 15-20 minutes and percent feeding represented totals over all replicates.

of replicates, in the absence of live adelgids. Eggs were laid on 60% of “diet” twigs (mean of  $1.3 \pm 1.6$  eggs/replicate) and 80% of “wool” twigs (mean of  $2.9 \pm 3.6$  eggs/replicate). A total of 32 eggs were laid on “wool” twigs and 12 eggs laid on “diet” twigs. There was no difference between numbers of eggs laid on “wool” twigs and “diet” twigs (Kolmogorov-Smirnov test, Dmn criterion value=0.2,  $p>0.05$ ).

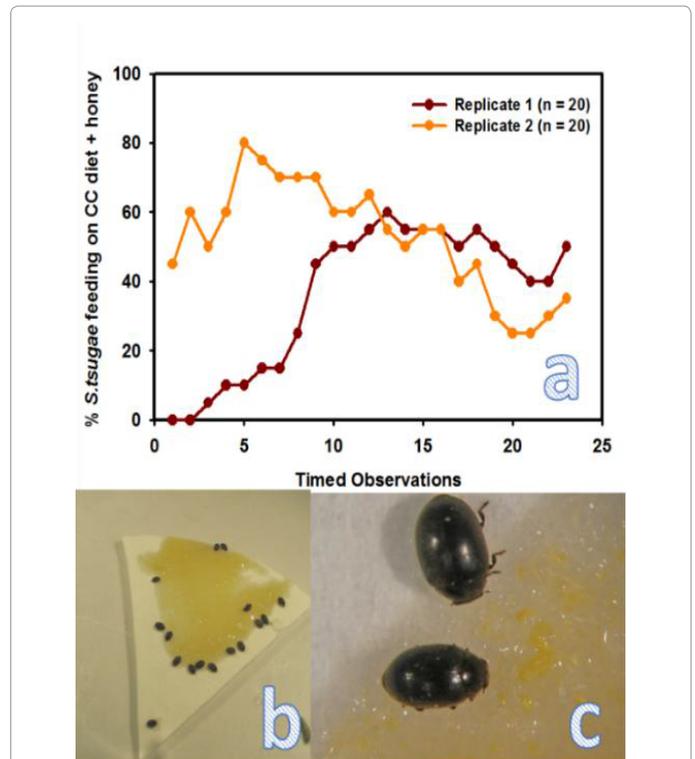
### CC Diets in honey

Adult *S. tsugae* readily responded to CC Diet presented as a honey paste (Figure 7a-7c). Feeding response was quick and robust as adults were attracted to fly or walk to feed on the CC Diet and honey

mixture. In less than 30 minutes, over 80% were feeding in Replicate 1 and 50% were feeding on CC Diet in Replicate 2 by the first hour (Figure 7a). In Figure 7, it is seen that once *S. tsugae* discovered the CC Diet, they continued to feed for 2 hours. Close-up observations (Figure 6c) revealed that the predators were actively feeding during the extended periods. Adults also preferred to feed on CC Diet and honey as compared to HWA N1-N2 (Figure 8, Mann Whitney U test  $Z=2.95$ ,  $p<0.01$ ).



**Figure 6:** Adult *Sasajiscymnus tsugae* switching from HWA nymphs and adults to feed preferentially on gelled CC Diet by the second day. Observations were made every 15-20 minutes over 2 days.



**Figure 7:** No choice timed observations of *Sasajiscymnus tsugae* feeding on CC Diet in honey (a) showing observations made at 15-20 minute intervals from start of the trials. The feeding arena is shown in (b), and a close-up of the feeding is shown in (c).

### Survival of *S. tsugae* on CC diets and HWA N1

When adults of different ages were confined for 41 days with gelled CC Diets only, survival of all ages and gender was very high (Table 2). Weights of individual female and male *S. tsugae* fed on different foods at the end of the experiment are also shown in Table 2. An analysis of variance showed that the gender of the adults was a significant factor influencing the weights recorded at day 20 and day 41 ( $F=6.36$ ;  $df=1, 105$ ,  $p<0.05$ ). Weights of newly emerged adult females (Group A) increased significantly on gelled diets between day 20 and by the end of the experiment on day 41 (Paired t-test;  $T=-1.97$ ,  $p<0.05$ ). Although older females from Group B also appeared to gain weight by the end of the experiment, differences were not significant (Paired t-test,  $T=-1.27$ ,  $p>0.05$ ). The weights of the younger males from Group A (Paired t-test;  $T=-0.80$ ,  $p>0.05$ ) and older males from Group B (Paired t-test;  $T=-0.98$ ,  $p>0.05$ ) were similar between days 20 and 41. As there was no loss of weight, this signaled the sustaining palatability and nutritional adequacy of the gelled CC Diets in sustaining the adults for over a month. At 70 days after this 41 day confinement on gelled CC Diets, survival on return to HWA remained high for younger Group A adults (90.9%) and older Group B adults (100%), indicating no detrimental effects on survival after an extended period fed on gelled CC diet alone.

