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Evaluation of the Parasitoid *Ooencyrtus mirus* (Hymenoptera: Encyrtidae) as a Potential Biological Control Agent of *Bagrada hilaris* (Heteroptera: Pentatomidae)

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

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by

Nancy Reisig Power

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Dissertation committee:

Dr. Thomas M. Perring, Chairperson
Dr. Timothy D. Paine
Dr. Mark Hoddle
Dr. Daniel Jeske

The Dissertation of Nancy Reisig Power is approved:

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Committee Chairperson

University of California, Riverside

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Dedication

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ABSTRACT OF THE DISSERTATION

Evaluation of the Parasitoid *Ooencyrtus mirus* (Hymenoptera: Encyrtidae)
as a Potential Biological Control Agent of *Bagrada hilaris*
(Hemiptera: Heteroptera: Pentatomidae)

by

Nancy Reisig Power

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, June 2020
Dr. Thomas M. Perring, Chairperson

In an effort to find a biological control agent of *Bagrada hilaris*, an invasive pentatomid pest on brassica crops in western North America, three hymenopteran egg parasitoids were recovered from brassica plant debris in Pakistan and sent to California, USA for evaluation. One of these, a uniparental species, has since been described as *Ooencyrtus mirus*. To evaluate *O. mirus* as a potential biological control agent, I investigated its host range and reproductive capacity. I also tested parameters for maximizing its reproduction, including temperature and the age of the parasitoid and host eggs. Finally, I examined *O. mirus*' ability to find host eggs on broccoli plants and in soil, where *B. hilaris* lays them. The results show *O. mirus* to be a generalist parasitoid species with a preference for *B. hilaris*. Under lab conditions *O. mirus* lays an average of 118 eggs and lives an average of 58 days. The immatures undergo quiescence at 14° or 16°C. They can be stored at these temperatures for at least 3 months with no loss of reproductive success. The immatures develop fastest at 36°C, but the best temperature for rearing is 30°C. Above 30°C, the second generation has an increase in the proportion of males, due to the high temperature killing the symbiotic *Wolbachia* bacteria that enable unfertilized

eggs to develop as females. Wasps of age 3-11 days have better reproductive success than wasps aged 0-2 days. Wasps are most successful on 0- and 1-day-old *B. hilaris* eggs, and they can reproduce on frozen eggs. The ability of *O. mirus* to find host eggs was poor in soil, and even worse on plants. These studies describe the biological characteristics of *O. mirus* that suggest it could be an effective biological control agent. However, more testing is needed on its host preferences under natural conditions to determine if it would be safe to release in the field; i.e., not a threat to native or beneficial host species.

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Chapter 1

Introduction

Bagrada hilaris **(Burmeister)**

Distribution

The painted bug, *Bagrada hilaris* (Burmeister), aka bagrada bug (= *B. cruciferarum* Kirkaldy) (Hemiptera: Heteroptera: Pentatomidae) (Fig. 1-1), is a pest on brassica and sometimes other crops worldwide. In its native range of Africa, Asia and

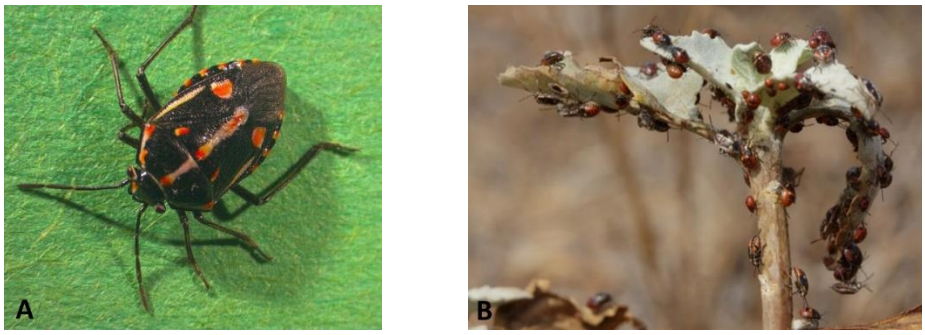

Fig. 1-1. *Bagrada hilaris*. **A**. Adult. **B**. Nymphs on native brittlebush, *Encelia farinosa*, Moreno Valley, California July, 2014. Waypoint: 33.87630N, -177.19337W. Photo by Daniel Pierce.

the Middle East (Howard 1907; Husain 1924), it is one of the major pests of leaf mustard (*Brassica juncea*) and oilseed brassica crops such as rapeseed and canola (*B. napus* L.) (Sachan & Purwar 2007; Abrol 2009; Bundy et al. 2018). *Bagrada hilaris* first appeared in the United States (U.S.) in Los Angeles County, California (Ca.) in 2008 (Arakelian 2008). Genetic analysis indicates that the resulting populations in North America are most closely related to those in Pakistan, and less closely related to those in South African (Smith et al. 2016). From Los Angeles County, *B. hilaris* spread southward to neighboring Orange County and continued to San Diego County within the same year. It

also spread west from Los Angeles into Ventura county and east into Riverside and Imperial counties in 2009. By 2010, it had reached adjacent San Bernardino and Kern counties, and three more states: Arizona, Nevada and New Mexico. By September 2012, it had moved west to Santa Barbara and north to San Luis Obispo counties and east into Texas. In 2013-2014 it continued spreading further north in California to Tulare, Fresno and Monterey counties, and to the Bay area by 2014. It has also appeared in Utah, Mexico and even Hawaii (Palumbo 2016; Matsunaga 2014). By 2015 it occurred in 27 total counties in California (Palumbo & Natwick 2010; Bundy et al. 2012; Vitanza 2012; Perring et al. 2013; Reed et al. 2013; Dara 2014) and southward into 6 states in Mexico (Sánchez-Peña 2014; Torres-Acosta & Sánchez-Peña 2016). Most recently, in 2016 *B. hilaris* appeared in Chile, its first report in South America (Faúndez et al. 2016). In Chile it spread quickly, not only as an agricultural pest, but also, for the first time, as a nuisance in homes and even "biting" a human (Faúndez et al. 2017; Faúndez 2018). In 2017, the Chilean Ministry of Agriculture imposed regulations to slow its spread (SAG 2017).

The potential range of *B. hilaris* includes all regions with a Mediterranean climate; i.e., coastal regions with hot, dry summers and cool, wet winters (Carvajal et al. 2018). The potential range is likely to change as the global climate changes, expanding to higher latitudes but shrinking in some parts of Europe, Asia and Africa (Carvajal et al. 2018). Among several factors analyzed, the strongest predictor of bagrada bug expansion was the human footprint. Other predictors included isothermality and precipitation in the warmest time, with drier air favoring *B. hilaris* (Carvajal et al. 2018).

Biology of *B. hilaris*

Bagrada hilaris is multivoltine, with the number of generations per year varying with temperature (Hill 1983). The females lay eggs on plants or in soil (Taylor et al. 2014). The egg stage lasts 3-6 days, depending on temperature. Of the five nymphal instars, the first four are orange after molting but get darker with age. The fifth instar looks similar to the adult. The life cycle, depending on temperature, takes 38-65 days. The mean fecundity per female is 95 eggs (range 36-173) (Halbert & Eger 2010).

Crop Damage

A warm-season insect on cool-season crops, *B. hilaris* lives on wild mustard weeds in the summer, and then infests cole crop seedlings in early fall. Growers have reported seeing the adults waiting for brassica seedlings to sprout from the soil. In California and Arizona, the feeding damage often kills the seedlings, reaching up to 60% mortality (Reed et al. 2013) and leading to incomplete stands of the crop (Palumbo & Natwick 2010; Palumbo et al. 2016). Seedlings that survive often have damage in the crown that results in unmarketable flower heads (Palumbo & Natwick 2010). *Bagrada hilaris* feeds primarily on new growth, including leaves, fruit, stems, and apical meristems, causing chlorotic spots on leaves, and stunting growth (Lambert & Dudley 2014; Palumbo et al. 2016). Besides physical damage, *B. hilaris* injects salivary enzymes that kill cells (Ahuja et al. 2008). It especially damages organic crops, sometimes causing complete stand losses (Lawrence 2012). *Bagrada hilaris* populations similarly often exceed the economic threshold on cole crops in Africa, India and the Mediterranean region (Hill 1975; Daiber 1992).

Bagrada hilaris plant hosts

Bagrada hilaris is found on *Brassica oleracea* L., including both direct-seeded and transplanted broccoli, broccoflower, cabbage, cauliflower, Chinese cabbage, collard and kale (Palumbo & Natwick, 2010). Other *Brassica* hosts include *B. rapa* L., *B. napus* L., and *B. juncea* (L.) Czem. (Palumbo & Natiwick 2010). Other Brassicales hosts include *Capparis spinosa* L., *Descurainia sophia* (L.) Webb ex Prantl, *Eruca vesicaria* (L.) and *Raphanus sativus* L. (Aalbersberg et al. 1989; Colazza et al. 2004; Palumbo & Natiwick 2010). *B. hilaris* can also infest Poaceae, such as *Chloris gayana* Kunth, *Cymbopogon distans* (Nees) J.F. Watson, *Cynodon dactylon* (L.) Persoon, *Cynodon plectostachyus* (K. Schumann) Pilger, napier hybrid *Pennisetum glaucum* (L.) Leeke (x *P. purpureum* Schumacher), *Pennisetum typhoides* (Burm.f.) Stapf & C.E. Hubb, *Saccharum officinarum* L., *Sorghum sudanense* (Piper) Stapf, *Triticum aestivum* L. and *Zea mays* L. (Aalbersberg et al. 1989; Cheema et al. 1973; Gupta & Gupta 1970; Rawat & Singh 1980; Rizvi et al. 1986). Legumes such as *Medicago sativa* L., *Phaseolus aureus* Roxb., *Phaseolus mungoI* Roxb. and *Trifolium alexandrinum* L. are also attacked by *B. hilaris* (Cheema et al. 1973; Gupta & Gupta 1970). The Euphorbiaceae species *Ricinus communis* L. (castor bean) and Amaranthaceae species *Chenopodium album* L. can also serve as hosts (Cheema et al., 1973). In California, *B. hilaris* is found on native plants such as buckwheat and brittlebush (Christiane Weirauch, 2017 and Daniel Pierce, 2017, respectively; personal communications; Fig.1-1 B) and especially on introduced wild mustards. As the mustards start to dry up at the end of the season, the bugs fly to the fresh, tender brassica seedling transplants on nearby farms.

Natural enemies of *B. hilaris* and other stink bugs in California

The records of natural enemies of *B. hilaris* globally are listed in Table 1-1. As *B. hilaris* invades more areas in California, the existing natural enemies of pentatomids may find it a suitable host. Hoffmann et al. (1991) found that several species of small indigenous hymenopterans (Scelionidae and Encyrtidae) parasitize eggs of indigenous Ca. stink bugs, and a few tachinid fly species were attacking nymphs and adults. The exotic species *Nezara viridula*, the southern green stink bug, was first reported in California in 1986 as a pest on tomatoes, soon spreading to other crops as well (Hoffmann et al. 1991). A study with 200 sentinel eggs revealed that no effective natural enemies were present, so in 1987 the European parasitoid wasp *Trissolcus basalis* (Wollaston) was released in Davis in northern California to control *N. viridula* (Hoffmann et al. 1991). In 2002, Ehler confirmed the success of *T. basalis* against *N. viridula* in northern California. His field studies with sentinel eggs showed that *T. basalis* was the major egg parasite of *N. viridula*, typically attacking 100% of eggs in an egg mass. Four other species of egg parasites were recovered from the sentinel eggs as well, but with lower rates of parasitism: two scelionids, *Gryon obesum* Masner and *Telenomus podisi* Ashmead; and two encyrtids, *Ooencyrtus californicus* Girault and *O. johnsoni* (Howard). Ehler also found that some sentinel eggs had been eaten by arthropod predators with chewing mouth parts. *Bagrada hilaris* is also a host for fungal pathogens such as *Zoophthora radicans* (Entomophthorales) (Torres-Acosta et al. 2016).

Table 1-1. Natural enemies of *Bagrada hilaris*, arranged by location.

Natural enemy species	classification	citation	location
Alophora (Hyalomya) pusilla Meigen	Diptera: Tachinidae	Cheema et al. 1973	Pakistan
Parellophora indica Mesnil	Tachinidae	Cheema et al. 1973	Pakistan
Chrysoperla carnea Stephens	Neuroptera: Chrysopidae	Cheema et al. 1973	Pakistan
spider, unidentified	Aranea	Cheema et al. 1973	Pakistan
Podisus maculiventris (Say)	Heteroptera: Pentatomidae	Grasswitz 2016	New Mexico, USA
Collops vittatus (Say)	(Coleoptera: Melyridae)	Bundy et al. 2012; Grasswitz 2016	New Mexico
Sinea diadema (Fabricius)	Heteroptera: Reduviidae	Grasswitz 2016	New Mexico
Ooencyrtus sp.	Hymenoptera: Encyrtidae	Walker Jones, personal communication, in Grasswitz 2016	New Mexico
Ooencyrtus lucidus Triapitsyn & Ganjisaffar	Encyrtidae	Triapitsyn et al. 2020	California, USA
Trissolcus basalis (Wollaston)	Hymenoptera: Scelionidae	Ganjisaffar et al. 2018 Felipe-Victoriano et al. 2019	California; Coahuila, Mexico
Trissolcus hyalinipennis Rajmohana & Narendran	Scelionidae	Ganjisaffar et al. 2018	California
Gryon myrmecophilum (Ashmead)	Scelionidae	Felipe-Victoriano et al. 2019	Coahuila
Telenomus podisi Ashmead	Scelionidae	Felipe-Victoriano et al. 2019	Coahuila
Idris elba Talamas	Scelionidae	Lomeli-Flores et al. 2019	Guanajuato, Mexico
Other predators, found with *B.hilaris* but not observed preying on them:			
big-eyed bug	(Hemiptera: Geocoridae	Grasswitz, personal communication, in Bundy 2012	New Mexico
carabid and staphylinid beetles, ants and spiders	Coleoptera, Hymenoptera, Aranea	Grasswitz 2016; Power 2017, personal observation of spider	New Mexico

Besides the field studies, Ehler did lab studies to test different life stages of 27 arthropod species for their ability to eat different life stages of *N. viridula* by putting one predator with 10 eggs, with 10 replications (100 eggs total) for each species. He only found a handful of species that preyed on *N. viridula* eggs: the malachiid beetle *Collops vittatus* (Say); the green lacewing *Chrysoperla carnea* (Stephens); two lygaeid bugs, *Geochoris punctipes* Say and *G. pallens* Stål, an *Oxyopes* sp. spider, and the isopod *Armadillidium vulgare* (Latreille). However, 23 of the 27 species tested preyed on the *N. viridula* nymphs. Of these, two reduviids, namely *Zelus renardii* Kolenati and *Sinea diadema* (Fabricius), consumed the most eggs: 73 and 83, respectively.

Pease and Zalom (2010) did a conservation biocontrol study in the Sacramento Valley in northern California on two stink bugs, *Euschistus conspersus* Uhler and *Thyanta pallidovirens* Stål. The egg parasitoids recovered from the field varied with the season, but a total of five parasitoids were found on *E. conspersus*: *Gryon obesum* Masner, *Trissolcus hullensis* (Harrington), *Trissolcus euschistus* (Ashmead), *Trissolcus utahensis* (Ashmead) and *Ooencyrtus johnsoni* (Howard). Of these, *G. obesum* was most abundant. They also found four predators on yellow sticky traps: *Geocoris atricolor* Montandon (Hemiptera: Lygaeidae), *Jalysus wickhami* VanDuzee (Hemiptera: Berytidae), *Hippodamia convergens* Guerin (Coleoptera: Coccinellidae), and *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae).

Economics of crops damaged by *B. hilaris*

The value of the brassica crop industry is substantial; the combined value of the broccoli (*Brassica oleracea* L. var. Italica), cabbage (*B. oleracea* L. var. Capitata) and

cauliflower (*B. olearacea* L. var. Botrytis) crops grown in the U.S. was $1,724,381,000 in 2017 (USDA NASS 2017). More than 90% of the fresh market brassica crops are grown in Ca. and Arizona, where *B. hilaris* is now present. A survey of brassica growers in Yuma, AZ, and the Imperial Valley, CA, estimated that 90% of broccoli acreage planted in 2010 and 2011 was infested with *B. hilaris* at some point in the growing season, and on average, this resulted in stand losses and plant injury exceeding 5 and 10% in cauliflower and broccoli crops, respectively (Huang et al. 2013). Millions of dollars of crop value are thus at stake in managing *B. hilaris* populations in the southwestern U.S.

Management of *B. hilaris*

Current management of *B. hilaris* includes an integrated approach of employing multiple strategies that may include transplanting instead of direct seeding, altering planting dates, destroying crop residues, and controlling mustard weeds in the summer (Palumbo et al. 2016). Other recommended techniques include frequent cultivation of the soil during the growing season to destroy *B. hilaris* eggs, reducing nitrogen fertilization, vacuuming the bugs, excluding them by growing the crops under screened tunnels or floating row covers, and using brassica trap crops (Palumbo et al. 2016; Bundy et al. 2018). However, the main control is by pyrethroid and neonicotinoid (neonic) insecticides (Palumbo 2015). Ahuja et al. (2008) found that sowing seeds treated with imidacloprid (a neonic) into dry soil, followed by irrigation, to be effective.

Relying mainly on chemical insecticides has drawbacks. Frequent application increases the risk and rate of pesticide resistance, as it applies more selection pressure on

the pest population (Kunz & Kemp 1994). Insecticides can deplete the populations of natural enemies, increasing the likelihood of an outbreak of the target or secondary pests. Neonics induce honeybee workers to abandon their social roles, leading to Colony Collapse Disorder (Kiljanek et al. 2016). The half-life of neonics in soil can surpass 1000 days, and even their metabolites are toxic, so repeated use can result in accumulation and exposure of non-target animals (Bonmatin et al. 2015). Worker safety and environmental concerns, along with the cost of using insecticides and the economic importance of brassica crops, are further factors that instigated an effort to expand integrated pest management (IPM) options for *B. hilaris*. A biological control agent, by adding another tool to the insect management toolbox and lessening the use of insecticides, can also help slow the development of insect resistance to insecticides, so the insecticides remain effective for a greater number of years. A classical biological control agent thus could be an important component of an integrated pest management program for *B. hilaris* and provide an effective agent for organic farmers.

To search for potential biocontrol agents of *B. hilaris*, R. Mahmood, B.E. Bajwa and K. Rashid collected *B. hilaris* eggs from brassica plant debris from two sites in the Toba Tek Singh District in the Punjab Plain of Pakistan in early April and early May, 2014, and placed the eggs in vials to see if parasitoids emerged. From the same places and similar times, they also collected *B. hilaris* adults from the wild, reared them in the lab to get eggs, and glued the eggs onto sentinel cards which they set out for four days to attract parasitoids. The eggs from canola plant debris yielded the platygastrids *Trissolcus hyalinipennis* (Rajmohana & Narendran) and *Gryon gonikopalense* Sharma as well as an

encyrtid, *Ooencyrtus* sp. (Mahmood et al. 2015). The latter has since been described as *Ooencyrtus mirus* Triapitsyn & Power (Triapitsyn et al. 2020). The sentinel eggs yielded only *T. hyalinipennis.* The population of *B. hilaris* was low in the area, suggesting that the parasitoids were keeping the pest population under good control. These three wasps comprised the first records of egg parasitoids of *B. hilaris* in Pakistan, and the first report of an *Ooencyrtus* attacking *B. hilaris* anywhere (Mahmood et al. 2015). However, *Trissolcus*, *Gryon* and *Telenomus* (also a platygastrid) species have been reported attacking *B. hilaris* eggs in neighboring India (Mahmood et al. 2015). Walker Jones, with the USDA Biological Control Laboratory in Stoneville, Mississippi, arranged for live specimens of the three hymenopteran parasitoids to be sent to the U.S. *Trissolcus hyalinipennis* and *G. gonikopalense* are under investigation at the United States Department of Agriculture (USDA) Western Regional Research Center in Albany, Ca. and at the European Biological Control Laboratory (USDA-ARS-EBCL) at Montferrier-sur-Lez, France. The focus of this dissertation is on the *Ooencyrtus* species, *O. mirus*.

Ooencyrtus mirus

Ooencyrtus mirus is a parthenogenetic egg parasitoid. Males occasionally emerge but are not needed for reproduction. The adult female *O. mirus* (Figs. 1-2 & 1-3) are about 1 mm long. The head and thorax are black. The posterior end of the abdomen is black but the anterior 2/3 is bright, translucent yellow/gold. The ventral side of the ovoid abdomen has a conspicuous, longitudinal groove that holds the ovipositor. The legs and antennae are yellow. The antennae are clubbed in the female but less so in the male. The male antennae have much longer, darker setae, giving them a hairy appearance. The

males on average are smaller and darker than the females, and have only dim gold bands on their more triangular-shaped abdomen (Figs. 1-4 & 1-5). The ventral abdomen has thin dark bands, and the aedeagus extends slightly past the abdomen and ends in a point.

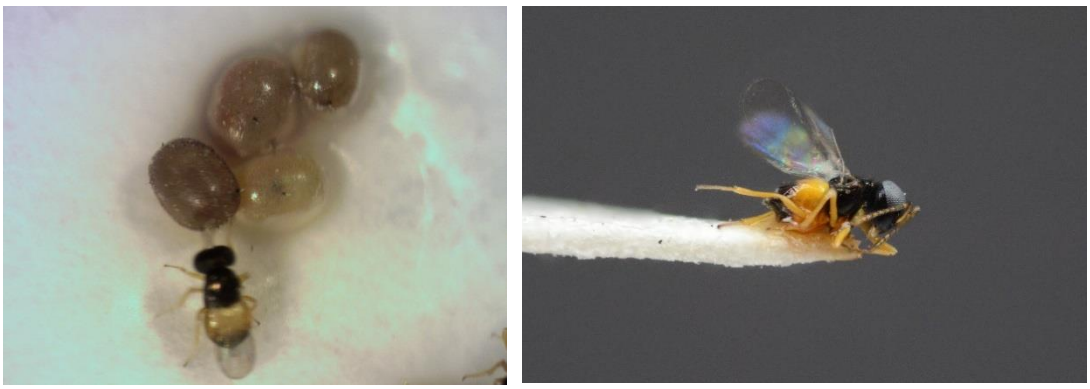

Fig. 1-2. Dorsal view of a live female *Ooencyrtus mirus* on *Bagrada hilaris* eggs (left) and lateral view of point-mounted, dead female (right). Photos by Ian Wright, 2017.

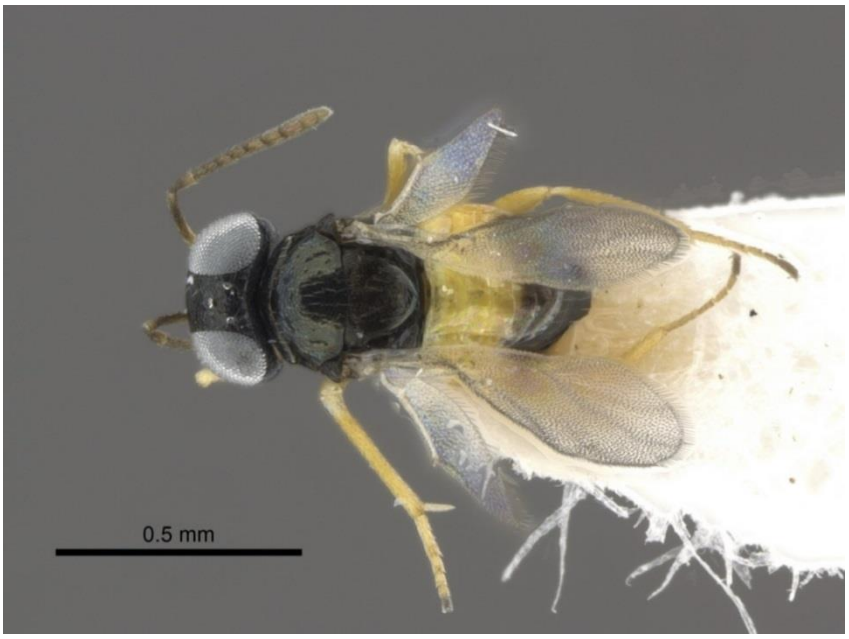

Fig. 1-3. *Ooencyrtus mirus* female, dorsal view; body length 0.83 mm. Photo by Rochelle Hoey-Chamberlain, April 2017.