Similar weights of males and females were recorded on different foods, whether fed gelled CC Diet only, or natural prey HWA N1, or supplementing HWA N1 with CC Diet and honey. Adults which were given HWA N1 only (Group C; Table 2) had survival between 66-73%, but this was improved to 92-95% with additional provision of the CC

Diet in honey paste for Group D (Table 2). Again, this attested to the sustainability of *S. tsugae* adults for extended periods on CC Diet. End weights of *S. tsugae* fed HWA N1 were compared with end weights of *S. tsugae* fed HWA N1 and supplemented with CC diet+honey (Table 2). There were no significant differences for females (Equal Variance  $T=-1.012$ ;  $p>0.05$ ) or males (Mann Whitney  $Z=-1.2$ ;  $p>0.05$ ).

For four pairs (male and female) of *S. tsugae* maintained on gelled CC Diets only for 30 days, normal feeding and oviposition was recorded on return to hemlock tips infested with HWA N2. Between 42-130 HWA N2 were consumed per pair during the first week and a mean of 6.7 eggs/week 1 and 6.5 eggs/week 2 recorded, indicating normal feeding and oviposition behavior after an extended period of confinement with gelled CC Diet only.

### Survival experiments for *S. tsugae* in large colony cages

The variable holding period or duration of period between foliage changes had an effect on cage survival (ANOVA;  $F=5.98$ , d. f. 1, 10;  $p<0.05$ ). Percentage survival decreased slightly with increase in the interval between foliage changes for cages provided with CC Diet (Figure 9) but was still superior to that of cages provided with Bug Pro®. Mean cage survival was  $85 \pm 10.7\%$  in cages provided CC Diet, which was significantly higher than mean percent survival ( $43.9 \pm 31.6$ ) in cages provided with Bug Pro® (Kolmogorov-Smirnov Test, Dmn criterion value=0.7;  $p<0.01$ ). Percent survival in cages with Bug Pro® was highly variable (Figure 9).

Results of colony cage survival for extended holding periods of foliage deterioration and HWA depletion are shown in Table 3. Survival in cages provided with CC Diet and honey was very high regardless of the age of the adults, the length of the holding period, and temperature, while survival was minimal (3%) when CC Diet was not provided.

### Survival experiments with *Laricobius nigrinus*

Survival of adults provided with HWA aestivating first instars and CC Diet was the highest (Figure 10). On day 56, 55% of adults, maintained on aestivating HWA first instars and supplemented with CC Diet, were still alive while only 20% adults provided with LLF (“Lacewing and Ladybug Food“ from Planet Natural) survived. At the end of the 78 day period, 55% of adults fed with CC Diet survived while only 15% of adults provided with LLF survived. For the control group, provided only HWA N1, there was 45% mortality at day 13 and by day 27, all control group adults were dead (Figure 10).

Mean weights of newly emerged *L. nigrinus* shipped from PABIL was  $0.88 \pm 0.20$  mg ( $n=60$ ) at the start of the experiment. Mean weight of control adults that were provided with only HWA first instar nymphs was  $0.69 \pm 0.13$  at day 13, with only 11 surviving then and only one adult was alive by day 19 (Figure 11). Weights of individual *L. nigrinus* were