Fig.1-4. Male *Ooencyrtus mirus*. Note hairs on antennae. Photo by Ian Wright, 2017.

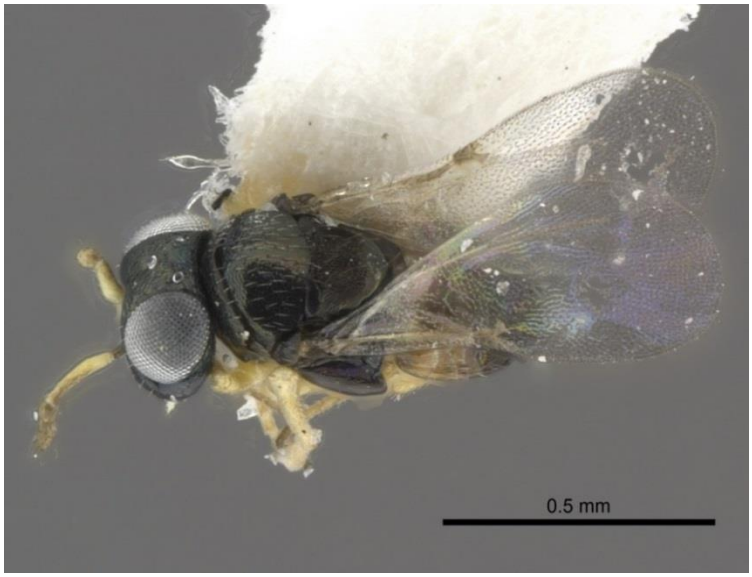

Fig. 1-5. *Ooencyrtus mirus* male, dorso-lateral view. Photo by Rochelle Hoey-Chamberlain, 2017.

Observations showed the behavior to be different between the sexes as well, with the males walking faster and moving their antennae faster. At normal rearing temperatures in the lab (23-26°C), males are rare, comprising only 1-3% of the

population. The females produce offspring without males. The males seem to be an artefact of when the species reproduced sexually in their evolutionary history.

As is typical for encyrtids, the eggs are inserted into the host egg, and part of the egg protrudes through the host chorion to the outside, serving a respiratory purpose (Maple 1937). The pedicels are easily visible under a stereomicroscope, enabling easy counting of the number of parasitoid eggs that have been laid.

Biological control

Biological control involves using live individuals of one species to manage a pest species. For herbivorous insect pests, hymenopteran parasitoids often are used as biocontrol agents because they can fly to search for prey and, compared to predators or tachinid fly parasitoids, they tend to be more host specific. Host specificity is important to avoid depleting populations of native insects or natural enemies of the pest. Egg parasitoids are especially desirable because they kill the hosts before the host can feed on and damage the crop plant (Peri et al. 2011).

Biological control agents can be used in three different ways, referred to as classical, augmentative and conservation biocontrol. Classical biocontrol involves finding out the origin of an exotic pest and importing one or more of its natural enemies with the intent for them to establish self-sustaining populations in the new region to keep the pest insect in check without further human effort. Augmentative control involves mass-rearing agents and releasing them in the field as needed. Conservation biocontrol involves creating environments that encourage beneficial insects, such as growing nectar-bearing flowers throughout the crop season to attract and provide food for beneficial

insects such as parasitoids. My research focuses on evaluating *O. mirus* as a potential classical or augmentative control agent of *B. hilaris*.

Biological control has been shown to be a good economic investment. Including unsuccessful attempts, classical biological control has yielded high returns on investment per dollar spent, ranging from 23:1 for control of weeds in Australia (McFayden 2008), to $364-729:1 for control of insect herbivores in urban trees in California (Tim Paine, University of California, Riverside, personal communication), with an estimated 30:1 return on average for all classical biological control programs as of 1995 (Hokkanen & Lynch 1995). Increasing regulations regarding screening for release have increased the cost since 1995, but since a successful agent will continue operating indefinitely, the return should prove to be worth the investment.

Part I. Reproductive Biology of *Ooencyrtus mirus*

Chapter 2

Effects of parasitoid age, host egg age, and host egg freezing on reproductive success of *Ooencyrtus mirus* on *Bagrada hilaris* eggs

Abstract

Bagrada hilaris (Burmeister) (Heteroptera: Pentatomidae) is a serious pest on brassica crops in many regions throughout the world. As part of our efforts to enhance biological control, we have been studying an egg parasitoid that was collected from *B. hilaris* eggs found on brassica plant debris in Pakistan. This species has recently been described as *Ooencyrtus mirus* Triapitsyn & Power (Hymenoptera: Encyrtidae). A major component of rearing biological control agents is understanding the relationship among host egg age, parasitoid age and reproductive success. To this end, we used a factorial design to evaluate all combinations of host egg ages 0-5 days and parasitoid ages 0-11 days. The results showed that the best combinations are 0- to 1-day-old host eggs with 3- to 11-day-old parasitoids. A further study using frozen host eggs showed that *O. mirus* can reproduce as successfully on frozen *B. hilaris* eggs as on fresh ones.

Introduction

One aspect of evaluating a potential biological control agent is to determine whether and how it can be mass-reared for field release. The Perring lab has reared enough *O. mirus* for research purposes for more than four years with no problems. The objective of this age study was to determine the relationship among *B. hilaris* host egg

age, parasitoid age, and the reproductive success resulting from the various combinations of these two parameters. Furthermore, we explored the reproductive success of *O. mirus* on frozen host eggs in comparison to fresh eggs.

Materials and methods

Host insect rearing

Painted bugs were reared in BugDorm® tents (BugDorm-2120, MegaView Science Col, Taiwan) in greenhouses set at 24-31°C. The insects were provided broccoli, canola and mizuna seedlings grown in 10 × 10 cm plastic pots as needed. Mating pairs of adults were brought into the lab weekly and placed in 15 cm diameter × 6.5 cm high, plastic Durphy® boxes (Durphy Packaging Co., Pennsylvania, USA) with two 2.5 cm diameter screen windows cut into the side for aeration. The insect boxes were placed in an insectary room set at 30 ± 1°C, 40-50% RH and 14:10 L:D. There the bugs were fed fresh, organic broccoli florets daily. The eggs from these adults were collected daily and glued to cardstock (1.27 × 4.23 cm) using a very thin layer of Elmer's® glue. These egg cards were used for *O. mirus* colony maintenance and for experiments.

Parasitoid rearing

Ooencyrtus mirus were reared in the quarantine facility at the University of California Riverside (UCR) in a room set at 23°C, with natural light from a window. The colonies were in upside-down ClickClack® containers, 10 cm diameter × 12.5 cm tall, with holes made in the side and top (Fig. 2-1). These holes were plugged with rubber stoppers allowing the addition and removal of insects. The bottom of the rearing container was sealed to the container with Parafilm® to prevent parasitoids from

escaping. Honey was streaked on the inside of the cage as needed to feed the adults. A very fine streak of the honey was required to prevent the small adults from becoming stuck while trying to feed. This was accomplished by using a streaking tool made of a single cat whisker which had the right taper and rigidity to provide the proper honey streak. The whisker was glued to the end of a thin wooden dowel that served as a handle.

Fresh bagrada bug eggs glued to 1.27 × 4.23 cm card stock were added to each cage for 24 hours and then removed and placed into 9.4 cm long × 2.2 cm diameter glass vials. The vials were kept on ridged plastic trays (Nordic Ware® 20.3 x 24.7 cm microwave bacon trays) until the *O. mirus* adults emerged about 3 weeks later. Some of the wasps that emerged from these vials were used for experiments and the rest were returned to the rearing cage.

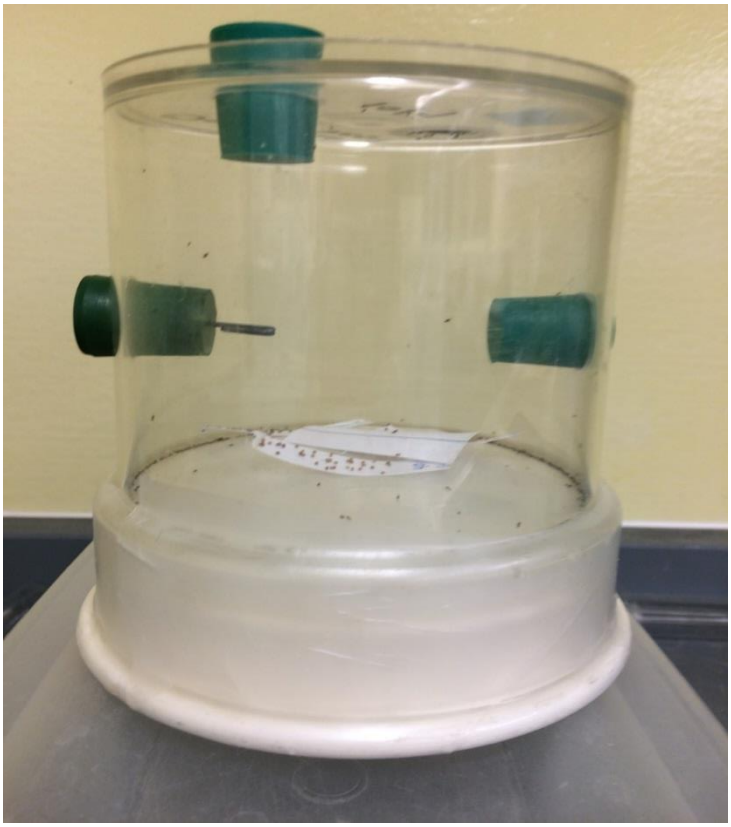

Figure 2-1. *Ooencyrtus mirus* colony cage with cards of *Bagrada hilaris* eggs.

Experimental Procedures

Creating parasitoids adults of different ages

Eleven days before the start of the experiment, a 9 cm long × 2 cm diameter glass vial was labeled with the number "11," representing the age in days these adults would be at the start of the experiment. The wasps that emerged from the rearing colony vials that day were added to the vial and the opening was plugged with a cotton ball. This was repeated for 11 more days, labeling the daily vial with the next smaller number each day through day 0. Honey was streaked in the vial as needed to provide food for the adults.

The wasps from all age groups were not exposed to host eggs until the start of the experiment on "day 0." These procedures were repeated for each of seven replicates.

Preparation of bug eggs of different ages

Five days before the start of the experiment ("day 0"), 5 bagrada bug eggs were glued to each of 13 cards (1.27 x 2.11 cm white card stock) above the printed numbers 1-2-3-4-5, and each card was labeled with the date in pencil. Once the glue was dry, all the egg cards were placed in a 13.5 cm diameter plastic Petri dish with white paper towel in the bottom, labeled with the date. A lid was then placed on the Petri dish to protect the eggs. A similar procedure was used for the other host egg ages, beginning the respective number of days ahead of the experimental start day. The Petri dishes of eggs and vials of wasps were kept in a growth chamber at 26°C.

Age experimental setup

All host eggs within the same replicate were started on the same day. On the start day for each of the seven replicates, one *O. mirus* adult and a card of 5 *B. hilaris* eggs were placed into a 9 cm long × 1 cm diameter glass vial, with one vial for each bug egg age (0-5) and parasitoid age (0-11) combination. Each vial was plugged with a cotton ball to prevent parasitoid escape while still allowing airflow. The 72 bug-egg/parasitoid age combinations in addition to 6 controls with no parasitoid resulted in a total of 78 experimental units per replicate arranged in a 6 × 13 factorial design. To assist in the management of the large number of combinations, vials were placed on ridged plastic trays, with each tray holding 13 vials of the same bug egg age. Each vial on the tray contained a wasp of one of the 12 wasp ages and the final vial was a no-wasp control

(Fig. 2-2). Each replicate had 6 of these trays to account for the 6 bug ages. The trays were placed in the growth chamber at 26 ± 0.5°C and 50% humidity for 24 hours, after which the wasps were removed. The vials of eggs then were returned to the growth chamber to allow for the development of bugs or parasitoids.

Figure 2-2. Two replicates of the factorial age experiment. Each tray has one age of *Bagrada hilaris* eggs and 12 different ages of *Ooencyrtus mirus* adult females, plus a control vial with no wasp. Each stack has one tray for each of six host egg ages (0-5 days). An empty tray was placed on top of each stack to keep the light even for all trays.

Data collection

As with other encyrtids, each *O. mirus* egg has a pedicel that protrudes through the host chorion. The number of pedicels on each egg was counted under a dissecting

scope and recorded. Each day, the number of *B. hilaris* nymphs or *O. mirus* adults that emerged from each vial and the sex of the latter were recorded, and the emerged insects were removed. The host eggs were examined (under a microscope if necessary) to determine from which egg the insect emerged so that each host egg could be tracked individually. After 28 days, the eggs were removed from the growth chamber and non-emerged eggs were dissected with fine forceps and a probe. The contents were recorded as "dead bug," "dead wasp," or "no insect." For the dead wasps, the life stage (larva, pupa or adult) was recorded.

Procedures for testing the viability of frozen host eggs for parasitization

A 10 cm diameter × 1.5 cm high Petri dish containing 40+ newly laid (0-day-old) *B. hilaris* eggs was placed in a freezer at -25.6 ± 5°C. After 24 hours, five of these frozen and now 1-day-old bagrada bug eggs were glued to each of eight cards (1.27 × 2.11 cm white card stock) above the printed numbers 1-2-3-4-5. To compare with fresh eggs, seven similar cards were assembled with 1-day-old *B. hilaris* eggs, and eight cards were created with 0-day-old *B. hilaris* eggs. Each card was placed in a labeled, 9 cm long × 1 cm diameter glass vial with a streak of honey. To each vial, a 3-day-old *O. mirus* adult female was added, and the vial was plugged with cotton. The wasp was removed after 24 hours. Data collection was the same as above except that the non-emerged eggs were not dissected.

In a separate study, two cards of five *Halyomorpha halys* Stål eggs were placed in the freezer at the same temperature as was done for *B. hilaris*. Seven days later, each card was placed in its own vial. The number of pedicels, number of *O. mirus* adults emerged

and developmental time were compared with those of fresh, 1-day-old *H. halys* eggs. As a control, a 3-day-old wasp was added to each of three fresh egg cards at the same time as the wasp was added to the frozen eggs. Additional controls were set up on different days: a 3-day-old wasp was added to each of two fresh egg cards on 7, 11, 14, 18, 21 and 26 days prior to testing the frozen eggs.

Statistical Analyses

For the age study, three of the responding variables had a binomial distribution: bug nymph emergence, wasp emergence per egg, and wasp survival per parasitized egg. These were analyzed using a generalized linear mixed model (glmer) fit by maximum likelihood (Laplace Approximation) for a binomial distribution with mixed effects, with wasp age and bug age as fixed effects and replication as a random effect. In addition, for bug nymphal emergence per host egg at each host egg age, the control group (no wasp) was compared to the test group by making a contingency table and comparing the groups with a Pearson's χ^2 test for homogeneity. Non-binomial data (number of pedicels per egg and developmental time) were analyzed using a linear mixed-effects model (lmer).

For the *B. hilaris* frozen egg study, no more than one wasp emerged from each egg, so the emergence data was binomial. These data were arranged in contingency tables, one table for each pair (control + 0-day-old, control + 1-day-old, and 0-day-old + 1-day-old eggs) and analyzed by Fisher's Exact Test for Count Data. Both the number of pedicels per host egg and the developmental times lacked a normal distribution for any of the three groups (0-day-old, 1-day-old, and frozen) but the groups had homogeneous variances. The data thus were analyzed with two-sample permutation t-tests. For the *H.*

halys study, due to the small sample size of frozen eggs, only the developmental time data was analyzed, using a two-sample permutation t-test. All analyses were performed in R (R Core Team 2019). The statistical tests are summarized in Table 2-1.

Table 2-1. Statistical tests employed for the age and frozen egg studies of the parasitoid *Ooencyrtus mirus* and hosts *Bagrada hilaris* and *Halyomorpha halys*.

Summary of statistical tests for age and frozen egg studies			
study	**responding variable**	**statistical test**	**R test**
age	number of pedicels per egg	linear mixed-effects model	lmer
	developmental time	linear mixed-effects model	lmer
	parasitized eggs	generalized linear mixed model	glmer
	B. hilaris emergence	generalized linear mixed model	glmer
	O. mirus emergence	generalized linear mixed model	glmer
	O. mirus survival	generalized linear mixed model	glmer
	B. hilaris emergence in test vs control group	Pearson's $\chi 2$	chisq.test
frozen egg	*O. mirus* emergence	Fisher Exact	fisher.test
	parasitized eggs	Fisher Exact	fisher.test
	number of pedicels per egg	2-sample permutation t	perm.t.test
	developmental time	2-sample permutation t	perm.t.test

Results

Host age, parasitoid age, and parasitoid reproductive success

From the 2494 host eggs exposed to a female *O. mirus*, a total of 1721 *O. mirus* adults emerged from 1713 host eggs; 8 eggs had two wasps emerge rather than just one. *Bagrada hilaris* nymphs emerged over the first 5 days of each replicate, and then no insects emerged for several days until the adult parasitoids began emerging on day 14. Egg chorion from which a bug emerged looked translucent and colorless, and the top

resembled a lid connected by a hinge (Fig. 2-3 A). If a wasp had emerged, the host egg had a large, irregular hole at a random location (Fig.2-3 B). Brown discs of frass lay inside and scattered on the card outside these parasitized host eggs. The *O. mirus* pedicels (Fig. 2.3 B), having a black base and white tip, could be seen protruding from the chorion of parasitized eggs.

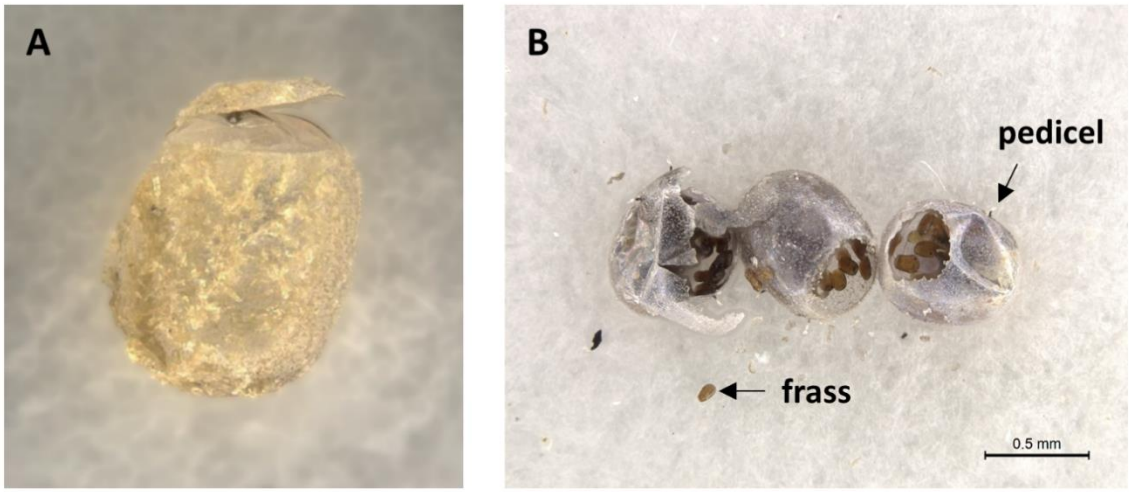

Fig. 2-3 A. *Bagrada hilaris* egg from which a nymph emerged. **B**. Three *B. hilaris* eggs from which an *Ooencyrtus mirus* adult emerged. Photos by Luke Kresslein.

Pedicels

On individual host eggs, the number of pedicels ranged from 0-6, with one being the most common number of pedicels (Fig. 2-4). The mean proportion of eggs laid by *O. mirus* per 5 host eggs ranged from 0.80 on 5-day-old host eggs to 0.95 on 2-day-old host eggs (Fig. 2-5 A). Among host egg ages 0-4, the mean proportion of pedicels laid per egg by *O. mirus* did not differ significantly ($P > 0.85$). Five-day-old eggs, on the other hand, had significantly fewer pedicels than 0 to 4-day-old eggs ($P < 0.05$), indicating that *O. mirus* females preferred the younger eggs. Across wasp ages, the mean proportion of

pedicels per egg increased significantly from 0-1, 1-2, and 2-3 days. From 3 to 11 days of age, the proportion did not differ significantly ($P > 0.05$) except that 6 and 11-day-old wasps laid significantly fewer eggs than did 8-day-old wasps ($P < 0.05$) (Figure 2-5 B).

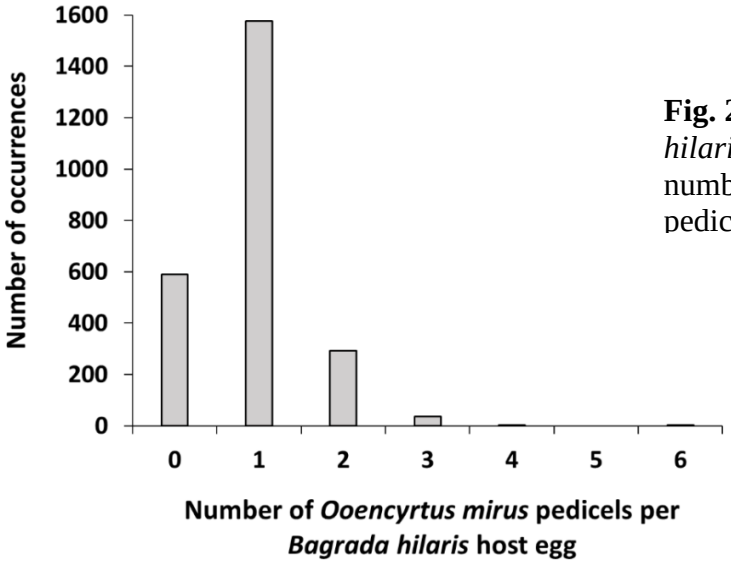

Fig. 2-4. Number of *Bagrada hilaris* host eggs with a given number of *Ooencyrtus mirus* pedicels.

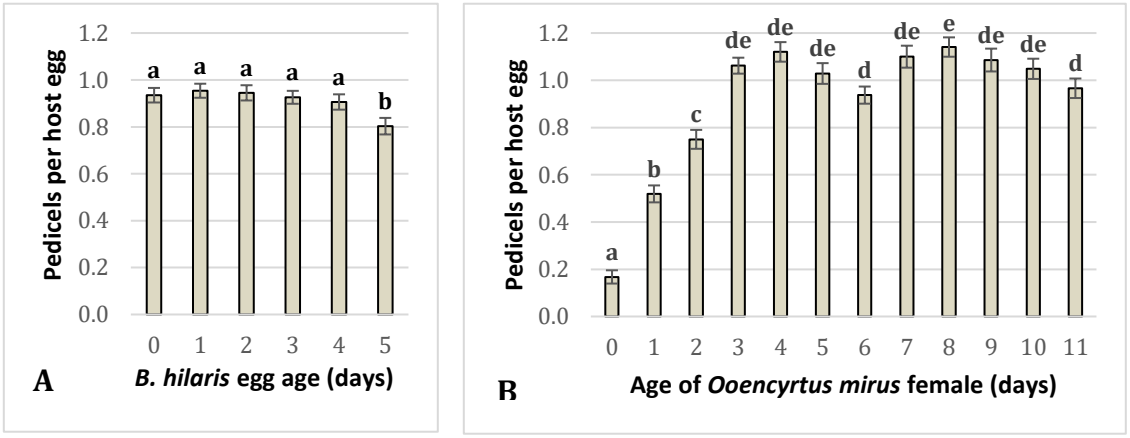

Fig. 2-5. Pedicels. **A**. Mean proportion of *Ooencyrtus mirus* pedicels per *Bagrada hilaris* host eggs according to *B. hilaris* egg age. **B**. Mean proportion of *O. mirus* pedicels per *B. hilaris* host eggs according to the age of the ovipositing *O. mirus* parent.