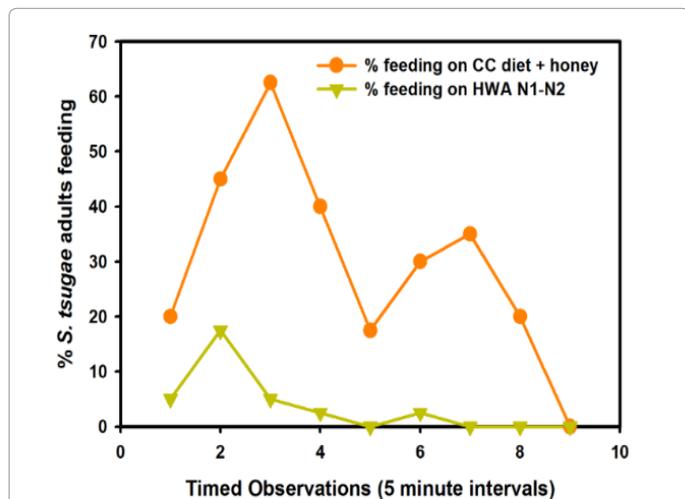


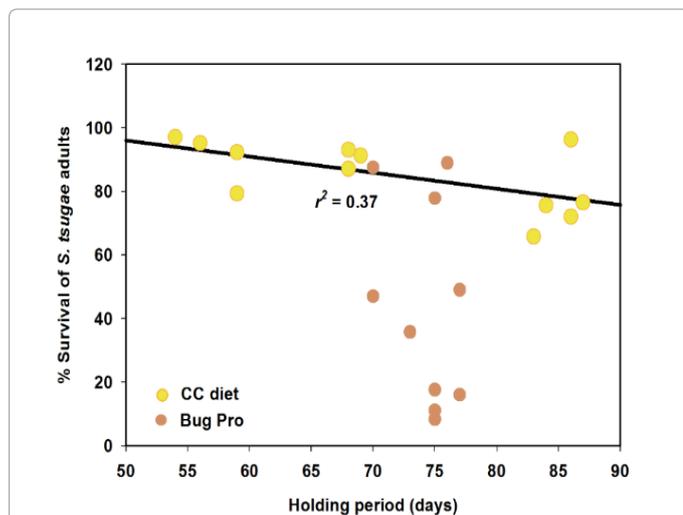
Figure 8: Adult *Sasajiscymnus tsugae* preferentially feeding on CC Diet and honey in the presence of immature HWA nymphs. Observations were made every 5 minutes for 7 hours.

Treatment	Group	Initial ♀	Initial ♂	Age at start (days)	% Survival and mean weights at experiment's end	
					Female	Male
Gelled CC Diet only for 41 d	A	12	15	8	91.7	86.7
Mean weight (mg)					$0.76 \pm 0.16$	$0.66 \pm 0.16$
Gelled CC Diet only for 41 d	B	12	16	42	100	93.7
Mean weight (mg)					$0.81 \pm 0.27$	$0.69 \pm 0.20$
HWA N1 for 32 d	C	19	15	42	73.7	66.7
Mean weight (mg)					$0.82 \pm 0.12$	$0.75 \pm 0.16$
HWA N1+CC Diet and honey for 32 d	D	13	19	42	92.3	94.7
Mean weight (mg)					$0.78 \pm 0.11$	$0.67 \pm 0.16$

Table 2: Comparative mean percentage survival and mean weights (mg) at experiments' end of adult *Sasajiscymnus tsugae* feeding on gelled CC Diet alone, or on natural prey, HWA N1, or HWA N1 supplemented with CC Diet+honey.

significantly higher when supplied with CC Diet compared to LLF over the 78 day period ( $F=40.45$ , d. f. = 1, 202;  $p < 0.0001$ ). Day of weighing was not a significant factor ( $F=1.52$ ; d. f. = 1, 202,  $p > 0.05$ ), signifying that adults supplemented with CC Diet were heavier throughout the experiment. Adults that were given CC Diet were heavier (mean weight over 78 days:  $0.97 \pm 0.19$  mg) than those given LLF (mean weight over 78 days:  $0.77 \pm 0.18$  mg). Adults that had access to CC Diet gained weight while adults which were fed LLF lost weight (Table 4). Mean individual weights of diet survivors and controls are graphed in Figure 11. Adult *L. nigrinus* on CC Diets were significantly heavier than those on LLF diets for 70% of weight measurements (Table 4).

Mean weights of adults that survived the 18 day holding period in



**Figure 9:** Comparison of percent survival of *Sasajiscymnus tsugae* adults over extended holding periods on deteriorating hemlock foliage and HWA in 7.5 L cages. Predators were provided with either CC Diet or Bug Pro® in honey-paste mixtures.

large containers was  $0.78 \pm 0.2$  mg ( $n=12$ ). Survival of *L. nigrinus* adults without HWA but provided water and CC Diet in honey paste is shown in Figure 12. Survival at 40 days was 58.3% (mean weight= $0.81 \pm 0.11$  mg). After Day 39, cumulative percent survival declined until a minimal of 25% were alive on day 68 (Figure 10). It appeared that CC Diet with honey alone can sustain newly emergent *L. nigrinus* adults with 33% mortality over 30 days (Figure 11). Weights of adults over time did not affect percent survival ( $F=0.95$ , d. f. = 7, 53;  $p=0.47$ ); surviving adults on day 68 averaged  $0.93 \pm 0.46$  mg compared to starting weights of 0.78 mg.