Emerged *B. hilaris* nymphs

Among *B. hilaris* eggs exposed to *O. mirus* adults, the mean proportion of *B. hilaris* nymphs that emerged was significantly higher in 5-day-old eggs than in the younger egg ages (Fig. 2-6 A, $P \leq 0.012$). The mean proportion of live *B. hilaris* nymphs that emerged did not differ significantly among bug egg ages in the vials that had no parasitoids (control) (Fig. 2-6 B, $P > 0.05$). For each host egg age, a significantly greater mean proportion of bugs emerged per egg in the control group than in the test group (Table 2-2, $P < 0.001$). The mean proportion of *B. hilaris* nymphs that emerged from eggs exposed to 0-day-old *O. mirus* did not differ significantly from the *O. mirus*-free control (Fig. 6B, $P = 0.97$). Compared to these two groups, significantly fewer *B. hilaris* emerged from eggs exposed to wasps aged 1-11 days ($P \leq 0.0001$). Eggs exposed to 1-day-old *O. mirus* yielded significantly more *B. hilaris* nymphs than eggs exposed to 3-11-day-old *O. mirus* ($P < 0.001$). Significantly more *B. hilaris* emerged from eggs exposed to 2-day-old wasps than from those exposed to wasps aged 3, 4 and 6-10 days ($P < 0.05$). Eggs exposed to wasps aged 3-11 days had the lowest *B. hilaris* emergence and did not differ significantly from each other ($P > 0.15$). No nymph emerged from any egg with a pedicel, regardless of whether a parasitoid emerged or not.

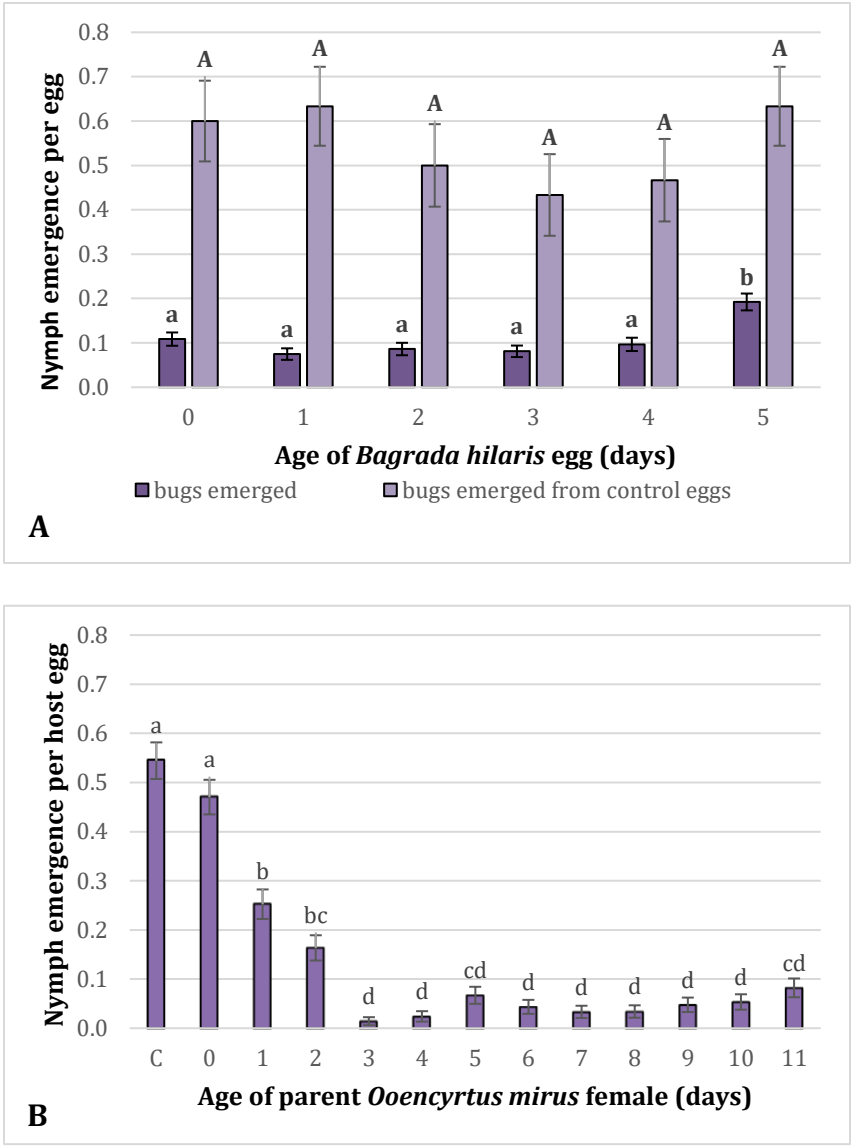

Fig. 2-6. *Bagrada hilaris* nymph emergence. **A**. Mean proportion of eggs from which a *B. hilaris* nymph emerged at each *B. hilaris* egg age. The darker bars represent eggs that were exposed to *Ooencytus mirus*; the lighter bars represent eggs that were not exposed to parasitoids. **B**. Mean proportion of eggs from which a *B. hilaris* nymph emerged according to the age of the ovipositing *O. mirus* female. Bars sharing the same letter do not differ significantly.

Table 2-2. Comparison of *Bagrada hilaris* nymph emergence from eggs exposed to an *Ooencyrtus mirus* parasitoid vs. eggs not exposed *O. mirus* (control) with Pearson's χ^2 test for homogeneity.

***Bagrada hilaris* egg age (days)**	**group**	**Emerged *Bagrada hilaris* nymphs**		**survival**	
		yes	**no**	**proportion**	***P***
0	parasitized	45	370	0.11	< 0.001
	control	18	12	0.60	
1	parasitized	31	384	0.07	< 0.001
	control	19	11	0.63	
2	parasitized	36	382	0.09	< 0.001
	control	15	15	0.50	
3	parasitized	34	386	0.08	< 0.001
	control	13	17	0.43	
4	parasitized	40	375	0.10	< 0.001
	control	14	16	0.47	
5	parasitized	79	332	0.19	< 0.001
	control	19	11	0.63	

Emerged *O. mirus* adults

The overall mean proportion of parasitoids emerged per host egg was 0.596, with a standard error of 0.010 (N = 2494). *Ooencyrtus mirus* offspring emergence per adult female was significantly higher from 0- and 1-day-old host eggs than from 3- to 5-day-old eggs (Fig. 2-7 A, $P < 0.003$). Although emergence from 2-day-old hosts did not differ significantly than emergence from 0-day-old hosts, it appears to be part of a downward trend in *O. mirus* emergence with increasing host age. Parasitoid emergence did not differ significantly among eggs aged 2-4 days ($P > 0.60$), but significantly fewer *O. mirus* emerged from 5-day-old eggs than from all the younger ages ($P < 0.01$).

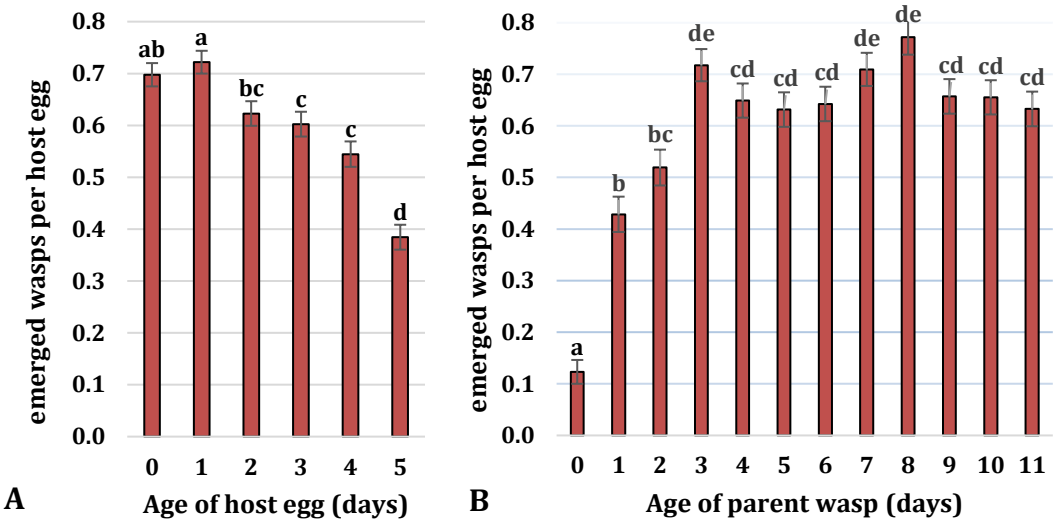

Fig. 2-7. Wasp emergence. **A**. Mean proportion of emerged *Ooencyrtus mirus* adults per *Bagrada hilaris* host egg, according to *B. hilaris* egg age. **B**. Mean proportion of emerged *Ooencyrtus mirus* adults per *Bagrada hilaris* host egg, according to age of the ovipositing *O. mirus* female. Bars sharing the same letter do not differ significantly.

The proportion of *O. mirus* offspring per host egg was significantly lower from the newly emerged (age 0) *O. mirus* adults than from the other ages (Fig. 2-7 B, $P < 0.0001$). Significantly more offspring emerged when the parent wasps were 1 and 2 days old compared to those 0 days old (Fig. 2-7 B, $P < 0.0001$), and 3-day-old parents produced significantly more offspring than 0- to 2-day-olds ($P < 0.001$). The *O. mirus* adult females reached full oviposition capacity at 3 days of age, and then emergence did not differ significantly among wasps 3-11 days old. Emergence from parasitoids aged 4-6 and 9-11 days did not differ significantly from that of 2-day-old wasps. The proportions of emergence for all the combinations of host egg age and parasitoid age are

given in a heat map in Figure 2-8. The combination with the highest emergence (without statistical significance) was 0-day-old *B. hilaris* eggs with 8-day-old *O. mirus*.

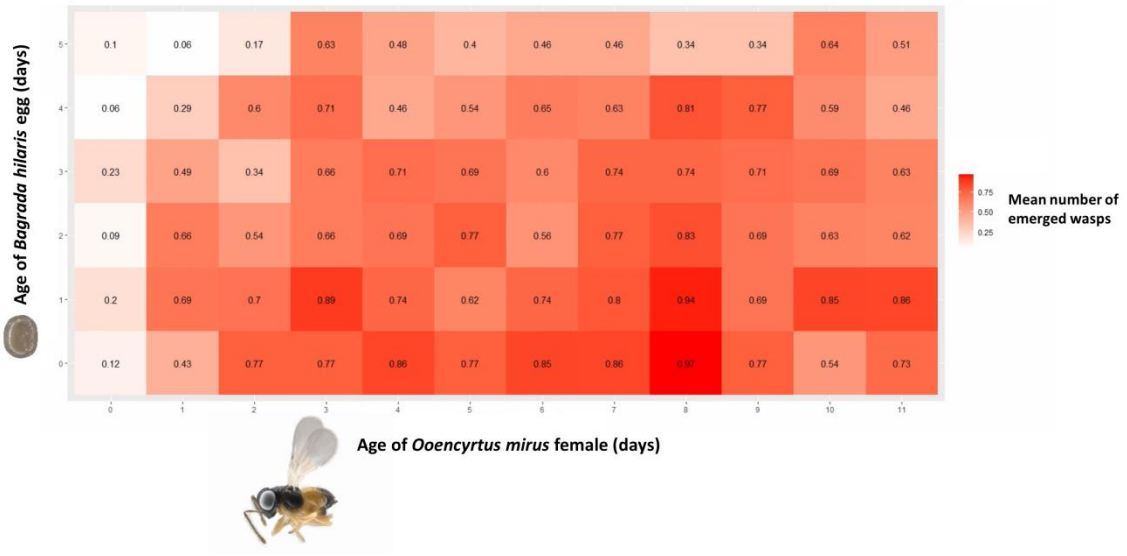

Fig. 2-8. Heat map of emerged wasps from a factorial experiment of host age and wasp age. The darker the square, the higher the mean emergence, with the highest of 0.97 by 8-day-old *O. mirus* parasitizing 0-day-old *B. hilaris* eggs.

Developmental time of immature *O. mirus*

The overall mean developmental time of immature *O. mirus* was 15.0 days, with a standard error of 0.03 (N = 1483). The developmental time did not differ significantly ($P > 0.08$) among host egg ages 0-4, but was significantly longer in 5-day-old host eggs (Fig. 2-9 A, $P < 0.0001$). Among adult *O. mirus* ages, developmental time was significantly greater in the offspring of 0-day-old wasps than in the offspring of 2- to 3- and 5- to 7-day-old wasps (Fig. 2-9 B, $P < 0.05$). The development of offspring of 11-day-old wasps took significantly longer than that of the offspring of 2, 3 and 5-day-old *O. mirus* ($P < 0.04$). Developmental time did not differ significantly among offspring of adults aged 2-10 days ($P > 0.10$).

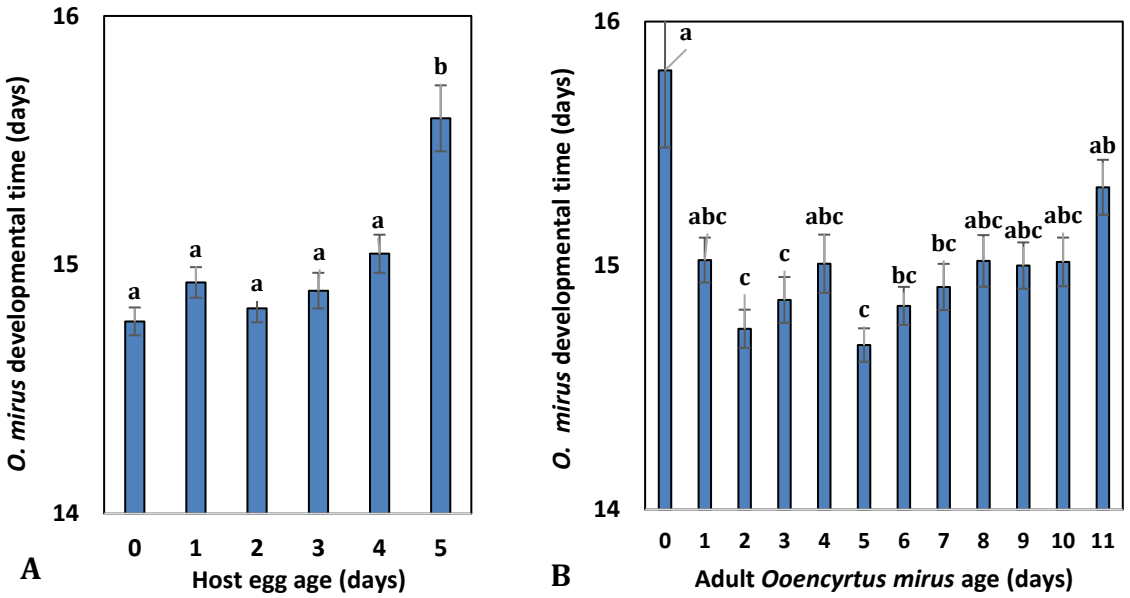

Fig. 2-9. Developmental time by **A**. *Bagrada hilaris* host egg age and **B**. *Ooencyrtus mirus* age.

Survival of *O. mirus* immatures

Ooencyrtus mirus survival was highest in 0- to 1-day-old host eggs compared to other ages ($P < 0.003$), followed by 2- to 4-day-old eggs (Fig. 2-10 A, $P < 0.01$). Survival was significantly lower in 5-day-old eggs. The age of *O. mirus* parents, however, had no influence on the survival of offspring (Fig. 2-10 B, $P > 0.86$). There were 8 instances where two *O. mirus* emerged from one egg, and in those cases, we increased the N for parasitized eggs by 1. The overall proportion of survival per parasitized egg was 0.77 with a standard error of 0.01 (N = 1905).

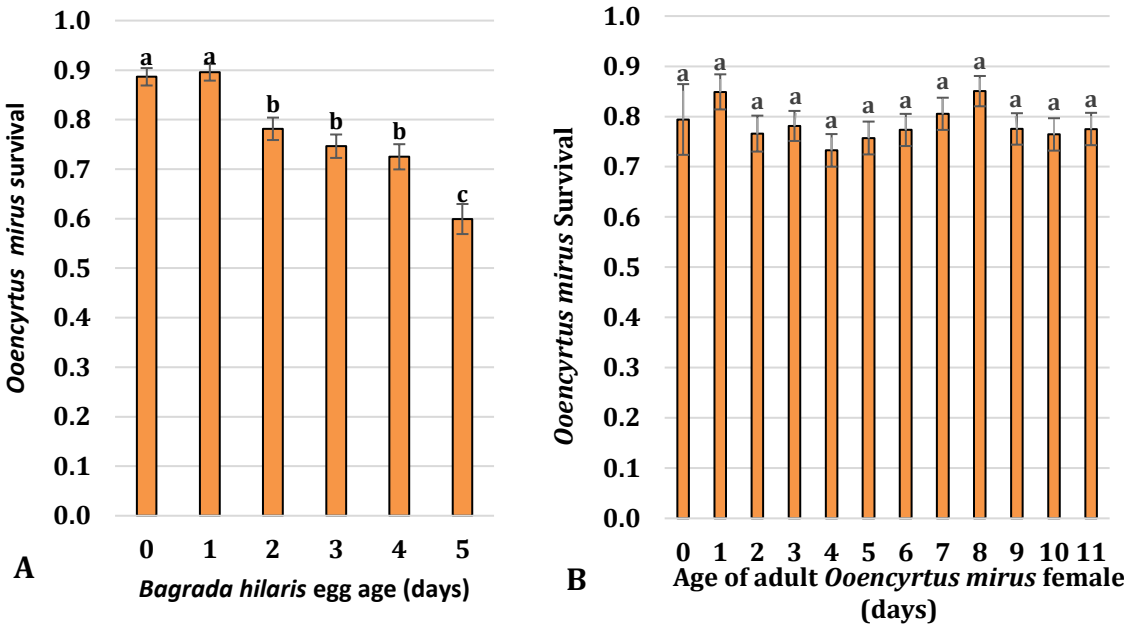

Fig. 2-10. Survival of *Ooencyrtus mirus* eggs. **A**. Proportion of *Bagrada hilaris* eggs parasitized by *Ooencyrtus mirus* from which adult *O. mirus* emerged, according to host egg age. **B**. Proportion of *Bagrada hilaris* eggs parasitized by *Ooencyrtus mirus* from which adult *O. mirus* emerged, according to age of the ovipositing *O. mirus* females. Bars sharing the same letter do not differ significantly.

Frozen egg study

When the reproduction of *O. mirus* on 0- and 1-day-old fresh *B. hilaris* eggs and frozen eggs were compared, no differences were seen in the number of pedicels per egg, percent of parasitized eggs, percent of host eggs from which wasps emerged, or developmental times (Table 2-3, $P > 0.40$ for all four responding variables). The developmental time ranges were 15-17 days for all treatments.

Table 2-3. *Ooencyrtus mirus* reproduction on 0- and 1-day-old fresh *Bagrada hilaris* eggs and frozen eggs. Data presented (± standard error of the mean) includes number of eggs tested (N), total number of pedicels (No. ped), mean number of pedicels per egg (No. ped/egg), percent of eggs that were parasitized (% par), percent of host eggs from which *Ooencyrtus mirus* emerged (Emerged), mean developmental time in days (Dev. Time), and range of developmental time in days (Dev. Range). Within each variable, means followed by the same letter are not significantly different ($P > 0.04$)

B. hilaris egg group	N	No. ped	No. ped/egg	% par	Emerged	Dev. Time	Dev. Range
0-day-old	40	39	0.975(±0.056)a	92.5 (±0.042)a	82.5 (±0.061)a	16.09 (±0.08)a	15-17
1-day-old	35	36	1.029 (±0.050)a	97.1 (±0.029)a	82.9 (±0.065)a	16.10 (±0.08)a	15-17
frozen	40	40	1.000 (±0.080)a	90.0 (±0.048)a	87.5 (±0.053)a	16.06 (±0.06)a	15-17

In *H. halys* host eggs, however, developmental time was significantly greater in frozen than in fresh eggs ($P < 0.001$) (Table 2-4). The development time range was 15-19 days for fresh eggs but 25-27 days for frozen eggs. Survival (% of pedicels that resulted in live wasps emerging) was 100% in the frozen eggs but only 73% in the fresh eggs. The contingency tables for the frozen vs. fresh egg comparisons for both *B. hilaris* and *H. halys* may be found in Figs. 2-5 and 2-6 (Appendix A).

Table 2-4. *Ooencyrtus mirus* reproduction on frozen vs. fresh *Halymorpha halys* eggs. No. = number; Peds = pedicels; Dev. = Developmental; std. = standard.

H. halys Egg Group	No. *B. hilaris* Eggs	No. Peds	Mean No. Peds per Egg	Mean No. Peds per Par. Egg	Mean No. Peds Laid per Parent *O. mirus*	No. Emerged *O. mirus*	% Success per Pedicel	Mean Dev. Time (days)	Dev. Time std. error	Dev. Time Range (days)
frozen	20	5	0.25	2.25	2.5	5	100	25.8A	0.37	25-27
fresh	150	33	0.22	1.86	2.2	24	73	16.0B	0.24	15-19

Discussion

Of the ages studied, the best for rearing *O. mirus* were 0- to 1-day-old *B. hilaris* eggs and 3- to 10-day-old *O. mirus*. These ages have a combination of a high number of pedicels, low *B. hilaris* emergence, high wasp emergence, short developmental time and high survival of immature parasitoids. The least successful host age was the oldest. These 5-day-old eggs had fewer pedicels, more nymphs emerging, the lowest *O. mirus* emergence, longer *O. mirus* developmental time, and the lowest *O. mirus* survival. Lower *O. mirus* emergence from 5-day-old *B. hilaris* eggs was due to both lower oviposition (as indicated by the number of pedicels) and lower survival on these older eggs. However, the variability in *O. mirus* emergence in 2- to 4-day-old host eggs was due to survival alone, not differences in oviposition. Low *O. mirus* emergence from young wasps (0-2 days old) was due only to lower oviposition and not to survival of the eggs that were laid by the young wasps**.** The least successful parasitoid age was 0 days.

The absence of nymph emergence from any parasitized eggs is consistent with our previous study of this species on both *B. hilaris* and alternate hosts (Chapters 2,4,5,6). This 100% mortality of host eggs combined with the current result of significantly lower survival rates of *B. hilaris* when exposed to *O. mirus* indicates that *O. mirus* may increase its biological control impact by killing the host even when parasitoid development is unsuccessful (Chapters 2,4,5,6; Abram et al. 2016).

Although some egg parasitoids show no difference in success on different age hosts (e.g., trichogrammatids on *Pieris rapae* (L.) (Godin and Boivin 2000)), most are more successful on young or medium age host eggs (Table 2-5). Of the 32 host-

parasitoid systems reported in Table 2-5, 13 showed the highest success on host eggs 2 days old or less, 3 on both young and medium age eggs, 6 on medium aged eggs, 6 on all but the oldest eggs, 3 with no difference among host ages and one on which success changed from high on young eggs to low on medium eggs to high on older eggs (Marston and Ertle 1969). *Ooencyrtus mirus* fits into the first group, with the highest emergence on *B. hilaris* eggs aged 0-1 days (Fig. 2-7 A).

Considering parasitoid age, different parasitoid/host systems vary considerably. Of the 11 host-parasitoid pairs reported in Table 2-6, success was greatest for 1-day-old wasps in four of the systems and for 2-day-old wasps in two of them. One pair each had greatest success at 3 and 4 days of age. Two parasitoids were more successful at 1 and 5 days than at 10 days of age, and one parasitoid increased its parasitism until 13 days and then declined. *Ooencyrtus mirus* increased its parasitism until day 3 and then stayed steady through day 11.

Only a few studies considered host egg age and parasitoid age at the same time. In the two factorial studies reported in Table 2-7, the parasitoids were like *O. mirus*, succeeding more on the youngest two host ages tested (Pizzol et al. 2012; Tunca et al. 2016). However, *Trichogramma cacoeciae* Marchal reached its highest success a day earlier and *Ooencyrtus pityocampae* (Mercet) two days later than *O. mirus*.

Frozen *B. hilaris* eggs were as good for reproduction of *O. mirus* as fresh eggs in all respects. This does not imply, however, that frozen eggs would be effective for sentinel cards in the field, where the eggs may be subjected to higher temperatures and have more time to decompose. This would have to be determined for the particular

region in which the eggs are being used. For rearing *O. mirus* in the lab, however, freezing the eggs might be a good option for ensuring that eggs are available when needed and to save on labor. Since this study used eggs frozen for 24 hours, additional tests should be done to determine the impact of longer term freezing on *O. mirus* success. Unlike in *B. hilaris* eggs, *O. mirus* development takes significantly longer on *H. halys* eggs.

Conclusion

O. mirus can be reared most efficiently on *B. hilaris* eggs that are 0-1 days old using adult *O. mirus* that are 3-10 days old. The parasitoid can be reared just as well on *B. hilaris* eggs that were frozen within 24 hours of emergence as on 0- or 1-day-old fresh eggs.

Table 2-5: Effect of host age on reproductive success of egg parasitoid wasps (in alphabetical order by parasitoid family)**.** Col. = Coleoptera; Het. = Heteroptera; Lep. = Lepidoptera; d = days; h = hours.

Authors	Parasitoid(s) (Hymenoptera)	Host	Host ages tested	Results or highest emergence or parasitism
Nechols et al. 1989	*Ooencyrtus anasae* (Ashmead) (Encyrtidae), *Ooencyrtus* sp. 'dark form', *Gryon pennsylvanicum* (Ashmead)	*Anasa tristis* DeGeer (Het.: Coreidae)	1, 2, 3, 4, 5, 6, 7, 8, & 9 d	Fewer progeny in the oldest eggs
Hofstetter & Raffa 1998	*Ooencyrtus kuvanae* (Howard)	*Lymantria dispar* (L.) (Lep.: Lymantriidae)	3,5,8,12 weeks	3,5 & 8 >12 weeks
Takasu & Hirose 1993	*Ooencyrtus nezarae* Ishii	*Riptortus clavatus* (Thunberg) (Het.: Alydidae)	0,2,4,7 d	# eggs laid per host decreased with host age. Survival higher in 2- & 4- than in 7-day-old host egg.
Binazzi et al. 2013	*Ooencyrtus pityocampae* (Mercet)	*Leptoglossus occidentalis* Heidemann (Het.: Coreidae), *A.tristis*	<1; 2,3,4,5,6,7 d	No difference
Fedde 1982	*Ooencyrtus trinidadensis* Crawford	*Leptoglossus corculus* (Say)	0,1,2,3,4,5 d	Most successful on 2-3-day-old host eggs.
Lashomb et al. 1987	*Edovum puttleri* Grissell (Eulophidae)	*Leptinotarsa decemlineata* (Say) (Col: Chrysomelidae)	0-8 no choice; 0,2,4,6,8 choice; 1,2,3,4,5 choice	Parasitism greater in host eggs ≤ 2 days than in eggs older > 2 days.

Ruberson et al. 1987	*Edovum puttleri*	*Leptinotarsa decemlineata*	1-5 d	Oviposited less frequently and produced smaller offspring in progressively older hosts. Survival higher in 1–2 day old than in 3–4-day old hosts.
Leibee et al. 1980	*Anaphes diana* Girault *[=Patasson lameerei* Debauche] (Mymaridae)	*Sitona hispidulus* (F.) (Col.: Curculionidae)	13.5-169.5 h in 12 h increments	Optimum: 2-day-old eggs; range of effective parasitism = 1-3-day-old eggs
Jacob et al. 2006	*Stethynium* sp. (Mymaridae)	*Zygina* sp. (Homoptera: Cicadellidae)	1,2,3,5,7 (choice); 8 & 9 (no choice)	No difference among 1,2,3,5&7; similar oviposition rate but higher success in 8 than 9-day-old eggs
Orr et al. 1986	*Telenomus calvus* Johnson (Platygastridae)	*Podisus maculiventris* (Say) (Het.: Pentatomidae)		Pre-imaginal development highest in ≤ 12-hour-old eggs
Peñaflor et al. 2012	*Telenomus remus* Nixon	*Spodoptera frugiperda* (JE Smith) (Lep.: Noctuidae)	1,2,3 d	Wasp chose 1- & 2-day-old over 3-day-old eggs; emergence: 1>2>3-day-old eggs
Navasero & Oatman 1989	*Telenomus solitus*	*Trichoplusia ni* (Huebner) (Lep.: Noctuidae)	30, 36, 42, 48, 54, 60, and 72 h	42 hours
da Rocha et al. 2006	*Gryon gallardoi* Brethes (Scelionidae)	*Spartocera dentiventris* (Berg) (Het.: Coreidae)	2, 3, 4, 5, 6, 7, 8, 12 d	2; emergence decreased with increasing host age
Peverieri et al. 2013	*Gryon pennsylvanicum* (Ashmead)	*Leptoglossus occidentalis* Heidemann (Het.: Coreidae)	<0.5, 2,4,6,8 d	No difference

Sousa & Spence 2001	*Tiphodytes gerriphagus* Marchal (Scelionidae)	*Limnoporus dissortis* Drake & Harris (Het.: Gerridae)	0,1,2,3,4,5,6,7 d	0,1,2,3
Ohno 1987	*Trissolcus plautiae* (Watanabe) (Scelionidae)	*Plautia stali* Scott (Het.: Pentatomidae)	1, 2, 3, 4, 5, 6, 6.5, 7, & 7.5 d at 22°C; 1, 2, 3, 4, 5, 5.5 at 25°C	No difference in parasitoid emergence except lower in the oldest, near-hatching eggs
Godin & Boivin 2000	Trichogrammatidae spp.	*T. ni*, *Pieris rapae* (L.) (Lep.: Pieridae) and *Plutella xylostella* (L.) (Lep.: Plutellidae)	Young, medium, old	Young eggs of *T. ni*, young or medium eggs of *P. xylostella*, and no difference among *P. rapae* eggs
Amalin et al. 2005	*Ceratogramma etiennei* Delvare (Trichogrammatidae)	*Diaprepes abbreviatus* (L.) (Col.: Curculionidae)	1,2,3,4,5 d (choice test)	2-4 days old were most acceptable in lab, and 1-4 days old in greenhouse
Pak et al. 1986	*Trichogramma* spp. (Trichogrammatidae)	*Mamestra brassicae* L., (Lep.: Noctuidae), *Pieris brassicae* L. and *P. rapae* L.	0 vs. 2 d 1 vs. 3 d	No difference except lower oviposition in 3-day-old *M. brassicae*
Reznik & Vaghina 2007	*Trichogramma buesi* Voeg. & *Trichogramma principium* Sug. & Sor.	*Sitotroga cerealella* (Olivier) (Lep.: Gelechiidae)	1-2 vs. 5-6 d	1-2 d

Liu et al. 1998	*Trichogramma dendrolimi* Matsumura	*Ostrinia furnacalis* (Guenee) (Lep.: Pyralidae)	0-6 h 18-24 h 30–42 h 48–72 h (no choice)	"the proportion of wasps that successfully parasitized host eggs, the number of host eggs parasitized, and the rate of parasitization all decreased by >50%" from the 0-6 h to 18-24 h age eggs and continued to decline with increasing host age
Tunçbilek & Ayvaz 2003	*Trichogramma evanescens* Westwood	*Ephestia kuehniella* Zeller (Lep.: Pyralidae) and *S. cerealella*	0-6 h 0-24 h 48-72 h 72-96 h	On *E. kuehniella*, the proportions of both parasitized eggs and adult emergence decreased with age. On *S. cerealella*, both measures were highest on 48-72 h old eggs.
Lewis & Redlinger 1969	*Trichogramma evanescens*	*Cadra cautella* (Walker) (Lep.: Pyralidae)	12,24,36,48,60 & 72 h	93-96% mean parasitism through 48 h, then 86% at 60 h and 82% at 72 h. Sometimes killed host even 3-4 h before host emergence.
El Sharkawy 2011	*T. evanescens, T. brassicae* Bezdenko & *Trichogrammatoidea bactrae* Nagaraja	*Pectinophora gossypiella* Saund. (Lep.: Gelechiidae), *Agrotis ipsilon* Hufn. (Lep.: Noctuidae) & *S. cerealella*	0,1,2,3 d	Number of parasitized eggs higher in 0,1; decreased with increasing host age
Marston & Ertle 1969	*Trichogramma minutum* Riley	*T. ni*	<11 h, 27 h, Close to eclosion	Nearly 100% < 11hr, 15% at 27 hr, almost 95% just before eclosion.

Honda & Luck 2000	*Trichogramma platneri* Nagarkatti	*Amorbia cuneana* Walsingham (Lep.: Tortricidae), *Sabulodes aegrotata* (Gueneé) (Lep.: Geometridae)	1,5,7,9 d in *A. cuneana;* 1,3,5 d in *S. aegrotata*	1,5,7 equally successful; 9 lower fec., shorter tibia 3 days > 1 day; no emergence from 5 d host
Taylor & Stern 1971	*Trichogramma semifumatum* (Perkins)	*T. ni*		Younger host eggs

Table 2-6: Effect of parasitoid age on reproductive success of egg parasitoid wasps Col. = Coleoptera; Het. = Heteroptera; Lep. = Lepidoptera; d = days; h = hours.

Authors	**Parasitoid(s) (Hymenoptera)**	**Host**	**Parasitoid ages tested (days)**	**Results or highest emergence or parasitism rate**
Akman Gündüz & Gülel 2005	*Bracon hebetor* Say (Braconidae)	*Galleria mellonella* (L.) (Lep.: Pyralidae) and *E. kuehniella*	1, 5, 10	For both hosts, total progeny were higher at 1 & 5 than at 10 days of age
Nechols et al. 1989	*O. anasae* (Ashmead), *Gryon pennsylvanicum* (Ashmead) (Scelionidae) *O.* sp. 'light form'	*A. tristis*	0-35	Number of parasitoid eggs laid peaked at 1 day old for *G. pennsylvanicum*, 2 days for *O.* sp., and 3 days for *O. anasae*, and then declined from there. *O.* sp. was more variable than the others.
Hofstetter & Raffa 1998	*O. kuvanae*	*Lymantria dispar* L. (Lep.: Erebidae)	1,4,7	1-day-old parasitoids had more offpring than 4- & 7-day-old ones

Aung et al. 2010	*Ooencyrtus nezarae* Ishii	*Riptortus clavatus* Thunberg (Het.: Alydidae)	1-20	4 days old
Lashomb et al. 1987	*Edovum puttleri* (Eulophidae)	*L. decemlineata*	constant exposure to eggs from 3 days old till death	Parasitism increased with age of parasitoid till 13 days, then declined.
Orr et al. 1986	*Telenomus calvus* Johnson (Scelionidae)	*P. maculiventris*	1-6	Production of female progeny peaked on the 1st day after adult emergence, then declined steadily until day 6
Garcia et al. 2001	*Telenomus cordubensis* Vargas & Cabello (Scelionidae)	*E. kuehniella*	1,2,3,4,5,6	1
Amalin et al. 2005	*Ceratogramma etiennei* Delvare (Trichogrammatidae)	*D. abbreviatus*	1-10 (the same females were given host eggs for 10 days in a row)	2 days old

Table 2-7. Literature review of the effect of host age and parasitoid age combined on reproductive success of egg parasitoid wasps, determined by factorial designs. Col. = Coleoptera; Het. = Heteroptera; Lep. = Lepidoptera; d = days; h = hours.

Authors	**Parasitoid(s) (Hymenoptera)**	**Host**	**Host ages tested (days)**	**Parasitoid ages tested (days)**	**Highest emergence or parasitism rate**
Pizzol et al. 2012	*Trichogramma cacoeciae* Marchal	*Lobesia botrana* Denis and Schiffermüller (Lep.: Tortricidae)	1-2 vs. 3-4 d	1,2,3,4 d	1-2 (host) and 2,3,4 (wasp)
Power et al. (current study)	*Ooencyrtus mirus* Triapitsyn & Power	*Bagrada hilaris* (Het.: Pentatomidae)	0,1,2,3,4,5	0,1,2,3,4,5,6,7,8, 9,10,11	0-1 (host) and 3-11 (wasp)
Tunca et al. 2016	*O. pityocampae* (Mercet)	*Philosamia ricini* Danovan (Lep.: Saturniidae)	1-2 vs. 3-4 d	1,3,5 d	1-2 (host) and 5 (wasp)

Chapter 3

Life History of *Ooencyrtus mirus*

Abstract

Ooencyrtus mirus Triapitsyn & Power is a newly described parasitoid that was brought to the U.S. from Pakistan for evaluation as a potential biocontrol agent for *Bagrada hilaris* (Heteroptera: Pentatomidae). To study the life history of *O. mirus*, newly-eclosed female *O. mirus* adults were held in individual glass vials streaked with honey for food. A card of *B. hilaris* eggs was added to each vial and replaced by a new one every day until the wasp died. The offspring were counted and sexed. The mean fecundity was 118 eggs, ranging from 27-259. The mean fertility was 106 offspring, ranging from 23-233. The mean longevity was 58.1 days, ranging from 37-72 days. The mean survival of *O. mirus* eggs was 88%. The percentage of male offspring increased from day 10 through day 49 and then dropped to 0. The increase in males was likely due to a depletion of *Wolbachia* bacteria in the female reproductive system, given the constant supply of host eggs. Compared to *B. hilaris*, *O. mirus* has a shorter life cycle and higher fecundity and longevity, indicating that *O. mirus* has the physiological reproductive capacity to manage *B. hilaris* populations.

Introduction

The recently described *Ooencyrtus mirus* Triapitsyn & Power is an idiobiont, thelytokous, egg endoparasitic hymenopteran, collected in Pakistan and sent to the U.S. for evaluation as a potential biocontrol agent for *Bagrada hilaris* (Heteroptera: Pentatomidae) (Mahmood et al. 2015). Originally from Africa and Asia, *B. hilaris* has invaded brassica crops in the southwestern U.S. (Palumbo et al. 2016), Mexico (Sánchez-Peña 2014; Torres-Acosta & Sánchez-Peña 2016), and Chile (Faúndez et al. 2016). *Ooencyrtus mirus* is a solitary parasitoid on a wide range of lab-tested hosts, but it is gregarious on the large eggs of *Halyomorpha halys* (Chapter 6).

Being an idiobiont from a hot climate and attacking the egg stage of the host are factors that can contribute to parasitoids having high juvenile mortality and thus a high fecundity (Blackburn 1991a). However, being an endobiont is associated with lower juvenile mortality and thus lower fecundity of adults (Blackburn 1991a). Based on all four factors (idiobiont, hot climate, egg parasitoid, endobiont), we expect the fecundity of *O. mirus* to be above average for Hymenopteran parasitoid species.

Fertility and longevity information can provide information for mass rearing as well as field releases, such as how many individuals to release, at what time and how frequently. Most importantly, it can help determine whether *O. mirus* might be an effective biocontrol agent against *B. hilaris* or other hosts. In this study, we determine the fecundity and longevity of *O. mirus* and compare it to female *B. hilaris*, which lay an average of 95 eggs (range 36-173) (Halbert & Eger 2010). Depending on temperature the mean life cycle of *B. hilaris*, is 38-65 days (Halbert & Eger 2010), whereas that of *O.*

mirus is 8-43 days (Chapter 4). Adult male and female *B. hilaris* are reported as living on average 14.3 and 15.8 days, respectively (Ghosal et al. 2006) and 20.9 and 25.9 days respectively (Singh & Malik 1993).

Methods

Bagrada hilaris eggs and *O. mirus* adults were reared by the same methods prescribed in the previous chapter. A *B. hilaris* egg cage is shown in Fig. 3-1.

Fig. 3-1. *Bagrada hilaris* egg cage: A Durphy box with 30 *B. hilaris* adults, fresh organic broccoli and paper towel lining the bottom. The sides of the cage have screen holes for air circulation.

The proportion of male offspring unexpectedly started increasing as the parent wasps got older. These were the males used for the longevity study. For all treatments, honey was applied using a cat whisker glued to the end of a 0.3 cm diameter x ~ 13 cm wooden dowel. The cat whisker made the streak thin enough so that the wasps did not get stuck

and die in the honey. Except for the daily check for emergence, the insects were kept in a Percival growth chamber (model I30BLL, Perry, Iowa, USA), set at 26°C, 50% RH, and 14:10 L:D.

Experimental Procedures

Longevity

To study longevity, 3 treatments were established. Treatment A consisted of unpaired males and females that were kept apart and provisioned only with honey provided as needed (i.e. no *B. hilaris* eggs). Males that emerged on the same day were aspirated into a single vial streaked with honey. The same number of newly-emerged females was put in a separate vial with streaks of honey. The vials were checked daily, and any dead wasps were recorded and removed. For Treatment B, male and female sibling pairs that developed from eggs oviposited on the same day by the same mother and emerged on the same day were placed together in a vial with honey. Some vials had two pairs that emerged from the same vial on the same day. No host eggs were added at any time. Treatment C consisted of only females supplied honey and host eggs. Each *B. hilaris* egg card was made from a 1.27 x 4.2 cm piece of white card stock with the numbers 1-10 printed on it. Ten 1-day-old *B. hilaris* eggs were glued to the card with 1 egg glued (Elmer's glue) above each number. For maintaining the adult female wasps, glass vials (9 cm long x 1 cm diameter) were labeled and streaked with honey. One newly emerged female *O. mirus* adult and one egg card were added to each vial, and the hole was plugged with cotton. The vials were stored on a ridged plastic tray (Nordic Ware® 20.3 x 24.7 cm microwave bacon tray). At the same time every day, the previous day's egg card

was replaced with a new one. More honey was added as needed. When a wasp was found dead, its day of death was recorded and the vial was removed from the tray. The suspected cause of death also was recorded, to separate "natural" deaths from ones caused by injury when replacing the egg card or the wasp getting stuck in honey.

Fecundity and Fertility

The fecundity and fertility were determined on insects in treatment C. The cards with parasitized eggs removed each day were placed in glass vials the same size as above and the vials were placed on the same type of tray in the growth chamber. When the egg cards were 6-11 days old, after all bug nymphs emerged but before the adult wasps emerged, the number of wasp pedicels per egg were counted under a stereomicroscope and recorded (fecundity). The vials of parasitized eggs were checked daily. Any emerged bug nymphs or adult wasps were recorded and removed (fertility). The sex of each emerged wasp also was recorded. Each vial was checked for 23 days after being removed from the parent vial.

Statistical analyses

Female longevities were compared among treatments A, B and C using one-way ANOVA followed by the nonparametric Dunn's Kruskal-Wallis test. For comparisons of longevities between non-paired and paired males, and between males and females in paired (B) and non-paired (A & C) treatments, data first were tested for normality using the Shapiro-Wilk test ($P < 0.05$), and then analyzed using the nonparametric Mann-Whitney U test or permutation t-test since the distribution was not normal. All data were analyzed in R (R Core Team 2019) with the alpha (α) significance threshold set at 0.05.

A life table for treatment C (non-paired females with daily supply of host eggs) was constructed using the survival and reproduction data according to Carey (1993). The Jackknife procedure (Meyer et al. 1986) was used to calculate the following population growth parameters and their mean and standard errors (SAS Institute 2016):

Net reproductive rate (R_0)	$\sum l_x m_x$
Intrinsic rate of population increase (r_m)	$\sum_{x=0}^{n} e^{-rx} l_x m_x = 1$ (in the above equation, r = r_m)
Mean generation time (T)	$\ln {R_0}/{r_m}$
Doubling time (DT)	$\ln {2}/{r_m}$
Finite rate of increase (λ)	e^{r_m}

Results

Fecundity & Fertility

The pre-oviposition period of the adult averaged 0.4 ± 0.1 days (Table 3-1). The oviposition period lasted another 40.4 days, followed by a post-oviposition period of 17.4 days. The mean daily fecundity increased to a maximum daily mean of 5.6 eggs on day 18, counting from the beginning of the parent's life as an egg (= day 5 of the adult's life) (Figure 3-2). Daily fecundity dipped during days 23-25, and then increased to a new maximum of 4.3 eggs on day 33. After that the mean daily fecundity slowly declined until the last day an egg was laid, on day 72. Excluding one wasp that had no offspring, lifetime fecundity ranged from 27-259 and the mean was 118.0 with standard error 11.9 (Table 3-1). The mean number of eggs laid daily by a female was 2.9 ± 0.3 eggs (Table 3-1). The number of eggs laid in a day by a single wasp ranged from 0 – 11. The mean

fertility (number of live offspring per female) was 106. With the no-offspring wasp included, fertility ranged from 0 to 233 live offspring, with a mean of 101.3 ± 11.6; without the no-offspring wasp, the mean fertility was 105.9 ± 11.1.

Table 3-1. Reproduction parameters (mean ± SE) of *Ooencyrtus mirus* on *Bagrada hilaris* eggs.

Parameters	**Non-paired females**
Pre-oviposition period (days)	0.4 ± 0.1
Oviposition period (days)	40.4 ± 2.0
Post-oviposition period (days)	17.4 ± 2.7
Total fecundity (eggs/female)	118.0 ± 11.9
Fecundity rate (eggs/female-day)	2.9 ± 0.3
Proportion of parasitism success (offspring/eggs/female/day)	0.88 ± 0.01
Proportion of female progeny/female-day	0.76 ± 0.04

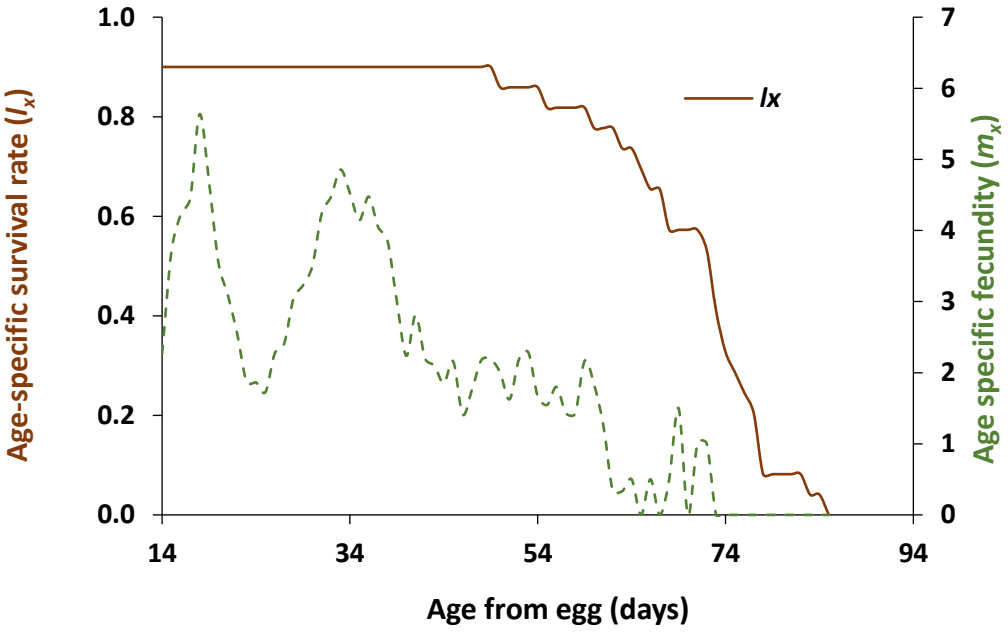

Fig. 3-2. Age-specific survival rate and age-specific fecundity of *Ooencyrtus mirus* reared at 26° with a continuous supply of host eggs.

Following the same pattern as fecundity, fertility reached an initial high from 5-day-old parents, dipped to a low at 9-12 days and then reached a second peak at 19 days before starting a slow decline until the end of the parent's oviposition period (Figure 3-3). The females laid eggs from day 0 to day 58, although few eggs were laid after day 48. The days recorded are the days that the eggs were removed after being in with the wasp for 24 hours.