Weights of individual diet survivors were also measured on return to developing HWA nymphs in November 2012 (Figures 11 and 12). Mean weights of survivors on CC Diet ( $n=9$ ) and on LLF ( $n=2$ ) on return to developing HWA nymphs did not significantly increase although feeding on HWA nymphs was observed. This indicates the comparative superior nutritional adequacy of the CC Diet over an extended period of 78 days in terms of percent survival and sustainment of healthy adult weights.

## Discussion

This research covers a very large scope of information that we developed in exploring ways to meet the feeding requirements of specialist predators of HWA. It reflects the difficulties in finding foods (fictitious and artificial diets) that would meet all the feeding requirements (palatability, nutritional value, bioavailability, and stability), and it is further complicated by the need to present diets in a suitable way—one that allows the food to remain palatable, retain moisture, resist microbial deterioration, and allow the very small mouthparts of the specialist predators to get access to the artificial diets. These problems in meeting such complicated needs are common to researchers who are trying to develop diets for specialists, especially ones with small mouthparts (including thrips, mites, and small beetles to mention a few [1]).

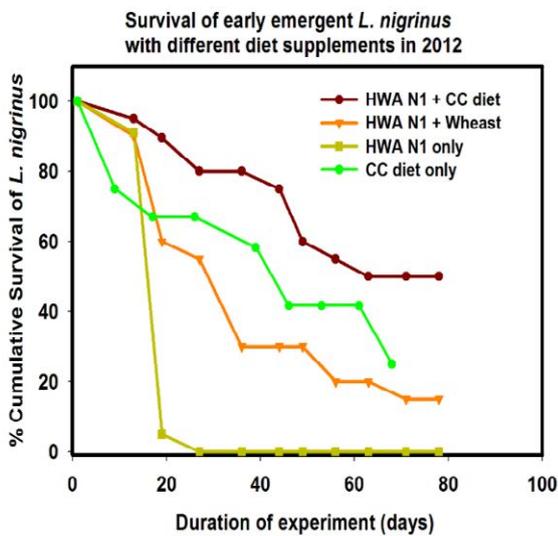
The life-history of HWA is extremely complex, and only a predator with a specially adapted life history could fit the pattern that would allow it to effectively suppress HWA populations. Hodek [32] reviewed

Treatment	No. of cages	No. of adults	Temperature °C	Mean age of <i>S. tsugae</i> (mo)	Holding period (days)	Mean% survival
CC Diet+HWA N1	16	412	14	$3.4 \pm 1.1$	$91 \pm 17.4$	$90.1 \pm 10.7$
CC Diet+HWA N1	9	253	14	$8.0 \pm 0.9$	$86 \pm 17.8$	$89.1 \pm 17.6$
HWA N1 only=Control	8	371	14	$2.7 \pm 0.5$	$71 \pm 5.1$	$3.1 \pm 2.9$
CC Diet+HWA N1	32	692	22	$4.3 \pm 0.7$	$60 \pm 9.5$	$94.7 \pm 6.5$
CC Diet+HWA N1	28	591	22	$7.9 \pm 1.5$	$64 \pm 6.7$	$92.5 \pm 9.2$

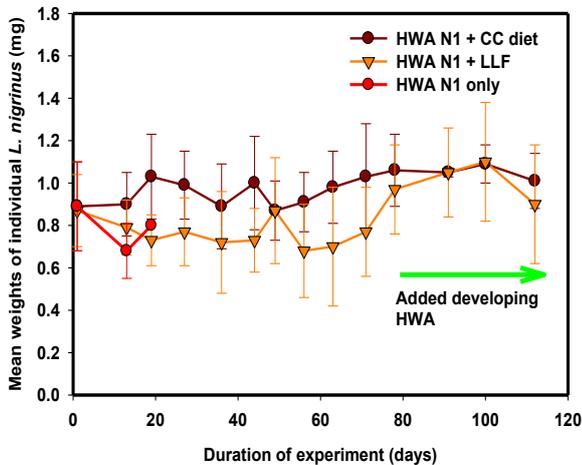
**Table 3:** Comparison of *Sasajiscymnus tsugae* percent adult survival with or without CC Diet and honey supplement at 14 and 22°C during extended holding periods of foliage deterioration and HWA depletion. Means  $\pm$  S.D. are reported.