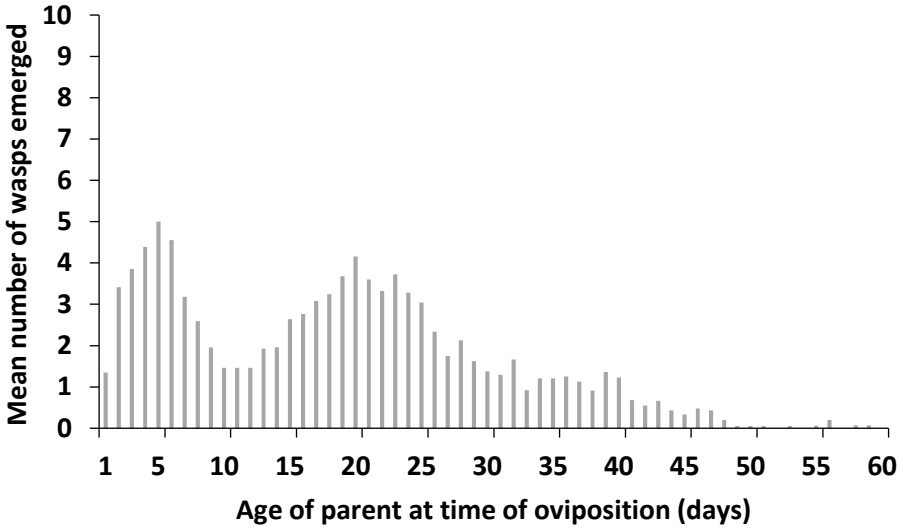

Fig. 3-3. Mean number of adult *Ooencyrtus mirus* emerged per 10 *Bagrada hilaris* host eggs according to age of the parent wasp

Longevity

Counting from adult eclosion, the mean longevity of wasps exposed to eggs was 58.1 days, and the range was 37-72 days. Treatment (not paired with males (A), paired with males (B), and not paired but exposed to host eggs (C)) had a significant effect on the longevity of *O. mirus* females ($F_{2, 90} = 7.566$, $P < 0.001$) (Table 3-2). Longevity did not differ between non-paired and paired females not exposed to eggs (Treatments A &

B) (P = 0.45, Fig. 3-4). However, longevity was significantly lower in non-paired females that were given host eggs than in females that were not given host eggs ($P < 0.01$). Females lived significantly longer than males in both non-paired and paired treatments with no host eggs ($P < 0.001$) (Figs. 3-4, 3-5 and 3-6). Longevity did not differ significantly between non-paired and paired males (P = 0.07, Fig. 3-4).

Table 3-2. Longevity of adult *Ooencyrtus mirus* in three different treatments (mean ± SE, measured in days). N = number of individuals. Means within the same column that do not share the same letter differ significantly (Dunn's Kruskal-Wallis multiple comparison, $P < 0.05$). * indicates significant differences between females and males within the same treatment. The longevity of "non-paired + host eggs" differed significantly from each group of males and from the combined male groups (Wilcoxon-Mann-Whitney test or t-test, $P < 0.05$).

	Treatments	**N**	**Female**	**N**	**Male**
A	Non-paired	45	69.3 ± 1.6 A*	45	54.4 ± 1.1 a
B	Paired	26	66.5 ± 2.5 A*	26	52.3 ± 1.1 a
C	Non-paired + host eggs	22	58.1 ± 1.8 B		

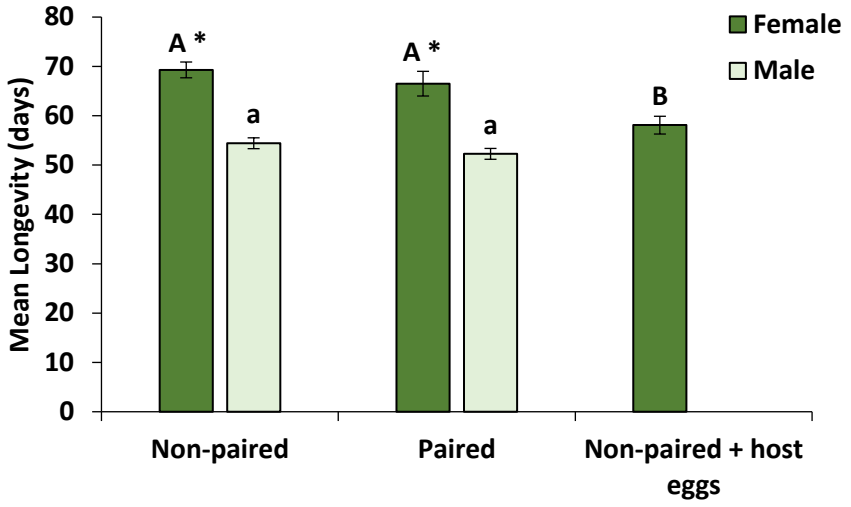

Fig. 3-4. Mean (± SE) longevity of *Ooencyrtus mirus* adults. Bars that do not share the same letter differ significantly. Significance is represented by capital letters for females and lower-case letters for males (Dunn's Kruskal-Wallis multiple comparison, $P < 0.05$). The asterisks (*) represent significant differences between females and males within each treatment for non-paired and paired females. The longevity of "non-paired + host eggs" also differed significantly from each group of males and from the combined male groups (Wilcoxon-Mann-Whitney test or t-test, $P < 0.05$).

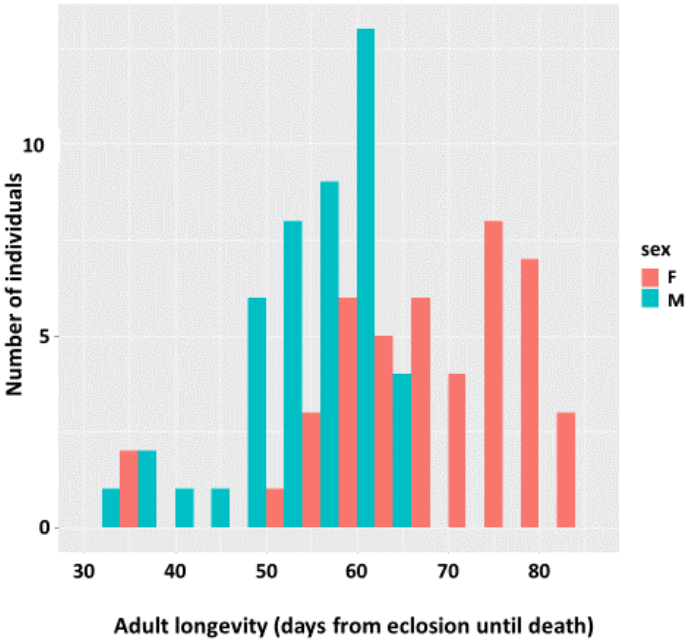

Fig. 3-5. Number of non-paired male and female *Ooencyrtus mirus* individuals that lived to given ages. A permutation t-test showed that males lived significantly fewer days than females ($P < 0.001$). Male mean = 54.4 days; female mean = 69.3 days.

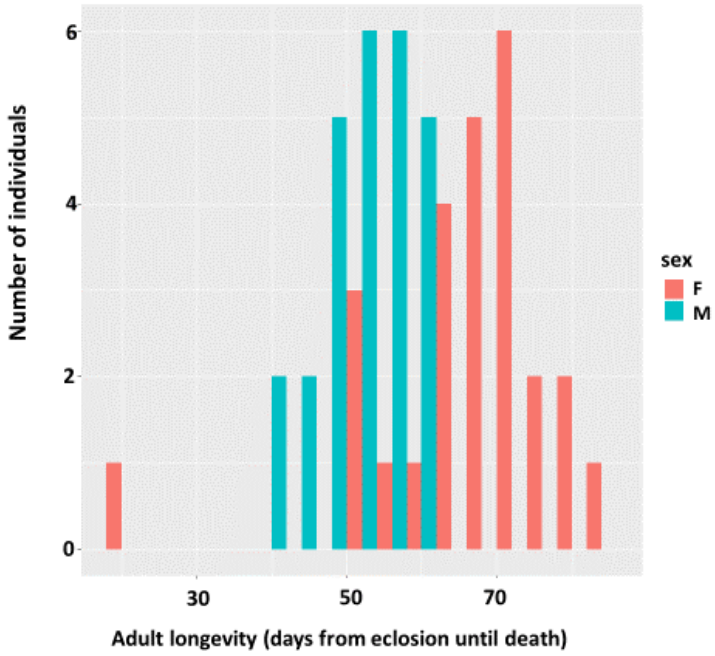

Fig. 3-6. Number of paired male and female *Ooencyrtus mirus* individuals that lived to given ages. A paired permutation t-test showed that males lived significantly fewer days than females did ($P < 0.001$). Male mean = 52.3 days; female mean = 66.5 days. The data were unpaired to make the histogram.

Sex ratio

The percentage of male offspring was 0-3% in the first 5 days (Fig. 3-7). On day 10, the percentage of males started increasing, with a general upward trend and did so until day 48. After this time, the number of males dropped to 0. Over the 51 ovipositional days, male offspring accounted for 0-80% of total. The mean percentage of males per female per day was 24 ± 3. The overall mean (total number of males/total number of offspring) was 19.5%. This contrasts the 1-3% male offspring typically found in our *O. mirus* colony.

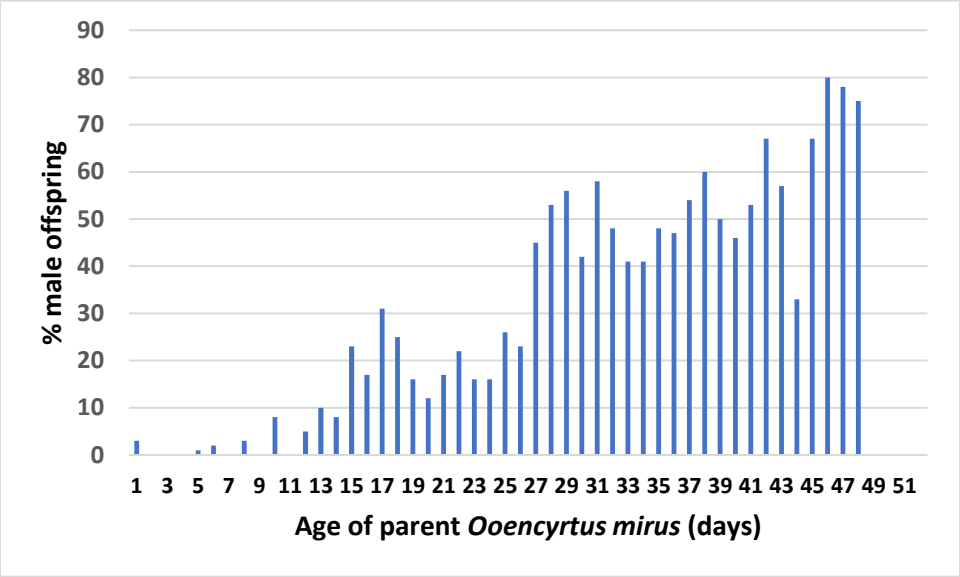

Fig. 3-7. Percentage of male offspring oviposited by parent *Ooencyrtus mirus* from the day of emergence until death.

Population growth parameters and survival of *O. mirus* eggs

The population growth parameters are shown in Table 3-3. The net reproductive rate ($R_{0)}$ was 106 females/female-generation, intrinsic rate of increase ($r_{m)}$ was 0.175 females/female-day, the finite rate of increase (λ) was 1.2 population multiplication/day, the mean generation time (T) was 26.7 days and the doubling time (DT) was 4.0 days. The overall mean success per *O. mirus* egg (i.e., survival) was 0.88, which did not decline with age (Fig. 3-8).

Table 3-3. Population growth parameters (mean ± SE) of *Ooencyrtus mirus* reared on *Bagrada hilaris* eggs at 26°C.

Parameter	**mean ± SE**
Net reproductive rate (R_0)	106.2 ± 22.3
Intrinsic rate of increase (r_m)	0.175 ± 0.010
Finite rate of increase (λ)	1.191 ± 0.012
Mean generation time (T) (days)	26.7 ± 1.3
Doubling time (DT) (days)	4.0 ± 0.2

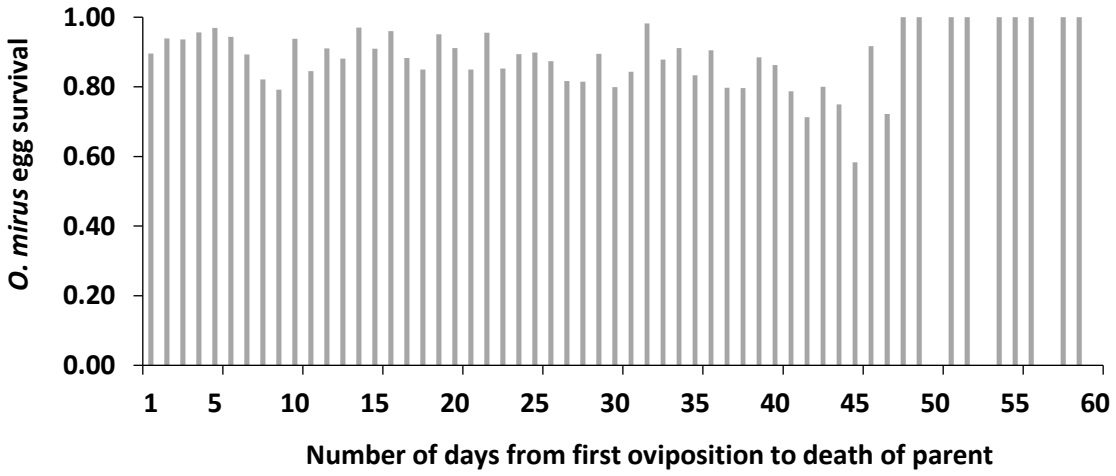

Fig. 3-8. Proportion of *Ooencyrtus mirus* eggs that survived to emerge as adults, according to age of the parent

Discussion

The increase in the percentage of male offspring over the course of the female ovipositional period was likely due to the depletion of *Wolbachia* bacteria. *Wolbachia* are known to induce parthenogenesis in hymenopteran parasitoids (Stouthamer et al. 1990). With constant daily exposure to host eggs, the *Wolbachia* in the female *O. mirus* ovaries gets used in wasp eggs faster than the *Wolbachia* can reproduce (Lindsey & Stouthamer 2017). Without the *Wolbachia* to make the haploid eggs female, the offspring are male. Since males are undesirable for mass rearing in the lab, exposure of

O. mirus adults to host eggs should be limited to alternate days or to only 1 hour/day. In *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae), another *Wolbachia*-impacted parasitoid, limiting the egg supply to alternating 24-hour periods resulted in a lower percentage of male offspring than from a continuous supply of eggs. Limiting exposure to eggs to one hour daily resulted in an even lower percentage of males. The differences correlated with *Wolbachia* titers from the female parents; the females with lower titers had a higher percentage of male offspring (Lindsey & Stouthamer 2017). *Ooencyrtus mirus* could be tested similarly to determine the optimum exposure to eggs to maintain high fecundity and minimize the percentage of male offspring.

The *O. mirus* life cycle in the laboratory takes 8-43 days, whereas the *B. hilaris* life cycle in the laboratory takes 38-65 days (Halbert & Eger 2010), both depending on temperature. The mean fecundity per female *B. hilaris* ranges from 36-173 eggs, with a mean of 95 eggs, compared to a range of 27-259, with a mean of 118, in *O. mirus*. The longevity of adult females is a mean of 58 days in *O. mirus* and 16 days in *B. hilaris*. Since *O. mirus* has a shorter life cycle, higher fecundity and greater longevity than *B. hilaris*, it appears to have the physiological reproductive capacity to manage *B. hilaris* populations. Further research would be needed on host-finding and survival of *O. mirus* in the field to determine if *O. mirus* might have the potential to establish and manage *B. hilaris* populations.

The longevity of *O. mirus* in the lab depends on having access to honey, applied to the glass in the vial in very thin streaks so the wasps do not get stuck and die in the honey. The best way we have found to apply the honey is with a cat whisker glued to a

thin dowel. Without honey, 100% of *O. mirus* adults die within 3 days. In the field, the adults would need access to nectar in flowers or a human-supplied carbohydrate source.

Table 3-4. Comparison of population and reproduction parameters among different *Ooencyrtus* species

	***Ooencyrtus* species**				
Parameter	***O. fecundus***	***O. kuvanae***	***O. lucidus***	***O. mirus***	***O. telenomicida***
R_0	198.1	136.25	103.8	106.2	53
r_m	0.253	0.768	0.171	0.175	0.154
λ	1.29	2.16	1.19	1.19	1.17
T (days)	20.9	65.75	27.1	26.7	26
DT (days)	2.74	0.9	4.0	4.0	4.5
Mean no. ♀ offspring/♀		136		118	84
Mean emergence rate			0.95	0.88	0.89
Mean ♀ longevity (days)		79.9	53.5	58.1	52.4

R_0 = net reproductive rate; r_m = intrinsic rate of increase; λ = finite rate of increase, T = mean generation time (days), DT = doubling time (days), no. = number. Sources: *O. fecundus* on *Eurygaster integriceps* Puton (Hem. Scutelleridae) – Ahmadpour et al. 2014; *O. kuvanae* on *Lymantria dispar* (L.) - Alzofon 1984; *O. lucidus* on *B. hilaris* - Ganjisaffar & Perring 2020; *O. telenomicida* on *Graphosoma lineatum* L. - Roversi et al. 2018.

Table 3-4 compares population and reproduction parameters to a few other egg-parasitic *Ooencyrtus* spp. for which data are available. Compared to *Ooencyrtus lucidus* Triapitsyn & Ganjisaffar and *Ooencyrtus telenomicida* (Vassiliev), *O. mirus* has a similar finite rate of increase and mean generation time. The net reproductive rates of *O. mirus* and *O. lucidus* are similar to each other at 106 and 104, respectively, and intermediate between *O. kuvanae* at 136 and *O. telenomicida* at 53. The intrinsic rate of increase is higher in *O. kuvanae* than in the other four species. The mean generation time is much greater in *O. kuvanae*, a temperate-climate species, and lowest in *O. fecundus*. The doubling time for *O. mirus* is the same as *O. lucidus* (4.0 days) and lower than *O.*

telenomicida (4.5 days), but higher than that of *O. fecundus* and *O. kuvanae*. Assuming that under normal circumstances almost all the *O. mirus* offspring would be female, the mean number of female offspring per female (118) is intermediate between *O. kuvanae* (136) and *O. telenomicida* (84). The mean longevity of females exposed to host eggs is 58.1 in *O. mirus*, lower than the 79.9 in *O. kuvanae* but higher than the 53.5 and 52.4 in *O. lucidus* and *O. telenomicida*, respectively. Of the four species, *O. kuvanae* would increase its population the fastest and *O. telenomicida* the slowest. The latter, however, is still being considered for augmentative control of *Haylomorpha halys* (Stål), another pentatomid like *B. hilaris*. *Ooencyrtus lucidus* may already be contributing to the control of *B. hilaris* in the field, along with *Trissolcus hyalinipennis* (Ganjisaffar et al. 2018). *Ooencyrtus mirus* is the only thelytokus species of these four. This, combined with the population and reproductive parameters reported herein suggest that it is a reasonable candidate for further evaluation as a classical or augmentative biological control agent of *B. hilaris* or possibly other pentatomids as determined in Chapter 6.

Chapter 4

Effect of temperature on the survival and developmental rate of immature *Ooencyrtus mirus*

Abstract

Bagrada hilaris (Burmeister) is an invasive pest of cole crops in the southwestern United States. To find potential biocontrol agents of *B. hilaris*, three egg parasitoids were imported from Pakistan, including *Ooencyrtus mirus*, a recently described uniparental species. We investigated the effect of temperature on survival and developmental rate in *Ooencyrtus mirus* from egg to adult. At 14°C and 16°C, no adults emerged unless the immatures were transferred later to a warmer temperature. At constant 18°C, a low percentage emerged, but again more emerged if the immatures were transferred to a warmer temperature. Survival ranged from 80-96% at 20°-37°C and did not differ significantly among these temperatures. No adults emerged at 38°C. Regardless of the amount of time the parasitized eggs were held at 14° and 16°C, the developmental times after returning the eggs to 26°C were similar, suggesting a quiescence process rather than simply slow development. At higher temperatures, the developmental rate increased linearly from 18° to 36°C, and then declined at 37°C. The Wang model provided the best fit of the data, and estimated a lower developmental threshold at 13.0°C, an optimal temperature at 35.6°C, and an upper developmental threshold of 38.3°C. The thermal constant for total immature development is 168.4 degree days. The results show 36°C to be the best temperature for rearing *O. mirus*, and that *O. mirus*-parasitized eggs can be

stored at 14°C for months without losing viability. These are crucial data to consider when mass rearing this biological control agent.

Introduction

Being poikilothermic, insects' developmental rate is dependent on ambient temperature. To find the temperature that minimizes developmental time in *O. mirus*, we test *O. mirus*' oviposition, developmental rate, survival and adult emergence at 13 different temperatures ranging from 14 - 38°C. Since our *Ooencyrtus mirus* colony is from a hot climate in Pakistan, we expect it to have a relatively high optimal temperature for development.

Materials and Methods

Host Egg Rearing

Bagrada hilaris were reared on the UCR campus in tent-style insect cages (BugDorm-2120, MegaView Science Co., Taiwan) in two greenhouses set at 30 ± 5°C with ambient humidity and light. The insects were provided with broccoli, canola (*Brassica juncea*) and mizuna (*Brassica rapa* var. *japonica*) seedlings grown for ~3.5 weeks in plastic pots (10 × 10 cm width × 9 cm depth). Adults for experiments were transferred to an insectary room with 30 ± 1°C, 40-50% RH and 14:10 L:D. Thirty bugs were placed into ovipositional cages, which were round, plastic Durphy boxes (Durphy Packaging Co., Pennsylvania, U.S.), 15 cm diameter × 6.3 cm height, with two, 2.5 cm screened holes opposite each other in the sides for ventilation. A piece of white paper towel (Brawny®) was cut to fit the round bottom of each Durphy box to absorb fecal material and excess moisture. Each day the adults were transferred to new cages and

supplied with fresh pieces of organic broccoli from a local grocery store. *Bagrada hilaris* laid their eggs on the plastic cage and on the paper towels. The eggs were gently removed from the cage with a paint brush and rubbed off the paper towel by hand for use in experiments.

Parasitoid Rearing

The rearing of *O. mirus* from Pakistan and all experiments were conducted in Quarantine on the UCR campus. Daily, approximately 40 *B. hilaris* eggs were glued onto a 1.3 cm × 4.2 cm piece of card stock. The egg card was placed in a 9.4 cm long × 2.2 cm diameter glass vial and ten 3-day-old adult parasitoid females were added. The open end of the vial was plugged with 100% cotton coil. After 24 hours, the adult parasitoids were aspirated out, and the vial of parasitized eggs was placed on a ridged plastic tray (Nordic Ware® 20.3 × 24.7 cm microwave bacon tray) and kept at room temperature (22-23°C) under ambient light until new *O. mirus* adults emerged from the eggs. Each day, newly emerged wasps were collected and placed into a fresh glass vial streaked with honey for a carbohydrate source. These vials of adults were placed in a growth chamber (Percival model I30BLL, Perry, IA, U.S.) at 26°C, 50% RH and 14:10 L:D until they were 3 days old and either used in experiments or returned to the colony.

Experimental Procedures

The survival and developmental time from parasitoid egg to adult was evaluated at constant temperatures of 14, 16, 18, 20, 23, 26, 28, 30, 32, 34, 36, 37 and 38ºC at 60 ± 10% relative humidity and 14:10 L:D photoperiod. The temperatures were chosen to provide more detail at the non-linear ends of the curve than in the linear middle of the

curve; hence the 3-degree intervals from 23 - 26ºC. For all temperatures, a Hobo data logger (Onset®, Bourne, MA, U.S.), was used to verify that the temperatures stayed within ± 1ºC. Based on the Hobo data, the growth chambers were adjusted daily to stay usually within 0.1ºC of the desired temperatures. Depending on the availability of growth chambers and host eggs, three temperatures at a time were started within a few days of each other. After we found that *O. mirus* developed most quickly at 36 ºC and died at 38ºC, we tested 37ºC to add detail at the peak of the developmental rate curve.

For all temperatures tested, one egg card was added to each of four glass vials, the same size as colony vials. Each egg card (1.3 cm × 4.2 cm) was made by gluing 25 one-day-old *B. hilaris* eggs in 3 rows of 8, 9 and 8 eggs with Elmer's Glue (Elmer's Products, Inc., Westerville, OH, U.S.). Five 3-day-old *O. mirus* adult females were added to each vial, for a ratio of 1 wasp: 5 *B. hilaris* eggs. The adults were allowed access to the eggs at 26ºC for 24 hours. After this time, the parasitoids were removed, and each host egg was examined under a Leica MZ75 dissecting microscope (50X) to determine if the egg had been parasitized. Like other encyrtid parasitoids, the eggs of *O. mirus* have a pedicel that protrudes from the host egg. *Bagrada hilaris* eggs with no pedicels or with more than one pedicel were discarded from the study. The remaining single-pedicel eggs were cut from the card and placed individually into gel caps (size 0, Capsuline®, Pompano Beach, Florida, U.S.). In this manner a minimum of 44 singly-parasitized eggs were used for each temperature, each egg representing one replicate in the analysis. The eggs for each temperature were placed on a ridged plastic tray and placed in a Percival growth chamber (model I30BLL, Perry, IA, U.S.) at the temperature being evaluated (Fig. 4-1).

The parasitized eggs in the gel caps were checked daily. Emerged wasps were counted and removed, and the number of days since oviposition was recorded as developmental time. Survival was measured as the proportion of eggs from which an adult parasitoid emerged. The sex of each emerged wasp was noted.

Fig. 4-1. Tray of gel caps each containing one *Bagrada hilaris* egg parasitized with one *Ooencyrtus mirus* egg, ready to be stored at 36°C.

Arrested Development (Quiescence)

For the 14° and 16°C treatments, there was no emergence for 93 days or 182 days, respectively, so the parasitized eggs in these temperatures were moved to 26°C to determine if the parasitoids had survived. Finding emergence of these parasitoids, we wondered if the immatures in the cold temperatures had undergone quiescence, so we conducted a study to investigate. Host eggs with one pedicel were obtained and placed in gel caps in the manner previously described. More than fifty single-pedicel eggs were kept at each of three temperatures (14°, 16° and 18°C) for 30 days, after which they were

moved to 26°C. During the original 30 days and post-transfer, the eggs were checked daily for emerged parasitoids. The emerged wasps were counted and sexed, and the day of emergence was recorded.

Statistical Analysis

The proportions of adults that survived at the different temperatures were tested for equality without continuity correction in R (R Core Team 2018) using the function "prop.test" ($P < 0.01$), followed by pairwise comparison of proportions ($P < 0.05$) for both the constant-temperature and cold-to-warm studies.

The developmental time data were not normal, so the impact of temperature on developmental times was tested for significant differences using the Kruskal-Wallis test (Kruskal & Wallis 1952) followed by Dunn's multiple comparison test ($P < 0.01$) (Dunn 1964) with Bonferroni adjustment (R Core Team 2018).

Using ArthroThermoModel (ATM) software developed by Mirhosseini et al. (2017) for use in MATLAB 2015, the relationship between temperature (T) and developmental rate (D_r, the reciprocal of developmental time) was estimated with a linear model and various nonlinear models including Logan 6 and 10 (Logan et al. 1976), Analytis 1 and Analytis 3 (Analytis 1977), Wang (Wang et al. 1982), Hilbert and Logan (Hilbert & Logan 1983), Ratkowsky (Ratkowsky et al. 1983), Beta (Yin et al. 1995), Lactin 1 and 2 (Lactin et al. 1995), Briere 1 and 2 (Briere et al. 1999), Janisch-Rochat (Janisch 1932; Rochat & Gutierrez 2001), Analytis 3-Kontodimas (Kontodimas et al. 2004), Janisch-Kontodimas (Janisch 1932; Kontodimas et al. 2004), Analytis 1-Allahyari (Allahyari 2005) and Performance 1 and 2 (Shi et al. 2011). The goodness of fit for each

model was assessed by the adjusted R^2 (R^2_{adj}) (Kvalseth 1985) and Akaike Information Criterion (*AIC*) (Akaike 1974) derived from the ATM software; models with the smallest *AIC* and the highest R^2_{adj} are considered the best fit. Based on these values, and the low difference between observed and predicted values, 5 nonlinear models were selected and their parameters presented (Table 4-1). In the linear model, the thermal minimum ($T_{\min}$) and the thermal constant (*K*) (cumulative degree-days) were calculated as $T_{\min} = -a/b$ and $K = 1/b$, respectively, where *a* and *b* are constants in the linear model (Campbell et al. 1974).

Developmental time for the *O. mirus* immatures after transfer from cold to warm temperatures was analyzed in pairs (14º vs.16º, 14º vs. 18º and 16º vs. 18ºC) using a Wilcoxon rank sum test in R (Wilcoxon et al. 1945).

Table 4-1. Estimated parameters of the linear and selected nonlinear thermal models with estimated values of their evaluation criteria for *Ooencyrtus mirus*.

Model (Reference)	Equation	Parameters	Estimations
Linear (Simpson 1903)	$D_r = a + bT$	a	0.08617
		b	0.0059395
		T_{min}	14.5
		K (DD)	168.37
		R^2	0.9629
		R^2_{adj}	0.9628
Wang (Wang et al. 1982)	$D_r = \frac{m[1 - exp(-K_1(T - T_{min}))][1 - exp(K_2(T - T_{max}))]}{1 + exp(-C(T - T_{min}))}$	m	5.564
		C	0.171
		K_1	0.001055
		K_2	1.244
		T_{min}	13.0
		T_{max}	38.3
		T_{opt}*	35.6
		SSE	0.0175
		R^2	0.9661
		R^2_{adj}	0.9658
		AIC	-5119.44
Lactin 2 (Lactin et al. 1995)	$D_r = exp(\rho T) - exp\left(\rho T_M - \frac{T_M - T}{\Delta T}\right) + \lambda$	ρ	0.00536
		ΔT	0.7493
		λ	-1.081
		T_M	39.83
		T_{min}*	14.5
		T_{max}*	38.2
		T_{opt}*	35.6
		SSE	0.0177
		R^2	0.9656
		R^2_{adj}	0.9654
		AIC	-5115.16
Analytis 1-Allahyari (Allahyari 2005)	$D_r = P\left(\frac{T - T_{min}}{T_{max} - T_{min}}\right)^n \left(1 - \left(\frac{T - T_{min}}{T_{max} - T_{min}}\right)^m\right)$	P	0.1443
		m	30.86
		n	1.02
		T_{min}	14.7
		T_{max}	38.2
		T_{opt}*	35.7
		SSE	0.0177
		R^2	0.9657
		R^2_{adj}	0.9654
		AIC	-5114.62
Performance 2 (Shi et al. 2011)	$D_r = m(T - T_{min})\left(1 - exp(K_2(T - T_{max}))\right)$	m	0.006262
		K_2	1
		T_{min}	15.0
		T_{max}	38.6
		T_{opt}*	35.6
		SSE	0.0181
		R^2	0.9649
		R^2_{adj}	0.9648
		AIC	-5105.63

Table 4-1 Notes: In all models, T is the rearing temperature (°C), and D_r is the developmental rate at temperature T (day^{-1}). T_{min} and T_{max} are the lower and upper temperature thresholds, respectively, and T_{opt} is the optimal temperature. Parameters a and b in the linear model, m, C, K_1, and K_2 in Wang model, P, m, and n in Analytis 1-Allahyari model, and m, and K_2 in Performance 2 model are constants to be fitted. In Lactin 2 model, ρ is a constant defining the rate of increase to optimal temperature, ΔT is the temperature range over which physiological breakdown becomes the overriding influence, λ forces the curve to intercept the Y-axis at a value below zero, and thus allows estimation of the lower temperature threshold, and T_M is the supraoptimal temperature at which $D_r = \lambda$. * Parameters that were calculated in Excel, not by the model.

Results

Parasitoid Survival at Different Temperatures

No adult *O. mirus* emerged at constant 14ºC or 16ºC. However, adults did emerge after the parasitized eggs were transferred to 26ºC after 3 months and 6 months, respectively (Table 4-2). Eggs transferred from 14ºC after 3 months had 85% emergence (not significantly different from the higher temperatures tested; pairwise proportion test with Bonferroni correction, $P = 1.0$) and eggs transferred from 16ºC after 6 months had 45% emergence (significantly lower than the higher temperatures) (pairwise proportion test with Bonferroni correction, $P \leq 0.01$ for all except 32°C) (Table 4-2). The cold temperature thus did not reduce survival within three months at 14ºC, but it did decrease survival by the end of six months at 16ºC. At a constant temperature of 18°C, emergence was 31%, which was significantly lower than emergence at 20°-37°C (pairwise proportion test with Bonferroni correction, $\chi^2 = 139.6$, $P < 0.001$). Because adults emerged in the constant 18ºC treatment, we assumed that the lack of emergence after 61 days was due to mortality and we did not transfer any of those eggs to 26ºC as we did in the 14º and 16ºC treatments. Since some parasitoids at constant 18ºC may have survived without emerging, I did not include this treatment in the analysis of survival in Table 4-2.

Table 4-2. Survival (proportion of *Ooencyrtus mirus* eggs that developed into emerged adults) for each temperature tested.

Temp (°C)	Total # Host Eggs Parasitized	Total # Emerged Parasitoids	Survival (%)
14*	46	39	85a
16*	47	21	45b
14†	52	38	73ab
16†	59	57	97a
18†	55	51	93a
20	51	45	88a
23	52	46	88a
26	49	47	96a
28	46	39	85a
30	52	47	90a
32	46	37	80ab
34	46	40	87a
36	53	49	92a
37	52	46	88a
38	44	0	0

Means within the same column followed by the same letter (Proportion test, $P < 0.05$) did not differ significantly.
*For 14° and 16°C, emergence of adults was subsequent to being transferred to 26°C after 3 and 6 months, respectively, at the colder temperature.
†Emergence of adults was subsequent to being transferred to 26°C after 30 days at the colder temperature.

A better comparison of survival at low temperatures was obtained when parasitized eggs were exposed to 14º, 16º and 18ºC for 30 days and then transferred to 26ºC. In this study, survival was 73%, 97% and 93% at 14º, 16º and 18ºC, respectively (Table 4-2). When these mortality estimates were included in the analysis from the original experiment, survival after 6 months at 16ºC was significantly lower than that from all other treatments except "30 days at 14ºC then 26 ºC" and "constant 32ºC." None of the other treatments differed significantly from each other ($P > 0.10$ for all combinations). At the constant temperatures between 20° and 37°C, the percent survival was 80-96%. At the highest temperature (38°C), no parasitoids emerged. After 23 days at this temperature, the eggs were moved to 26ºC for another 23 days, and still no adults emerged, confirming that the parasitoids did not survive at this temperature.

Developmental Times at Different Constant Temperatures

Parasitoids did not emerge at constant temperatures of 14° and 16°C. The time to first emergence declined from 43 days at 18°C to 8 days at 34° and 36°C before rising to 9 days at 37°C (Table 4-3 and Figure 4-2). The mean developmental time was 47.3 days at 18ºC, decreasing linearly to 8.1 days at 36ºC and then increasing to 9.1 days at 37ºC.

Table 4-3. First day of emergence, range of emergence (days), and average developmental times ± SE (days) of *Ooencyrtus mirus* at different constant temperatures.

Temp (˚C)	Initial Egg Number	1st Day of Emergence	Range of Emergence (days)	Average Developmental Time (days) *
14	46	-	-	-
16	47	-	-	-
18	59	43	9	47.3 ± 0.52a
20	51	29	6	30.4 ± 0.14a
23	52	21	6	22.3 ± 0.23ab
26	71	14	5	14.5 ± 0.11bc
28	46	11	4	12.2 ± 0.10cd
30	68	10	4	10.6 ± 0.09de
32	62	9	3	9.5 ± 0.08ef
34	62	8	6	8.8 ± 0.18fg
36	75	8	2	8.0 ± 0.05g
37	52	9	3	9.1 ± 0.06f
38	44	-	-	-

* Means within the same column followed by the same letter (Kruskal-Wallis test, $P < 0.05$) were not significantly different.

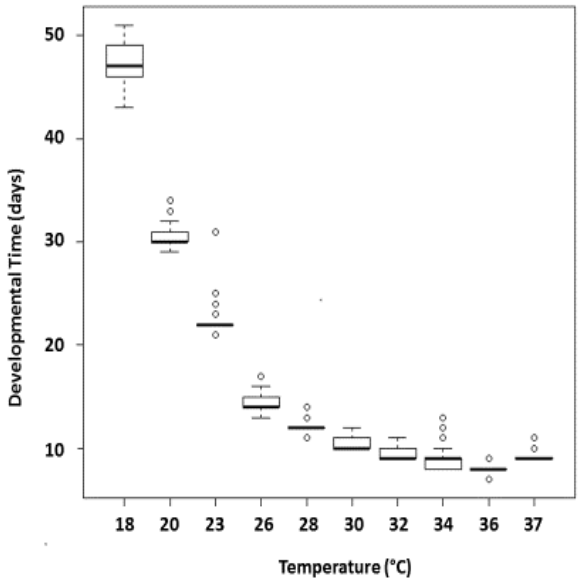

Fig. 4-2. Developmental time (days) of *Ooencyrtrtus mirus* immatures at different temperatures (°C). No adults emerged at constant 16° or 38°C**.**

Of the thermal models tested in the ArthroThermalModel program (Mirhosseini et al. 2017), four non-linear models fit the data most closely: Wang, Lactin 2, Analytis-1-Allahyari, and Performance 2 (Figure 4-3). All these models captured the linear rise, the decrease at 37°C and the zero development at 38°C equally well. All the models estimate a thermal minimum that is lower than 16°C. Our data showed that larvae held as low as

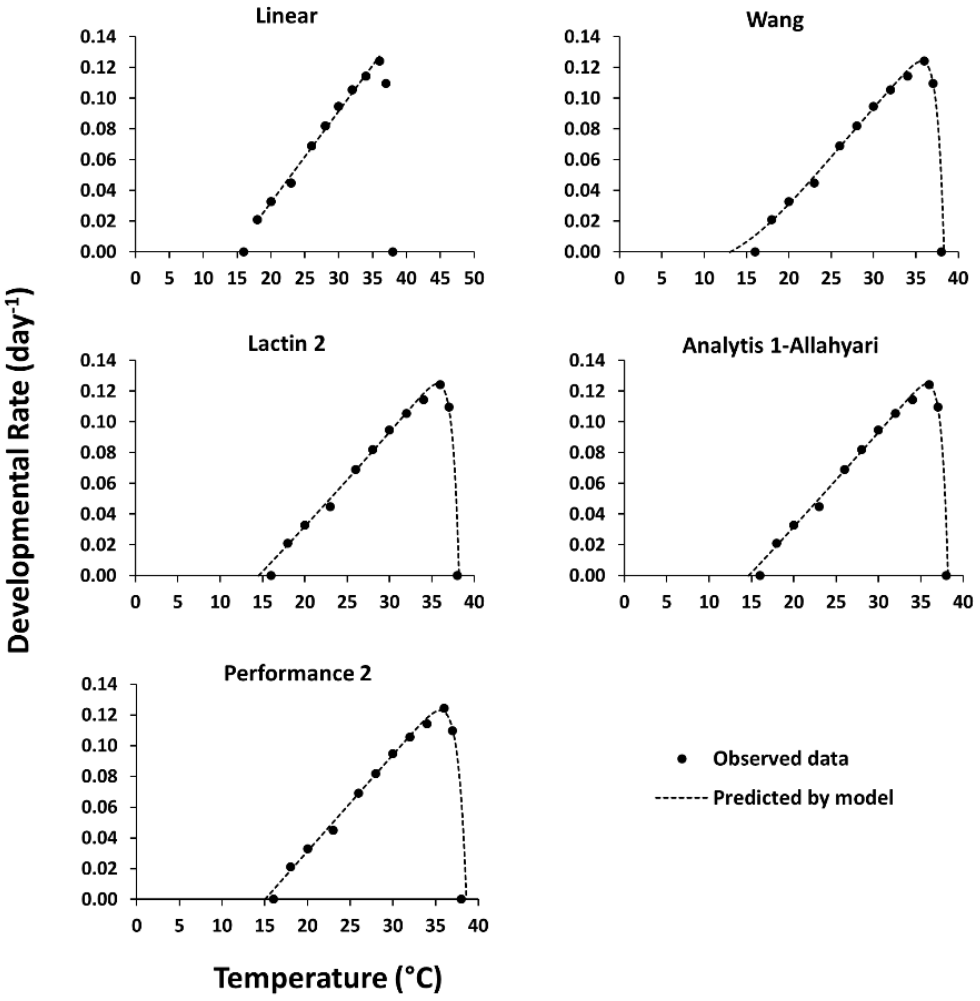

Fig. 4-3. Linear model and the four non-linear models identified by the ArthroThermalModel program as best fitting the temperature-development data for *Ooencyrtus mirus*.

14°C survive and continue to develop once they are transferred to 26°C. The Wang model is the only one that estimates T_{min} below 14°C (Table 4-1), indicating a good fit for our data. In addition, this model had the smallest *AIC* and the highest adjusted R^2, further confirming the best fit to the data.

The linear model fit the linear part of the curve from 18°-36°C. Differences between the predicted mean developmental times and the actual mean developmental times varied from 0.9ºC to -2.4ºC (Table 4-4).

Table 4-4. Observed mean developmental times ± SE (days) compared to times predicted by the linear model for *Ooencyrtus mirus* at temperatures on the linear part of the developmental curve.

Temp (°C)	Observed Developmental Time (days)	Predicted Developmental Time (days)	Difference (Predicted - Observed)
18	47.3 ± 0.52	48.2	0.9
20	30.4 ± 0.14	30.7	0.2
23	22.3 ± 0.23	19.8	-2.4
26	14.5 ± 0.11	14.7	-0.2
28	12.2 ± 0.10	12.5	0.3
30	10.6 ± 0.09	10.9	0.3
32	9.5 ± 0.08	9.6	0.1
34	8.8 ± 0.18	8.6	-0.1
36	8.0 ± 0.05	7.8	-0.2

Arrested Development (Quiescence)

The parasitized eggs transferred after 30 days from 14° to 26°C emerged on consecutive days 10-15 with a mean post-transfer developmental time of 11.7 days (Table 4-5). When parasitized eggs were transferred from 16° to 26°C, three adults emerged on day 7, one emerged on day 9, and 53 emerged on days 10-12. For the parasitized eggs transferred from 18° to 26°C after 30 days, 38 adults emerged over days

4-6. No individuals emerged for the next 4 days, but a second batch of 13 adults emerged on days 10-13 days (Table 4-5). The apparent split in emergence times for the 16° and 18°C treatments, which was absent in the 14°C treatment, indicates differential development related to quiescence. The data suggests that all the parasitoids at 14°C entered an arrested state of development and resumed development after being placed at 26°C, and thus were labeled "post-quiescence" (Table 4-5). At 16°C, only four individuals emerged early after transferring to 26°C (average emergence of 7.5 days)

Table 4-5. Developmental times of *Ooencyrtus mirus* at 26°C after 30 days at 14°, 16° and 18°C.

Temp (°C)	Post-cold development time (days)	# Males emerged	# Females emerged	Mean dev. time (days)	Std. error	Status
14		0	0			no quiescence
14	10	0	4			
14	11	0	10			
14	12	0	22			
14	13	0	0			
14	14	0	1			
14	15	0	1	11.7 a	0.147	post-quiescence*
16	7	1	2			
16	9	0	1	7.5	0.500	no quiescence
16	10	0	19			
16	11	1	11			
16	12	0	22	11.3 b	0.071	post-quiescence*
18	4	2	11			
18	5	0	23			
18	6	0	2	4.7	0.092	no quiescence
18	10	0	1			
18	11	0	3			
18	12	0	8			
18	13	0	1	11.7 a	0.178	post-quiescence*
	total	4	142			

*****The post-quiescence data were analyzed in pairs in R using the Wilcoxon rank sum test. The resulting W test statistics were 700, 453 and 248.5, respectively. The *P*-values were 0.002, 0.034 and 0.98, respectively.

suggesting that those four did not enter quiescence, but continued to develop slowly at 16°C. The majority of 16°C individuals emerged on days 10-12, suggesting they did enter quiescence, but then resumed development after transfer to the warmer temperature (post-quiescence). At 18°C, most of the individuals emerged on days 4-6, indicating that they continued to develop at 18°C (no quiescence), and they developed faster than the non-quiescent immatures held at 16°C (mean 4.7 days at 18°C compared to 7.5 days at 16°C). After transfer from 18°C to 26°C, the remaining parasitoids developed (post-quiescence) and emerged in an average of 11.7 days, which was statistically similar to the post-quiescent times for 14°C ($P = 0.98$, Table 4-5). The post-quiescent development time for insects held at 16°C was significantly shorter than those held at 14°C ($P = 0.002$) and at 18°C ($P = 0.034$, Table 5). In summary, all the parasitoids at 14°C entered quiescence, most at 16°C did, and only a few did at 18°C.

Sex Ratio

In our experience with raising *O. mirus* for the past 3 years, we rarely find males in our colonies. In the present study, just 3 males emerged out of 476 adults (0.6%) reared at constant temperatures ranging from 18–37°C. All 3 of those males were reared at 36°C. In the cold-to-warm study, 4 out of 146 emerged adults (2.7%) were males. Of these, 2 were started at 16°C and 2 at 18°C; no males emerged from those reared at 14°C.

Discussion

Effect of Temperature on Survival and Emergence of *Ooencyrtus mirus*

The temperature range for emergence of *O. mirus* was 18°-37°C, although at a constant temperature of 18°C the proportion of emerging parasitoids was significantly

lower. The results at 14° and 16°C indicate that some of the non-emerged immatures held at 18°C may have survived and resumed development if they had been transferred to a warmer temperature. At 14°C and 16°C, immature *O. mirus* survived in an arrested developmental state for months until they were transferred to 26°C, at which time they resumed development and emerged as adults. The proportion of emergence of individuals kept at 14°C for 3 months and then transferred to 26°C did not differ significantly from that of immatures reared at any of the constant temperatures tested between 20°-37°C. However, the proportion of emergence after 6 months at 16°C was significantly lower. The subsequent study showed that immatures maintained at 14°, 16° and 18°C could resume development with survival similar to that of individuals reared at higher temperatures. The studies in which parasitoids were transferred from cold to warm temperatures indicate that immatures can be stored at cold temperatures for at least three months with no loss of survival, but with significant loss of survival by 6 months. This information would be useful if this parasitoid were being mass-reared for release but were not needed at certain times, such as during hot or cold seasons or when the *B. hilaris* population is below the economic threshold. It also indicates that the parasitoids could be shipped safely at these temperatures.

Between 20-37ºC, *O. mirus* survival was between 80 and 96%, and did not differ significantly among the temperatures tested. No individuals survived at 38°C. This survival pattern is similar to that of *Ooencyrtus anasae* (Ashmead) and *Ooencyrtus* sp. nr. *anasae,* egg parasitoids of squash bugs (*Anasa tristis* DeGeer, Heteroptera: Coreidae); the survival of *O. anasae* ranges from 86-91% at 20.8º, 23.0º and 26.6ºC (Tracy & Nechols

1987). *Ooencyrtus telenomicida* (Vassiliev), a species morphologically very similar to *O. mirus* (Triapitsyn et al. 2020) shows a similar pattern but the proportion of emergence is low (40.0%) at 22°C and then higher at 26° and 30°C (84.2% and 87.0%) (Roversi et al. 2018). Survival in the *O. telenomicida* rearing colony at 26ºC was 89.6% (Roversi et al. 2018); these proportions of emergence are in the range of *O. mirus* and overlap with the range of *O. anasae* (Ashmead) and *Ooencyrtus* sp. nr. *anasae.* In this latter species, immatures did not survive at ≥ 32.5ºC (Rahim et al. 1991), giving it a lower thermal maximum than the 37ºC in *O. mirus* In *Ooencyrtus trinidadensis* Crawford, survival ranged from 91-100% between 18º and 33ºC. At 36ºC, some development but no emergence occurred. This species thus has a lower thermal maximum for emergence than the *O. mirus* in the present study.

Cold Temperature Adaptations

According to Burges (1959), "Quiescence is an arrest of development caused by unfavourable environmental conditions and ended as soon as favourable conditions return." While diapause is also an arrest of development in response to environmental cues, it is more complicated, continuing even under conditions that would be suitable for growth, until certain well-defined changes occur in the environment and the insect undergoes a series of physiological changes (Burges 1959). These changes may not themselves allow growth, but they may permit growth to resume once conditions favor it (Burges 1959). One consequence is that all individuals under the same conditions may not end diapause at the same time (Burges 1959). In the present study, however, the *O. mirus* that survived did resume development at the same time as each other after 3

months at 14ºC and after 1 month at 14º, 16º and 18ºC once they were moved 26ºC, indicating they underwent quiescence rather than diapause. The quiescence is facultative in that, at constant warm temperatures, the colony does not undergo quiescence. Our *O. mirus* colony at UCR, for example, has reproduced for 59 generations over 45 months with no arrestment of development. A quiescent period thus is not necessary in the *O. mirus* life cycle, making it possible to rear this species continuously.