Diet Supplement	Mean Weights (mg) of individual <i>L. nigrinus</i>				
	Day 13	Day 19	Day 27	Day 36	Day 44
CC+HWA N1	$0.90 \pm 0.15$ $n=19$	$1.03 \pm 0.2$ $n=17$	$0.99 \pm 0.16$ $n=16$	$0.89 \pm 0.89$ $n=16$	$1.0 \pm 0.22$ $n=15$
	$T=2.36^*$	$T=4.56^{***}$	$T=3.38^{**}$	$Z=-1.7^*$	$T=2.67^{**}$
LLF+HWA N1	$0.79 \pm 0.13$ $n=18$	$0.73 \pm 0.12$ $n=12$	$0.77 \pm 0.16$ $n=11$	$0.72 \pm 0.24$ $n=6$	$0.73 \pm 0.15$ $n=6$
	$T=0.27$	$T=2.52^*$	$T=2.37^*$	$T=1.65$	$T=0.79$
CC+HWA N1	$0.89 \pm 0.15$ $n=12$	$0.91 \pm 0.14$ $n=11$	$0.98 \pm 0.17$ $n=11$	$1.03 \pm 0.25$ $n=10$	$1.06 \pm 0.17$ $n=10$
LLF+HWA N1	$0.87 \pm 0.25$ $n=6$	$0.68 \pm 0.22$ $n=5$	$0.7 \pm 0.28$ $n=4$	$0.77 \pm 0.21$ $n=3$	$0.97 \pm 0.21$ $n=3$

**Table 4:** Difference in weights (mg) over time of early emergent *Laricobius nigrinus* provided with dormant settled first instar nymphs of HWA supplemented with either CC Diet or commercial "Lacewing and Ladybug Food" (LLF) in a honey paste. Means  $\pm$  S.D. are reported.



**Figure 10:** Cumulative percent survival of early emergent *Laricobius nigrinus* on dormant first instar HWA in late summer and early fall 2012: supplemented with either CC Diet or Bug Pro ("Wheat") mixed with honey or on CC Diet and honey only.



**Figure 11:** Patterns of mean individual weight (mg) trends of early emergent *L. nigrinus* maintained on dormant first instar HWA (a low quality food), with or without artificial diet supplements, CC Diet or Lacewing Ladybug (=LL Food from Planet Natural). Weights of survivors are also shown after return to developing HWA nymphs for feeding (indicated by green arrow).

the complex life histories of the Coccinellidae that have evolved in the context of complex prey life histories and ecological relations such as sporadic availability. Similarly, the predatory derodontid, *L. nigrinus* has special features of its life history that dispose it to be an efficient specialized predator of HWA [28,31]. Figure 1 shows the life cycle of HWA is complex in the context of the specialist predators, *S. tsugae* and *L. nigrinus*. The figure shows progreddiens and sistens generations, along with phases of development where the HWA actively feed, develop, and reproduce (including a winged adult phase), and other periods when HWA is inactive as aestivating first instar nymphs.

It should be noted that in the summer aestivation phase of the

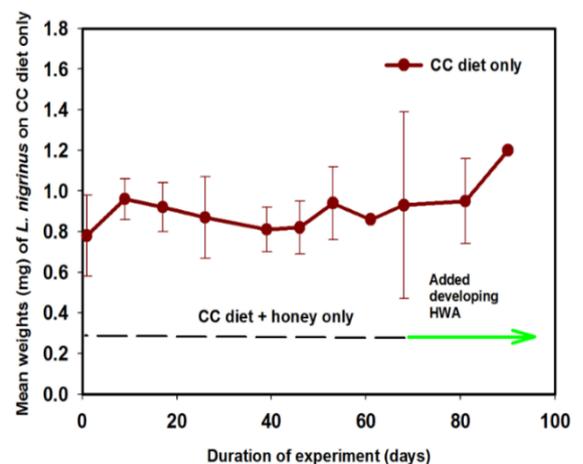
HWA life cycle, there is no prey biomass available to maintain specialist predators of the *Laricobius* genus, which survive the period as pupae in the soil. *Sasajiscymnus tsugae*, however, is adapted to feed and survive on this dormant first instar stage in the field and laboratory [23-25]. Along with the morphological and behavioral differences in HWA during these different phases, there are also biochemical differences. Our preliminary work on lipid, carbohydrate and protein content indicates that during certain periods, HWA nutritional value greatly drops, especially during the late phases of their torpor and early stages of feeding activity onset. We have found the nutritional composition of HWA in their inactive phases drops to less than half of what is present in actively feeding individuals [33,34]. This helps explain the periods of dormancy in predators that are specially adapted to feed on HWA, where their nutritional needs must mesh with their hosts. This concept of predator/prey ecological meshing is seen in Figure 1. In Figure 2, our observations of normal feeding activity is illustrated with *S. tsugae* feeding on live eggs (Figure 2a) and comparisons of feeding on artificial diets are illustrated in Figure 2c. These Figure 2a-2c help demonstrate the behavioral standards of comparison that we had to establish to evaluate a broad spectrum of artificial diets and factitious hosts.

We divided our experiments and observations into three major categories to cover all aspects of our research on development of artificial diets and rearing systems for predators of HWA:

- Feeding on Natural Hosts (Prey)
- Feeding on Factitious Hosts
- Feeding on Artificial Diet
- Various artificial diets
- Diet presentation techniques
- Factors in diet stability

### Natural hosts

The different life stages of HWA offer different nutrient rewards to predators, especially with respect to 1) overall biomass, 2) protein content, 3) lipid content, 4) carbohydrate content, and 5) vitamins and minerals. We have begun analysis of these factors in several of the life-



**Figure 12:** Trends in mean individual *Laricobius nigrinus* adults, maintained only on CC Diet and honey and water for 68 days. Weights on return of survivors to developing HWA nymphs is also indicated (green arrow notes return to high quality HWA nymphs on Day 70).

stages, but because these studies are preliminary, we can provide only partial results. Using the analytical techniques described by Cohen and Patana [35], with modifications of the protein analysis method [34], we measured protein, carbohydrate, and lipid content of HWA individuals. We have found that eggs that have been oviposited, as well as eggs that are inside females, have a high lipid content with approximately 50% of the dry weight being lipid. The protein content ranges from about 30-40% of dry weight and the carbohydrate content is less than 10%, leaving about 4-5% ash (minerals) and a small biomass (less than 3%) composed of other components such as nucleic acids and components derived from host plants. These findings are in accord with the findings of Cohen and Patana [35] regarding the nutritional composition of eggs, except that the adelgid eggs and neonate larvae have a higher lipid content than comparable lepidopteran eggs and neonates.

We found that a great deal of lipid, especially oil, is stored in the eggs and remains present in the neonate crawlers. The oil is present as a storage material providing energy and bio-materials for crawlers as they settle on host plants and begin their feeding forays. We learned from analysis of natural prey that HWA predators, especially when they use adults with eggs, derive a large lipid component in their diet [34].

### Factitious prey

Use of factitious hosts has a long been a successful strategy in rearing entomophagous insects (illustrated in Figure 13; [36,37]). For example, the Mediterranean grain moth *E. kuehniella* (Pyralidae) and the Angoumois grain moth *S. cerealella* (Gelechiidae) have been used extensively in commercial predator and parasitoid production of green lacewings and egg-parasites such as *Trichogramma* spp. [13,38]. Eggs and other life stages of factitious hosts and prey have been used for decades to support development of predators [38-43], and they have even been used in mass-rearing of generalist predators such as lacewings and big-eyed bugs [1,35,42,44].

We did not anticipate the failure of factitious prey in light of the numerous cases where predators, including coccinellids, were shown to feed robustly on non-target insect eggs [38,39,41,42]. In fact, one of the most remarkable successes in rearing a so-called specialist coccinellid was demonstrated with *S. parcesetosum* reared on pink bollworm eggs by the USDA, APHIS Pink Bollworm Rearing Facility in Phoenix, AZ from 1992-1997, by Robert T. Staten (personal observation, Cohen).



**Figure 13:** Eggs of *Ephesthia kuehniella* are shown that were exposed to *S. tsugae* and *L. nigrinus*. In Figure 13a, an egg that had been utilized by *S. tsugae* is seen with its characteristic depletion because the materials had been removed by the predator, and the egg is also dark due to the action of the polyphenol oxidase that takes place as part of the predators' extra-oral digestion [36,37]. Because the use of eggs themselves elicited few feeding bouts, we also tried to "disguise" the *Ephesthia* eggs as HWA by placing fresh adelgid wool and exudates (Figure 13b) to determine whether or not the HWA materials would enhance predation. There was no evidence of enhanced feeding in the presence of adelgid wool or fecal exudates, so efforts to disguise factitious prey were discontinued.

However, our efforts to use factitious prey with various species of Lepidoptera eggs, as well as other potential hosts, including various aphid species met with no success at stimulating feeding by HWA predators.

### Feeding on artificial diets

**Diet presentation techniques: Packaging:** We tested techniques used previously (and with considerable success) with several predaceous insects, including encapsulation [15,45,46]. These techniques were designed for predaceous insects that were thought to be liquid feeders, and the diets were strictly liquid in texture. We also tested diets that were very viscous suspensions or slurries. We packaged these diets in Parafilm® as described and reviewed by Cohen and Smith [13]. Such packaging included stretching the Parafilm® to several (3-6) times its original dimensions, which resulted in weakening the film and thinning it to less than its original thickness. Therefore, starting with Parafilm® with a consistent thickness of 125 µm [13-15], we stretched the diet covering to less than 25 µm [13-15].