Based on the results in Table 4-5, we hypothesize that at 18ºC, 75% of the emerged adults had not quiesced as immatures, but had continued developing slowly. The other 25% had arrested development, were not as mature as the non-quiescent group at the time of the transfer to 26°C, and therefore took longer to complete development. At 16ºC only 7% of the immatures did not enter quiescence, and at 14ºC all the immatures quiesced. Therefore, within the 14-18°C range, the colder the temperature, the sooner quiescence occurs and for a greater percentage of individuals. Compared to the 16°C treatment, the 18°C group had a much higher percentage (73%) of early-emerged adults and these adults emerged sooner than the 16°C early-emerged group (4.7 vs. 7.5 days, respectively). These data show the transition from most individuals continuing to develop (i.e. not quiescing) at 18°C to 100% of the individuals entering quiescence at 14°C.

The developmental time of the "post-quiescent" group (10 days or later) at 16° was significantly shorter than at either 14° or 18°C. This may indicate that emergence of the non-quiescent and quiescent groups overlapped at this intermediate temperature; i.e., perhaps some of the individuals that emerged on day 10 had not undergone quiescence

and were more mature at the time of the transfer to the warmer temperature than were the 14° and 18°C individuals that had definitely undergone quiescence.

Compared to *Ooencyrtus ennomophagus* Yoshimoto, the elm spanworm egg parasitoid, *O. mirus* enters an arrested state of development at a lower temperature (16° instead of 18°C). Once *O. mirus* is returned to 26°C, it took 11.7 days to develop compared to 14 days when this species was raised at constant 26°C. This suggests that development is arrested in an early larval stage. If development were arrested at a later stage, we would expect that the number of days to emergence after transfer to 26°C would be fewer. In contrast, *O. ennomophagus* arrests growth at a late larval stage (Anderson & Kaya 1974; Kaya & Anderson 1976). *Ooencyrtus nezarae* Ishii females undergo diapause instead of quiescence as adults (Numata 1993). In *Ooencyrtus kuvanae* (Howard), a parasitoid of gypsy moth in the northeastern U.S. forests, survival of immatures decreases with the length of exposure to cold temperature (Kamay 1976). If the immatures are transferred before the late pupal stage, survival is much higher than if they are transferred during the late pupal stage (Kamay 1976). This species overwinters as an adult in forest debris (Hitchcock 1972, Kamay 1976). It uses supercooling (the ability to stop ice from forming with anti-freeze chemicals) to survive cold winters. *Ooencyrtus kuvanae* from New Jersey (U.S.) had a significantly higher supercooling point than those from Maine (U.S.), which is at a higher latitude and colder (Griffiths & Sullivan 1978). These comparisons show that *Ooencyrtus* species display a variety of strategies for surviving cold seasons. *Ooencyrtus mirus* fits in the warmer end of the

climate spectrum, with no need for diapause or supercooling in the warm climate of the Toba Tek Singh district in the Punjab province of central Pakistan.

The ability of *O. mirus* to arrest development at cold temperatures could be useful for mass rearing. The immatures could be stored at low temperatures when they were not needed, with development resumed when parasitoids were needed. With this option, parasitoids could be reared on demand with only 11 days' notice. Putting the colony in quiescence when not needed would save on the labor of continuously rearing new adults.

Effect of Temperature on Developmental Time of *Ooencyrtus* Species

Ooencyrtus mirus can develop between 18º and 37ºC with the rate of development increasing linearly from 18-36 ºC. The Wang model estimated the thermal minimum (T_{min}) for *O. mirus* at 13°C, the thermal maximum (T_{max}) at 38.3°C and the thermal optimum (T_{opt}) for immature development at 35.6°C. The thermal constant (K) for total immature development was estimated from the linear model to be 168.4 degree-days. The actual maximum temperature for development was below 38°C, lower than what the models predicted. From 20°-36°C, the actual mean developmental time determined in our studies was similar to the developmental times predicted by the linear model, suggesting a good fit of the model (Table 4-4).

Compared to *O. mirus*, *O. kuvanae*, has a lower T_{min} of 10.5°C and a higher K of 250.7 degree days (Wang et al. 2012). In *O. anasae* and *O.* sp. nr. *anasae*, the developmental rate similarly increased between 20.8° and 26.6°C. In *O. telenomicida*, the developmental rate also increased from 22° to 26°C and from 26° to 30°C (Roversi et al. 2018). In female *O. telenomicida*, the developmental times declined from 21.2 days at

22°C to 15.8 days at 26°C and 11.6 days at 30°C, all of which differed significantly from each other (Roversi et al. 2018). At 26° and 30°C, developmental times were about one day faster in *O. mirus* than in *O. telenomicida* (14.7 vs. 15.8 days at 26°C and 10.3 vs. 11.6 days at 30°C, respectively) but the developmental time for *O. mirus* at 23°C was longer than the time for *O. telenomicida* at 22°C (22.3 vs. 21.2 days, respectively). These differences may reflect that *O. telenomicida* is from a cooler climate in Russia and the Ukraine compared to the Punjab Plain in Pakistan. In *Ooencyrtus papilionis*, higher temperatures yielded faster rates of development between 15° and 30°C (Rahim et al. 1991). Likewise, in *O. kuvanae*, the developmental rate increases with temperature, in this case from 18°C through the optimal temperature of 32°C (Kamay 1976). At 32°C the average *O. kuvanae* developmental time was 14.0 days, compared to only 8.1 days in *O. mirus* at its optimal temperature of 36°C. The mean developmental time at 18°C was 38.5 days in *O. kuvanae* compared to 47.3 days in *O. mirus*, again showing adaptation to a cooler climate. At 13°C, *O. kuvanae* larvae could develop through the early pupal stage. At 35°C, no development occurred past the egg stage (Kamay 1976). In *O. mirus*, the larvae developed slowly at cold temperatures and finish developing if transferred to higher temperatures, but 38°C, rather than 35°C, was lethal. In *O. trinidadensis* from the Caribbean and southeastern U.S., developmental time decreased from 46.0 days at 18°C to 13.6 days at 33°C (Fedde 1982). Some development but no emergence occurred at 36°C. In summary, among all the *Ooencyrtus* species mentioned, developmental rate increases with temperature, but the range of developmental temperatures varies according to the climate from which the species originate, with *O. mirus* at the high temperature

end. Comparing developmental times among the species, *O. mirus* has the highest optimum temperature and a shorter developmental time than *O. anasae*, *O*. sp. nr. *anasae, O. kuvanae, O. telenomicida* and *O. trinidadensis* at their respective optimum temperatures.

Sex Ratio

In contrast to the high (97-99%) percentage of females emerged in our study regardless of temperature, for *O. telenomicida* the percentage of females increases from 49.1% at 22° to 75.5% at 26° and 77.6% at 30°C (Roversi et al. 2018). Unlike *O. mirus, O. telenomicida* is biparental. In *Ooencyrtus kuwanai* Peck (synonym of *Ooencyrtus kuvanae* (Howard), (Zhang et al. 2005)), another biparental species, the sex ratio was constant among four different temperatures (13°, 18°, 24° and 30°C), but at 35°C the male:female sex ratio was lower than at the other temperatures (Kamay 1976). The *O. mirus* sex ratio is lower than the sex ratio of these species, and less subject to change at different temperatures.

Implications of Findings for Use of *Ooencyrtus mirus* in Biological Control

Temperature can impact parasitoids and their primary hosts in ways that influence biological control in the field. For *O. mirus* and its host *B. hilaris*, our study suggests that this parasitoid could provide control over a wide range of temperatures. *Bagrada hilaris* does not develop below 20°C, but it does survive at temperatures from 22°- 40°C, with the highest survival at 24° - 35°C (Reed et al. 2017, Deep et al. 2014, Singh & Malik 1993). *Ooencyrtus mirus* survives and develops between 18° and 36°C, 2 and 4°C cooler than its host's low and high temperatures, respectively (Reed et al. 2017).

Developmental rates of both the parasitoid and host rise linearly as temperature increases from 20° to 36°C.

Ooencyrtus mirus enters an arrested state of development at low temperatures, which may enable it to survive cool seasons in the field. Both its new range in the southwestern U.S. and its home range in the Toba Tek Singh district of Pakistan have cool winters. The two coolest months of the year in both places are December and January. In Toba Tek Singh the average January temperature is 12.5°C. The hottest month, June, averages 34.8°C, with highs in the low 40°C's (Climate-Data.org). In the Imperial Valley of California (Imperial County), a hot desert region where brassicas are grown in the cool winter season, the hottest month is July, with an average high of 41°C and an average low of 24°C. The coolest month is December, with an average high of 21°C and an average low of 4°C (U.S. climate data). Although the average highs are above *O. mirus*' thermal maximum of 38°C, the temperature through most of the day is lower than the high temperature. These lower daily temperatures, in addition to the adult parasitoid's ability to seek cooler microhabitats (for example on transpiring plants, under plant debris or near irrigation water) would allow the parasitoid to survive.

In contrast to the desert, one of the most concentrated brassica-growing areas in the U.S. is Monterey County, Ca. This county has an average high of 14.3° and 14.6° in December and February, respectively, and an average low of 6.6° and 6.7°C for those same months. Even summer months are cool, with the warmest month, September, having an average high of 20.9°C and an average low of 11.6°C (U.S. climate data). *Bagrada hilaris* has been present in this area since 2013 (Grettenberger et al. 2016),

although it does not develop the devastating densities seen in the desert. Given the results of the present study, we would not expect *O. mirus* to be an effective natural enemy in this or other cool climate areas, because its survival and developmental rates are low at these temperatures.

Chapter 5

The impact of high temperature mortality of *Wolbachia* on the sex ratio of host *Ooencyrtus mirus*

Abstract

Wolbachia bacteria occur in more than half of all insect species. In Hymenoptera, *Wolbachia* often manipulates its host insect reproduction to *Wolbachia*'s advantage. *Wolbachia* is likely the reason that males are rare in the uniparental *Ooencyrtus mirus* (Hymenoptera: Encyrtidae). The percentage of male offspring can be increased by giving female parents a continuous supply of *Bagrada hilaris* (Heteroptera: Pentatomidae) host eggs for 2-3 weeks, by feeding the parents antibiotics, or by rearing parent wasps at high temperatures. The purpose of the current study is to determine whether thelytoky in *O. mirus* is due to *Wolbachia*, and if so, at what time in development the sex change occurs. We also see if *Wolbachia* removal results in gynandromorphy, as in some other hymenopterans. Finally, mating behavior is observed to see if and where it breaks down as a result of the species becoming thelytokus. Parent *O. mirus* were reared at 26, 30, 31, 32, 33, 34 and 36°C. The sex ratio of their offspring, reared at 26°C, was determined. Next, the parents were switched between 26° and 36°C during development to narrow down the critical period at which the sex change occurred. The sex ratio changed in the offspring of *O. mirus* parents reared at high temperature, even if the offspring themselves were reared at a normal temperature. The constant temperature at which the percentage of males starts to increase after 2 generations is 31°C, rising to 39% males at 33°C and

100% males at 34° and 36°C. The critical period for the change in sex ratio was toward the second half of the parent's development. The critical period was more than 48 hours in duration. Genetic tests to detect the 16S rRNA gene confirmed that female *O. mirus* contain *Wolbachia* and males do not. Examination of preserved males and male/female pairs under a microscope showed no signs of gynandromorphy. Observation of the mating behavior of live *O. mirus* under a microscope showed that males initiate courtship by drumming their antennae at a female's antennae, but after a few seconds, the females typically turn and walk away. However, a few instances of possible copulation were noted. As hypothesized, the results indicated that thelytoky in *O. mirus* is mediated by *Wolbachia* bacteria. To maximize the population growth rate without generating males, the best temperature for mass rearing this species is 30°C, not the 36°C noted in Chapter 4.

Introduction

The current study investigates whether the thelytoky in *O. mirus* is due to *Wolbachia* bacteria. This hypothesis is based on the expectation that *O. mirus* evolved from an arrhenotokus species, but the presence of live *Wolbachia* in the female reproductive organs makes the unfertilized eggs female. Transmitted from a female host to her offspring, *Wolbachia* are gram-negative α-proteobacteria (Rickettsiales: Anaplasmataceae) that occur in more than half of all insect species (de Oliveira et al. 2015). In Hymenoptera, the presence of *Wolbachia* has been found to manipulate reproduction in a way that favors propagation of the bacterium (Stouthamer et al. 1999). In some parasitoids it does this by stimulating the host to produce more females than are produced by uninfected hosts (Hunter 2020), thereby leading to parthenogenesis (Stouthamer et al. 1990). Such induction of parthenogenesis has evolved multiple times (Werren et al. 1995). In thelytokus species, the mechanism of action has been shown to be *Wolbachia* causing the failure of chromosomes to separate in the first round of mitosis, making haploid eggs diploid and thus female (Kose & Karr 1995).

If parthenogenesis in *O. mirus* is due to *Wolbachia*, then removal of the *Wolbachia* should result in a higher proportion of males. *Wolbachia* can be removed in at least three ways. The first is by feeding antibiotic-laced honey to the adult female hosts (Stouthamer et al. 1990). The second is by maintaining the host colony at a temperature that is high enough to kill the *Wolbachia* but not the insects (e.g., Stevens 1989; van Opijnen & Breeuwer 1999). A third way is to provide the parasitoids a

continuous supply of host eggs, which depletes the *Wolbachia* supply (Lindsey & Stouthamer 2017).

Besides increasing the proportion of males, in some species the removal of *Wolbachia* causes gynandromorphy to arise in some of the host offspring; e.g., in the sawfly *Diprion pini* (L.) (Hymenoptera: Diprionidae) (Pistone et al. 2014) and the moth *Ostrinia scapulalis* (Walker) (Lepidoptera: Crambidae) (Sakamoto et al. 2008). Gynandromorphy is the presence of both male and female body regions within one individual, at both the genetic and phenotypic level. While gynandromorphs are more common in Hymenoptera than in other insect orders besides Lepidoptera, they are still rare (Wilson 1962).

In this study, we checked for the effect of *Wolbachia* removal on the sex ratio by rearing *O. mirus* at high temperatures. We narrowed down the temperature at which the change in sex occurs and the critical developmental time period in which it occurs. The presence of *Wolbachia* in females and its absence in males is verified by genetic sequencing of both sexes. Male and female *O. mirus* were examined under a microscope to look for evidence of gynandromorphy. Lastly, the mating behavior of male and female *O. mirus* was observed to see how it compares to that of sexual species.

Materials & Methods

Insect rearing

Ooencyrtus mirus and *B. hilaris* were reared according to the procedures in Chapter 2. The *O. mirus* colony was established in quarantine at UCR in January, 2016 from individuals sent from the Toba Tek Singh District in the Punjab Plain of Pakistan

(Mahmood et al. 2015). The *B. hilaris* colony was started from, and occasionally refreshed with, individuals field-collected in Riverside, Ca. The *B. hilaris* colonies were reared in tent-style cages (BugDorm®-2120, MegaView Science Col, Taiwan) in greenhouses set at 24-31°C and fed fresh broccoli, canola and mizuna seedlings grown in 10 × 10 cm plastic pots. As needed for egg collection, adults were brought to an insectary room where they were kept in round Durphy cages (Durphy Packaging Co., Pennsylvania, USA) at 30 ± 1°C, 40-50% RH and 14:10 L:D and fed organic broccoli. From these cages, eggs were collected daily, glued to small pieces of card stock, and transferred to the *O. mirus* colony in quarantine.

Effect of temperature on sex ratio

Forty 1-day-old *B. hilaris* eggs were glued to a 1.27 x 4 cm piece of white card stock using Elmer's glue (Elmer's Products, Inc., Westerville, Ohio, U.S.) and placed in a 9.4 cm long × 2.2 cm diameter glass vial. Fifteen 3-day-old *O. mirus* females (F_0 generation, reared at 26°C) were added to the vial for 3.5 hours and then removed. Typical of Encyrtidae, *O. mirus* eggs have a pedicel that protrudes through the host chorion (Chapter 6, Fig. 6.4). A stereomicroscope was used to identify host eggs with exactly one pedicel. Each egg was cut out on its section of card stock and put in a gel cap (size 0, Capsuline®, Pompano Beach, Florida, U.S.). The resulting gel caps were divided evenly onto two separate ridged trays (Nordic Ware® 20.3 × 24.7 cm microwave bacon tray). One tray was placed in a growth chamber at 26°C (normal rearing temperature) and the other at 36°C. The 36°C temperature was chosen because a prior study showed it to be the temperature at which development is quickest in *O. mirus* (Chapter 4). These

procedures were repeated on two subsequent days, yielding a total of 22 single-parasitized eggs in each of the two temperature groups (26° and 36°C). The gel caps were checked once per day for parasitoid emergence. The emergence date and sex of each offspring (F_1 generation) was recorded. Males were removed. When the offspring were three days old, five new 1-day-old *B. hilaris* eggs were added for each female *O. mirus* in order to rear a third (F_2) generation of *O. mirus*. Again, single-pedicel eggs were selected. Each egg was reared at the same temperature at which its parent was reared (26° or 36°C).

Based on the results at 36°C (100% males in the F_2 generation), similar tests were conducted at 34°, 32° and 30°C to see if the percentage of males would be lower and to find the highest temperature at which mostly females emerged. For each temperature, a card of twenty-five 1-day-old *B. hilaris* eggs was placed in a vial with 25, 3-day-old female wasps (reared at 23°C and then kept at 26°C for 3 days) for 3 hours in a growth chamber at 26°C. This yielded enough single-parasitized eggs for 18 eggs per temperature. The single pedicel eggs were placed in a vial in a growth chamber at the designated temperature (one chamber at each of 34°, 32° and 30°C), all on the same day. The eggs were checked daily and when the offspring emerged, the number and sex were recorded, and the females were transferred to new vials streaked with honey for food. At 3 days of age, the females were supplied with host eggs at the rate of 5 eggs per female for 24 hours. As with the previous generation, these new eggs were checked daily and the number and sex of offspring were recorded. Based on the results at these temperatures, further tests at 31° and 33°C were conducted using the same procedures.

Results for the temperatures that yielded intermediate percentages of males (not 0 or 100%) were compared using Pearson χ^2 tests in R (R Core Team 2018).

Critical time period for sex determination

The following two tests were conducted to narrow down the critical period when exposure of a developing *O. mirus* female wasp to high temperature (36°C) changes the sex of her progeny from female to male.

Critical Period Test 1.

Three-day-old female wasps (F_0 generation) were combined with one-day-old bagrada eggs glued to card stock at a ratio of 13 wasps: 25 bug eggs in a 9.4 cm long × 2.2 cm diameter glass vial for 2.25 hours. The egg card was removed and, under a microscope, eggs with a single pedicel (F_1 generation) were selected and divided evenly into 7 groups. These procedures were repeated for two more days until each of the 7 groups had eleven single pedicel eggs. Parasitized eggs from different days were kept separate. For each collection day, the vials were labeled 1 through 7, along with the date. The #1 vial was placed in a 36°C growth chamber and the other six vials in a 26°C growth chamber. Forty-eight hours later, the #1 vial was transferred to 26°C and the #2 vial was transferred to 36°C. At the same time at each subsequent 48 hours through day 12, the vial at 36° was returned to 26° and the next numbered vial was transferred to 36°. Since the developmental time is 14-15 days at 26°, this gave each group a chance to be at 36° for a different 48 hours, or about 1/7 of development time.

Once the adult wasps emerged (mostly or all female), the number and sex were recorded. After 3 days they were provided 1-day-old *B. hilaris* eggs at the rate of 5 host

eggs/female wasp for 24 hours. Again, the eggs with one pedicel (F_2 generation) were separated out. They were reared in glass vials at 26°C until the offspring emerged. The number and sex of these F_2 adults was recorded.

Critical Period Test 2.

Because the F_2 generation of Critical Period Test 1 was 100% female, a second critical period test was conducted, with 1/2 instead of 1/7 of the developmental time at 36°C. The F_1 single pedicel eggs were divided into four groups: A) 36°C constant; B) 36°C for four days (half the total developmental time at 36°C, per Chapter 4), and then transferred to 26°C; C) 26°C for seven days (half the developmental time at 26°C) and then transferred to 36°C; and D) 26°C constant. Procedures for the F_2 generation were the same as in the first critical period test, with the F_2 generation reared at 26°C.

***Wolbachia* detection**

We screened for the presence of *Wolbachia* in male and female *O. mirus* using a diagnostic polymerase chain reaction (PCR) based on the bacterial 16s gene. Male and female *O. mirus* adults were obtained from a separate study on the fecundity of this species (Chapter 3). Additional males were obtained by rearing parents at 36°C, and other males emerged from *Euschistus conspersus* host eggs in the alternate host study (Chapter 6). The reason why *E. conspersus* host eggs gave rise to males is not known, and may be random. The wasps were euthanized in 95% ethanol in microcentrifuge tubes. DNA was extracted using an established Chelex resin-based method. The wasps were moved from the ethanol onto sterile filter paper to let the ethanol evaporate, after which they were placed, three per tube, into clean tubes containing 2 µl proteinase K

(>600 mAU/ml; QIAGEN #19131) and ground using a sterile glass pestle. Following maceration, 60 µl of a 5% suspension of Chelex® 100 (Bio Rad, Hercules, CA) in water was added. The reactions were incubated in a water bath at 55°C for 1 hour and then in a second water bath at 99ºC for 10 minutes. Each sample then was spun at 14,000 rpm for 4 minutes to pelletize the Chelex resin, and 50 µl of the DNA-containing supernatant was transferred to a new tube. Extracted DNA was stored at -20°C until used in the PCR.

Diagnostic PCRs were conducted in 25 µl volumes containing 1x Thermopol Buffer (New England Biolabs, Ipswich, Massachusetts, U.S.), 1.5 ml BSA (10 mg/ml; New England Biolabs), and 0.4 µM each of the primers W-Specf/W-Specr (Werren & Windsor 2000). A master mix was prepared using 108 µl dd H_2O plus the following µl volumes of 10 µM solutions: 25 buffer, 15 BSA, 10 each of $MgCl_2$, 2 Taq polymerase and 50 dUTP. The $MgCl_2$ primers and buffer were vortexed for 1 second before adding. Master mix (23 µl) was added to each supernatant tube plus to another tube as a negative control and another to which a known DNA sample was added as a positive control. The tubes were centrifuged and then incubated for 2 minutes in the thermocycler (Eppendorf Mastercycler) at 94ºC. They were further incubated for 42 cycles of denature/anneal/extension, starting at 95/60/68°C, respectively. The annealing temperature was decreased in each successive cycle. The presence/absence of PCR products was determined using standard gel electrophoresis.

Gynandromorphy

Forty-one males and 28 male-female *O. mirus* pairs, all sourced from adult *O. mirus* females exposed to a continuous supply of *B. hilaris* eggs for two weeks or more, were euthanized in 95% ethanol in microcentrifuge tubes. Within each pair, the male and female were siblings that emerged on the same day in the same vial. Each preserved individual was checked under a Leica Wild M10 stereomicroscope for gynandromorphy. The features checked included setal length on both antennae, abdomen coloration, and genitalia. Male antennal setae are longer than the antenna is wide, whereas female setae are shorter than the antennal width. The female abdomen has "at least the proximal half of the gaster [bright] yellow" (Triapitsyn et al. 2020), whereas the male abdomen has subtle bands of black and pale yellow. The female ovipositor rests in a longitudinal groove in the abdomen, whereas the male genitalia does not. The triangular tip of the ovipositor extends only slightly beyond the tip of the abdomen, whereas the male genitalia extends well past the tip of the abdomen.

Mating behavior

Males and females were separated within 24 hours after emergence. Three male *O. mirus* were added to a vial with three female *O. mirus* and observed under a stereoscope for 10 minutes. This was repeated for a total of 12 observation times with fresh males and females. The females were 3 days old but the males were different ages in different observation times, including 0, 1, 2, 3 and 4 days old. At least two sets of three males of each age were observed.