**Diet presentation techniques: Texture modification of diets.** Although many insect diets are synthesized with gelling agents (usually hydrocolloids such as agar or carrageenan), we found in early testing that the firm gels based on these gelling agents were not accepted by the predators. After failing to induce feeding with Parafilm® packaged diets, we followed a strategy of providing diets that were modified internally by increasing viscosity with several kinds of macromolecules such as dissolved proteins (e.g. whey proteins) and other hydrocolloids, including locust bean gum, xanthan gum, gelatin (from modified collagen), pectin, carrageenan, alginates, and chitosan. In the case of alginates and chitosan, the viscous diets could be treated with surface modifications to form a film or skin over the surface of the diet. For example, diets that contained alginate could be modified to form a layer of strongly gelled film by application of calcium salts such as calcium chloride or calcium lactate. The duration of exposure to alginate-containing diets with calcium solutions on their surface determined the thickness and strength of the film. We also used chitosan as a film-forming agent, and both with alginate and chitosan, the predators fed readily through the film. However, our goal of preserving or stabilizing the diet to prevent desiccation and microbial attack was not achieved with the simple formulations that we used (e.g. 1% sodium alginate or 1% chitosan), so we did not pursue this approach in the current series of studies reported here. However, there is an increasing body of literature on the use of alginate and especially chitosan as edible film in the food industry as reviewed by Elsabee and Abdou [47]. Elsabee and Abdou [47] point out that the desirable properties of edible films include 1) reduction of microbial deterioration, 2) reduction of oxidative degradation, and 3) loss of moisture. Their review includes several publications on using mixtures of hydrocolloids such as starches, milk proteins, soy proteins, and oils to help enhance the three desirable qualities mentioned here. There seems to be great promise in these approaches for insect diets, especially in light of the fact that films can be made to be thin enough to permit insects with very small mouthparts to penetrate them and that such films are well-documented to have antimicrobial properties. This discussion leads to the next section on improvement of diet stability in interim diets.

**Factors in diet stability:** Cohen [1] describes the four characteristics that must be operant for a diet to be fully successful: 1) palatability, 2) complete nutritional value, 3) bioavailability, and 4) stability. The rationale behind these characteristics are 1) the insect must eat the diet robustly to thrive on that diet, 2) all the insect's nutritional

requirements must be met to support the range of metabolic pathways, 3) the mere presence of a nutrient does not guarantee that the insect will be able to digest, absorb, transport, or otherwise utilize that component: so it must meet bioavailability demands, and 4) even if a diet meets all of the first three requirements, if it becomes degraded due to microbial, oxidative, or other deterioration factors, the diet will soon fail to support healthy growth. Therefore, all other aspects of diets that make them useful in supporting healthy insects become unimportant if stability failures take place. Such failures can manifest themselves as microbially-derived deterioration, oxidative damage to nutrients or taste components, separation of particles or liquids with immiscible phases, enzymatically-induced changes, or excessive water loss.

One of the most commonly used techniques to prevent microbial growth is addition of antimicrobial agents, especially antifungal agents [1,48]. The most serious drawback of such chemical agents is the potential harm that they can cause to the target insect, including direct toxicity and feeding deterrence [48,49]. In our current studies, we found that the predators were very sensitive to antifungal agents, and this compounded the problem of frequent occurrences of microbial, especially mold, outbreaks. Therefore we tried to use alternative techniques summarized by Cohen [1] such as 1) reduced water activity, 2) lowering pH below threshold of growth of common microbes, and 3) heating (pasteurizing) diets, and 4) combining two or more of these strategies. We tried to lower water activity to under  $a_w$  of 0.70 using glycerol and proline, but the predators failed to feed well on these humectants when they were added in quantities that would suitably lower water activity. Mixing the diet with honey to form a paste gave very satisfactory results, but if we mixed hydrated diet to mix with honey (whose water activity is  $<0.55$ ),  $a_w$  would approach or exceed 0.90, and the microbial growth problem would recur. This prompted our strategy to adhere the diet to freeze dried, powdered chicken breast and then freeze dry the composite slurry. We would then grind the freeze dried diet in a cutting mill and store the product in sealed 50 ml centrifuge tubes until the diet was to be used. We found that by mixing the diet with honey, the resulting  $a_w$  was less than 0.50. The mixture with its paste-like consistency could be applied to foliage, sides of the rearing containers, or on clean filter paper and placed in cages and would remain mold-free for weeks, as long as it was not wetted by routine spraying of foliage with water (a tactic frequently used by insectary workers who are trying to keep the beetles and the foliage hydrated).

As has been pointed out [1], the most crucial determinant of stability in insect diets is water activity, with activities of less than 0.50  $a_w$  being resistant to diet degradation due to microbial growth, free-radical formation, and enzymatic processes. Therefore, we designed the diet to be freeze dried as a final production step. After being freeze-dried, the diet is routinely ground to a powder (with particle sizes ranging from 50-200  $\mu\text{m}$ ) and stored in either vacuum-sealed bags or 50 ml plastic centrifuge tubes. It is preferable to store diet with packets of oxygen scavenger and desiccant (e.g. FreshPax<sup>®</sup> from <http://www.multisorb.com/products-and-systems/freshpax-oxygen-absorber-packets-and-strips/>). Desiccants such as active silica gel can also be obtained in convenient packets (e.g. ULINEX, [http://www.uline.com/BL\\_1004/Silica-Gel-Desiccants](http://www.uline.com/BL_1004/Silica-Gel-Desiccants)).

### Concluding general comments

Although we tested well over a hundred formulations of artificial diets and factitious prey, we observed feeding only with the chicken egg-based diet that we called FDFE3 Diet, the CC Diet (a combination of FDFE3 adhered to freeze dried, ground chicken breast meat), and

a commercial formulation called Lacewing and Ladybug Food (from Planet Natural <http://www.planetnatural.com>). Of these three foods, both *S. tsugae* and *L. nigrinus* fed more readily and survived longest on the CC Diet and FDFE3 Diet, but we consider all three artificial diets as examples of “interim” diets. Survival on gelled CC diet alone was very high for *S. tsugae* after 41 days (mean 89.2%; Table 2) and also for *L. nigrinus* on CC diet in honey paste ( $>50\%$  on day 56; Table 4) as compared to 100% mortality by day 27 for adults given HWA N1 alone. Survival of adult *S. tsugae* for more than 2 months on deteriorated hemlock foliage and in the absence of live high quality HWA was superior and consistently much higher when provided CC diet in honey paste than with commercial supplements, again demonstrating the nutritional utility of this unique artificial diet. The conclusion is that the CC diet in either formulation is highly effective in extending the survival of adult HWA predators, especially those produced during mass rearing for biological control releases and could be extremely useful for storage of predators until release, or when HWA prey is unavailable, scarce or of low nutritional quality. Most important was the observation that CC diet-fed *S. tsugae* quickly exhibited normal feeding behavior and reproduction when returned to its natural prey, live HWA.

In reviewing approaches to artificial diet development over the past 100 years Cohen [1] discussed the various strategies, which included models of diets based on analysis of natural foods of target insects, nutrient self-selection or similar behavioral studies, using mixture approaches such as geometric models, heuristic procedures (which use rationale-driven trial and error), derivation of diets from existing formulations, and an eclectic approach, which incorporates two or more of these approaches. The current work falls mainly into the heuristic category where we used rationale-driven tests of materials that were expected to improve feeding and nutrition (based on observations of our target insects and special features of components). The approach used here was largely incremental, where a partial success with one formulation led to modifications of components or relative quantities. In some instances, the efforts were more saltatory with rather abrupt changes in formulation. Also, however, it must be noted that many of the ideas for testing a type of diet came from the literature on previously reported successes. A small amount of our work was based on analysis because most of the nutritional profile of HWA as a prey was already known, and efforts to use extracts from HWA “wool,” exudates, and cuticular chemicals failed to produce positive results.

As an example of how our process was carried out in some of the more successful iterations, we used egg yolk based on previous studies where this component was found to be useful to various predators [1,45,46,49]. The heating process and mixing egg yolk with water, fructose, vitamins, and antimicrobial agents came from a previous study of a diet for multiple species of predators [13,14]. Using betaine HCl also was derived from the literature [27]. The kind of iteration process is then illustrated by this scenario: the heated (pasteurized) egg diet elicited feeding but was too runny, so we used hydrocolloids (locust bean gum and xanthan gum, for example) to add viscosity, and the instability of the diet once it was synthesized led to a strategy of freeze drying. The efficacy of using re-hydrated freeze dried diet had to be determined by trials, and the error that came from excessive re-hydration had to be corrected in a trial and error (heuristic) manner. It is emphasized here that the heuristic approach has been used in many diet-development projects, and they are more powerful as problem-solving tools because they involve rationale-driven trials.

Finally, it is important to recognize that although the body of this work did not meet the desired objectives of developing a rearing system that would allow continuous, convenient, economical and unlimited production of HWA predators, we did design a diet and presentation technology that supports survival and biomass maintenance of specialist predators. We believe that this diet and presentation technology will serve currently operating mass rearing programs for HWA, and that this diet will also be useful for other predators as a food supplement or interim medium.

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