Results

Effect of temperature on sex ratio

Female parents reared at constant 26° and 30°C produced 0% males (Fig. 5-1). A low percentage of males emerged from parents reared at 31° and 32°C, not significantly different from each other (P = 0.098, df =1). The percentage of males at 33°C was significantly higher than at both 31° and 32°C (P = 0.009 and 7.09e-06, respectively; df = 1). Parents reared at 34° and 36°C produced 100% male offspring. In summary, the percentage of male offspring increased from 0 to 100% as the parental rearing temperature increased from 30° to 34°C.

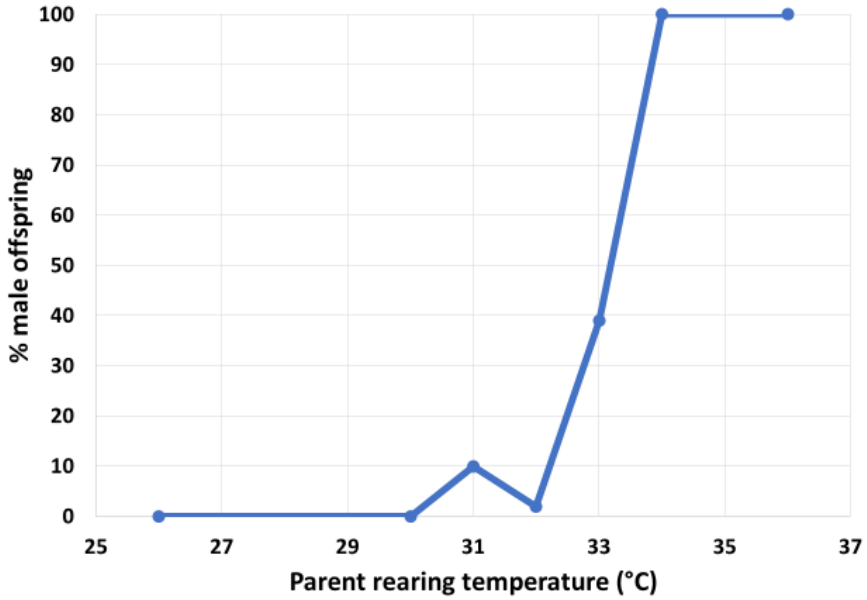

Fig. 5-1. Percent male *Ooencyrtus mirus* offspring from parents reared at different temperatures.

Critical time period for sex determination

For the first critical period test, the F_2 generation sets #1-7 produced 47, 48, 29, 41, 39, 49 and 27 females, respectively, and 0 males. In the second test, the Group A F_2 (from F_1 reared at constant 36°C) was 100% male (Table 5-1). The Group B F_2 (from F_1

reared at 36°C for the first half and 26°C for the second half) was 76% female and 24% male. The Group C F_2 (from F_1 reared at 26°C for the first half and 36°C for the second half) was 100% male, and the Group D F_2 (from F_1 reared at constant 26°C) was 100% female.

Table 5-1. *Ooencyrtus mirus* Critical period test #2 results: Number of female and male offspring of parents that were reared under different temperatures. Parent rearing temperatures: **A**. constant 36°C. **B**. 36°C for the first half of development and 26°C for the second half. **C**. 26°C for the first half of development and 36°C for the second half. **D**. constant 26°C. The offspring (F_2) were reared at 26°C in all four groups.

group	Number of female offspring	Number of male offspring	% female	% male
A	0	26	0	100
B	34	11	76	24
C	0	20	0	100
D	25	0	100	0

Wolbachia detection

Whether they were sourced from 36°, a continuous supply of eggs, or *E. conspersus*, the males tested negative for *Wolbachia* (Fig. 5-2 A). None of the 9 male lanes shows a blot at the same position as the positive control for *Wolbachia*. The females tested positive for *Wolbachia.* The female lanes in Fig. 5-2 B all show blots at the *Wolbachia* positive control position.

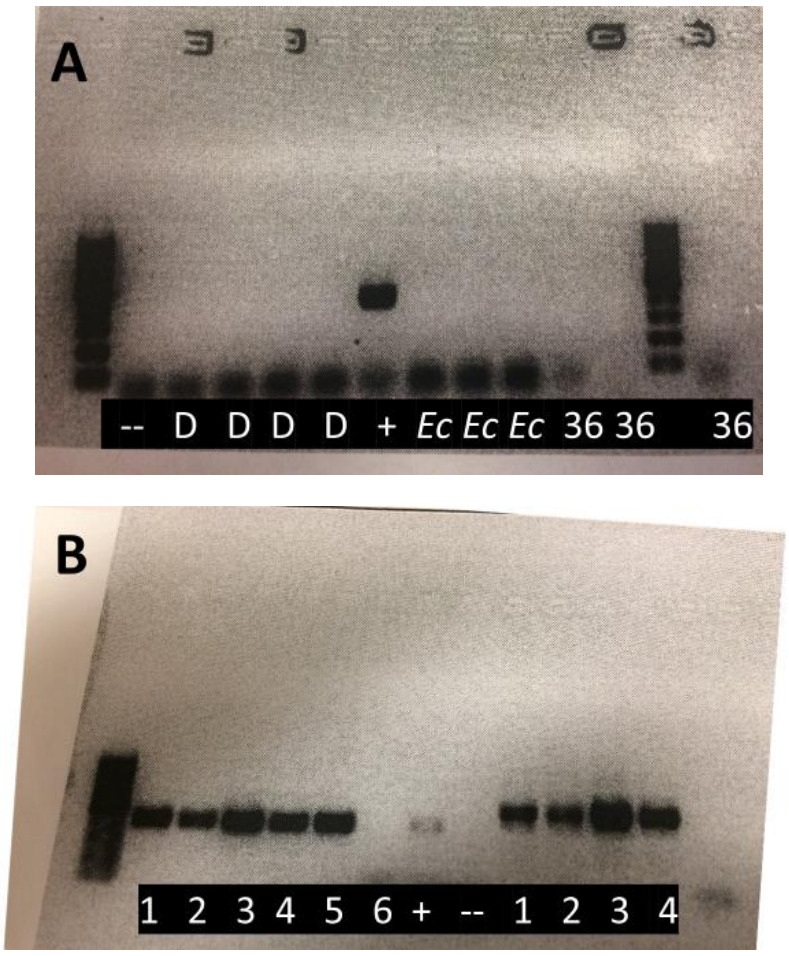

Fig. 5-2. Gel plates testing for *Wolbachia* 16S gene in **A**) Male and **B**) Female *Ooencyrtus mirus*. **D** = depleted *Wolbachia* (males from females reared with constant supply of host eggs for 2+ weeks), + = positive control, -- = negative control, ***Ec*** = males reared on *Euschistus conspersus* host eggs, **36** = males reared at 36°C for two generations.

Gynandromorphy

For each trait examined, all males had male characters and all females had female characters. No gynandromorphy was observed in the 97 individuals (69 males and 28 females) examined.

Mating behavior

The most common interaction was for a male to approach a female head-on and drum his antennae on or under the female's antennae. Within less than a second or up to 6 seconds, the female turned and walked away. Sometimes the male would chase the female as she walked away. On one occasion, when two males were pursuing the same female, one male tried to push the other away. In another observation, a male rocked side-to-side while antennating the female. Yet another time, two males antennated each other. Sometimes a male would pursue a female and drum his antennae on her abdomen from behind.

The newly emerged males had shorter and less frequent encounters with females than the older males did. On two different occasions, a male attempted to rear-mount a female. Nine different times, a male was observed touching the tip of his abdomen to the tip of the female abdomen for a second or two, at 180° angle to the female with his head pointed away from her. After one of these times, the male moved backward quickly, antennae facing downward and still, and then stood in place and twitched for a few seconds, followed by grooming. The behavior preceding grooming starkly contrasted the usual male behavior, in which the males move forward, antennae facing forward and drumming rapidly.

Discussion

Effect of temperature on sex ratio

This study suggests that *Wolbachia* is active in *O. mirus* at temperatures up through 32°C (Fig. 5-1). Based on the increase in males at 31-33°C, the *Wolbachia*

started to die off or were impaired in their ability to manipulate the sex of the parasitoid offspring at these temperatures. The *Wolbachia* did not survive at 34°C and higher. According to the Chapter 4 study on temperature, *Ooencyrtus mirus* survives up through 37°C; thus it tolerates higher temperatures than its symbiotic *Wolbachia*. The sex ratio results differ from those for *Ooencyrtus submetallicus* (Howard) in Florida, U.S. in which unmated *O. submetallicus* produced males (Buschman & Whitcomb 1980). However, our results parallel those of Wilson & Woolcock (1960) for *O. submetallicus* (Howard) sourced from Trinidad Island in the Caribbean Sea, except that the critical temperature for *O. mirus* is much higher. In *O. submetallicus*, the second generation was 100% female at 28.0°C and 100% male at 29.4°C. *Ooencyrtus mirus* had a wider transition range, with both sexes of offspring from mothers reared at 31-33°C, and 34°C the lowest temperature that produced 100% males. As with *O. mirus*, the *O. submetallicus* parent generation reared at the high temperature was still mostly, or all female; only the offspring of that generation were male. The *Ooencyrtus mirus* - *Wolbachia* temperature relationship is more similar to that of *Ooencyrtus pityocampae* (Merc.) in Israel. The offspring of *O. pityocampae* larvae kept at constant temperatures through 32°C were female, with both male and female offspring arising from larvae kept at 32.5 - 33°C, and only male offspring emerging from larvae at kept at 34°C or higher (Halperin 1990). Likewise for *Trichogramma semifumatum* (Perkins), but at lower temperatures, the offspring were almost all female when the parents were reared below 25.6°C, but 97% sterile males and 3% gynandromorphs when the parents were reared at 32.2°C (Bowen & Stern 1966). Thelytokus *Trichogramma pretiosum* Riley have 1% male offspring at

28.26°C and 40% males at 31°C (Pintureau & Bolland 2001), whereas thelytokus *Trichogramma cordubensis* Vargas & Cabello produce 29% males at 28.26°C and 100% males at 31°C (Pintureau & Bolland 2001). In summary, heat-killing of *Wolbachia* can occur in many hymenopteran species, but the minimum lethal temperature varies among *Wolbachia* in different host species. For all the aforementioned species, the temperature during the female parent development determines the sex of her offspring, with high temperature leading to males and moderate temperature for each species leading to females. This implies that the offspring start to form even while the parent is a larva or pupa itself. In spite of this, however, *O. mirus* does not start ovipositing immediately after eclosion, and does not reach full oviposition capacity until 3 days after eclosion, even though the eggs laid before 3 days of age have as high survival as eggs from parents aged 3 days and older (Chapter 2).

The effect of heat on *Wolbachia* can explain why, within the same or closely related species, the male:female ratio often tends to be higher in warmer latitudes (Pelesneer 1926). One example of this, noted by Wishart (1938) is that *Macrocentrus ancylivorous* populations was 42% male in New Jersey, USA (Holloway 1934) but only 35.5% male in higher latitude Ontario, Canada populations (WE van Steenburgh 1934, unpublished data, in Holloway 1934). In the lab, Wishart (1938) found that mated *Chelonus annulipes* Wesm. (Hymenoptera: Braconidae) parents reared at 32.2°C had 85% male offspring, but parents reared at 21.1°C had only 34% male offspring. *Ooencyrtus mirus* fits with this pattern, having a higher male:female ratio at higher temperatures, at least in the lab.

Critical period

The critical period appears to be more than two days in duration, as seen by the first critical period test yielding all female offspring. The critical period also seems to be toward the middle or end of the developmental time, since all of the F_2 offspring of parents subjected to 36°C in the second half of development were male, but only about 1/3 of offspring of parents subjected to 36°C in the first half of development were male. While beyond the scope of this study, more tests with varying times at 36°C could further narrow down the critical period. Bowen and Stern (1966), for example, narrowed down the critical period for *Trichogramma semifumatum* (Perkins) to the pupal stage of the parent. The need for more than two days of high temperature to induce production of males may explain how the species can survive (i.e., not become 100% male) in the hot climate in Pakistan. Even in the hottest months, the average low temperatures are below 29°C (Weather Spark) due to the dry air. However, the average daytime high has a summer peak at 41°C, so *O. mirus* may go dormant or seek out cooler microclimates during that time, unless they can endure short periods at such high temperatures. They die at a constant temperature of 38°C in the lab (Chapter 4).

Halperin (1990) took the temperature transfer a step further with *O. pitycampae*. Females bred at 34°C were transferred to 30°C for oviposition, presumably over multiple days. First males emerged, then both males and females, and then females only. Laraichi (1978) did a similar study on *Ooencyrtus fecundus* Ferriere & Voegele. Like *O. mirus*, *O. fecundus* is an egg parasitoid of a pentatomid, in this case *Aelia cognata* Fieber. (Laraichi 1978). Parents were reared at 30° or 35°C and then switched to the opposite

temperature after emergence. The rearing temperature determined the offspring sex for the eggs laid by the parent during her first three days of oviposition. After that, though, the opposite sex offspring appeared in increasing proportions. Since the *O. mirus* in our study were given access to eggs for only one 24-hour period, it remains to be determined if the F_2 sex ratio would stay the same if the females had access to eggs on subsequent days. In *O. kuvanae*, the opposite temperature effect was seen by Kamay (1976). Initial exposure to 35°C, followed by rearing at 24°C resulted in only 21% males, compared to 45% males reared at constant 24°C. The effect was within the generation exposed to high temperature, not on the offspring. In this case, a different physiological mechanism than the suppression of *Wolbachia* appears to be at work.

As occurs in *Trichogramma*, the *Wolbachia* likely makes haploid eggs female by inhibiting chromosome separation during anaphase of the first round of mitosis (Stouthamer & Kazmer 1994; Kose & Karr 1995). *Ooencyrtus mirus* likely evolved from an arrhenotokus species that shifted to theyltoky because *Wolbachia* induced greater proportions of female offspring than were produced by uninfected *O. mirus* hosts (Hunter 2020).

Gynandromorphy

Out of 816 *O. submetallicus* first through fifth generation offspring reared at 21.1°, 26.7° and 28.1°C (70°, 80° and 82.5°F) that were sexed, Wilson and Woolcock (1960) found less than 1% males and no gynandromorphs. At 29.4°C (85°F), however, they found 20 gynandromorphs along with 78 males and 18 females in the second

through fifth generations. In contrast, *O. mirus* had no gynandromorphs out of 41 males and 28 male/female pairs, all 2nd generation, that were checked.

Mating behavior

Compared to *O. kuvanae*, a haplo-diploid species, the *O. mirus* male typically undergoes only the first step of the courtship ritual, engaging his antennae with the female's antennae. The timing of the antennation is shorter in *O. mirus*, usually 0-4 seconds and maximum 6 seconds before the female turns and walks away; in *O. kuvanae*, antennation lasts 4-23 seconds (Ablard et al. 2011). The antennation itself is different in that *O. kuvanae* male antennae surround and lock the female antennae . In response, the female's antennae drop and point downward. After leg strikes by the male, the female goes into a "trance" (Ablard et al. 2011). In *O. mirus*, the antennae of both sexes keep moving and no "trance" is induced. Earlier observations on the mating behavior of *O. kuvanae* by Alzofon (1984) included the male contacting the female with his antennae and then walking around her until they were facing each other and touching antennae. The female would turn side-ways and the male had to follow her, remaining head-to-head, or she would walk away. In *O. kuvanae*, the male mounts the female from behind while still facing forward. In *O. mirus*, the male sometimes went tail-to-tail with the female for a few seconds. In spite of the quickness, sperm may have been transferred, if *O. mirus* males are as quick as *Trichogramma evanescens* Westwood (Hymenoptera: Trichogrammatidae) males. In the latter species, the mean duration for the first time a male copulates is under 2 seconds, and the mean is under 3 seconds for the next 7 copulations (Damiens & Boivin 2005). The unusual behavior of one male after being

tail-to-tail with a female (walking backwards, putting antennae down and not drumming, and twitching its body) may indicate that copulation occurred. The post mating somewhat resembles that of *Cephalonomia tarsalis* (Ashmead) (Hymenoptera: Bethylidae). In this species, after the male dismounts, the two wasps separate from each other immediately, and then begin grooming (Cheng et al. 2004). In the parasitoid *Cotesia urabae* Austin & Allen (Hymenoptera: Braconidae), as well, copulation is usually followed by stationary or grooming behavior (Avila et al. 2017). Further study on *O. mirus* could show whether it is still capable of reverting to arrhenotoky, as shown in four *Trichogramma* spp. (Stouthamer et al. 1990), or whether, as in most natural populations having parthenogenesis-inducing *Wolbachia*, sexual reproduction is no longer possible (Stouthamer et al. 2010).

Conclusions

The increase in the male: female sex ratio of *O. mirus* offspring from parents reared at high temperatures, combined with the presence of *Wolbachia* in females but not in males, confirm that the thelytoky in *O. mirus* is due to reproductive manipulation by *Wolbachia*. The change occurs mainly during the second half of the development of the parent wasp. Unlike in some parasitoid species, the loss of *Wolbachia* does not cause gynandromorphy in *O. mirus*.

Although *O. mirus* reproduces most quickly at 36°C (Chapter 4), the current study indicates that, above 30°C, the male: female ratio increases due to the depletion of *Wolbachia*. Since the species is parthenogenetic, males are undesirable. The best temperature for mass rearing this species thus appears to be 30°C. Further testing

generations would be needed to ensure that the offspring continue to be mostly female in successive days and subsequent generations at this temperature.

The mating behavior and males in *O. mirus* appear to be artefacts from when the species was haplo-diploid. Further study could determine if mating can still occur and if the eggs can be fertilized; i.e., if sexual reproduction is still possible in *O. mirus*.

Part II. Ecology of *Ooencyrtus mirus* as a potential classical biological control agent

Chapter 6

Evaluation of the physiological host range of *Ooencyrtus mirus*

Abstract

In an effort to find a biological control agent of *Bagrada hilaris* (Burmeister) (Heteroptera: Pentatomidae), an invasive pest on brassica crops in North America, three hymenopteran egg parasitoids were recovered from brassica plant debris in Pakistan and sent to California, USA for evaluation. One of these has recently been described as *Ooencyrtus mirus* Triapitsyn & Power (Hymenoptera: Encyrtidae). Adult females of this species were exposed to the eggs of eight alternate pentatomid host species, two non-pentatomid heteropterans, and two lepidopterans, in choice and no-choice tests. Although it was more successful on *B. hilaris* than on the other species, *O. mirus* was able to reproduce on all the alternate hosts except for one of the lepidopterans, whose eggs appeared too small for this parasitoid. The results show *O. mirus* to be a generalist parasitoid species with a preference for *B. hilaris*. The implications of this study on the release of *O. mirus* for the control of *B. hilaris* are discussed.

Introduction

The painted bug, also known as bagrada bug, *Bagrada hilaris* (Burmeister) (Hemiptera: Heteroptera: Pentatomidae) is a serious pest on brassica crops (Reed et al. 2013). In its native range of Africa, Asia and the Middle East (Howard 1907; Husain 1924), it is one of the major pests of leaf mustard (*Brassica juncea* (L.)) and oilseed brassica crops such as rapeseed and canola (*Brassica napus* L.) (Sachan and Purwar

2007; Abrol 2009; Bundy et al. 2018). *Bagrada hilaris* first appeared in the United States (U.S.) in Los Angeles County, California (Ca.) in 2008 (Arakelian 2008). By 2015, it had spread to 24 other counties as well as four contiguous states plus Hawaii (Palumbo and Natwick 2010; Bundy et al. 2012; Vitanza 2012; Perring et al. 2013; Reed et al. 2013; Matsunaga 2014) and southward into 6 states in Mexico (Sánchez-Peña 2014; Torres-Acosta and Sánchez-Peña 2016). Active mainly in the warm season, *B. hilaris* nonetheless infests cool-season crops by attacking cole crop seedlings in early fall after living on wild mustard weeds in the summer. The feeding damage often kills the seedlings, reaching up to 60% mortality (Reed et al. 2013) and leading to incomplete stands of the crop (Palumbo and Natwick 2010; Palumbo et al. 2016).

Current management of *B. hilaris* integrates multiple strategies that may include transplanting instead of direct seeding, altering planting dates, destroying crop residues, and controlling mustard weeds in the summer (Palumbo et al. 2016). Other recommended techniques include cultivating the soil frequently during the growing season to destroy *B. hilaris* eggs, reducing nitrogen fertilization, vacuuming the bugs, excluding them by growing the crops under floating row covers, and employing brassica trap crops (Palumbo et al. 2016; Bundy et al. 2018). The principal control method, however, is by application of pyrethroid and neonicotinoid insecticides (Palumbo and Carriere 2015). Frequent application of insecticides increases the risk of pesticide resistance, as it applies more selection pressure on the pest population (Kunz and Kemp 1994). Insecticides also can deplete natural enemy populations, increasing the likelihood of an outbreak of the target or secondary pests. Worker safety, environmental concerns

and harm to honeybees (Kiljanek et al. 2016), along with the cost of using insecticides and the economic importance of brassica crops, are further factors that instigated an effort to expand integrated pest management (IPM) options for *B. hilaris*. For this purpose, a search for egg parasitoids useful in biological control was initiated. Egg parasitoids are particularly desirable because they decrease the pest population before it damages the crop plants.

In 2014, Walker Jones (Research Entomologist, United States Department of Agriculture-Agricultural Research Service (USDA-ARS) Biological Control Laboratory, Stoneville, Mississippi, U.S.), collaborated with three Pakistani entomologists to send parasitoids obtained from *B. hilaris* eggs in Pakistan to the U.S. The parasitoids were from bagrada eggs recovered from plant debris of leaf mustard and canola in the field (Mahmood et al. 2015). Three hymenopteran egg parasitoid species were sent from Pakistan to Jones' lab. One of these, recently described as *Ooencyrtus mirus* Triapitsyn & Power (Triapitsyn et al. 2020) is herein evaluated as a potential biological control agent.

For an exotic species to be released in the field in the U.S., a permit must be obtained from the USDA Animal and Plant Health Inspection Service (APHIS). The agency considers host specificity as a major factor in deciding whether to allow the release of a potential biocontrol agent. To that end, the objective of the current study was to determine the host specificity of *O. mirus* by exposing adult females to the eggs of *B. hilaris* and alternate host species.

The first step in determining the overall host range of a potential control agent is to understand the physiological host range; i.e., species on which the parasitoid can survive and develop successfully under controlled conditions in the lab (McEvoy 1996; Onstad and McManus 1996). This may be followed by behavioral and ecological host range studies to determine which of the physiological hosts the parasitoid may choose and succeed on in the field (Onstad and McManus 1996). The current study was conducted to determine the physiological host range as well as whether *O. mirus* chooses *B. hilaris* over other host species under laboratory conditions.

Materials and methods

Host sources and rearing

Bagrada hilaris

Bagrada hilaris colonies were reared on the UCR campus in tent-style insect cages (BugDorm-2120, MegaView Science Col, Taiwan) inside two greenhouses at 30 ± 5°C with ambient humidity and light. They were fed broccoli (*Brassica oleracea* L. var. *italica* Plenck), canola (*Brassica napus* L.) and mizuna (*Brassica rapa* L. var. j*aponica*) seedlings grown in 10 × 10 cm square plastic pots. Adults for experiments were transferred to an insectary room set at 30 ± 1°C, 40-50% RH and 14:10 L:D. Thirty bugs were placed into each of 8-10 round, 15 cm diameter × 6.3 cm height plastic Durphy boxes (Durphy Packaging Col., Pennsylvania, U.S.), with two, 2.5 cm screened holes opposite each other in the sides for ventilation (Chapter 3, Fig. 3-1). A piece of white paper towel (Brawny®) was cut and placed in the bottom of each box. Daily, the adults were transferred to new cages and supplied with fresh organic broccoli florets. *Bagrada*

hilaris eggs for experiments were removed gently from the plastic cages with a paint brush and gently rubbed off the paper towel by hand.

Alternate hosts

The alternate hosts studied included nine pentatomid species: the invasive pests *Halyomorpha halys* Stål, *Murgantia histrionica* (Hahn), and *Nezara viridula* (L.), the beneficial predator *Podisus maculiventris* (Say) (Fig. 6-1), the native pests *Chlorochroa ligata* (Say), *Chlorochroa uhleri* (Stål), *Euschistus conspersus* Uhler, *Euschistus servus* (Say), and *Thyanta pallidovirens* (Stål). Two other heteropterans, *Jadera haematoloma* (Herrich-Schäffer) (Rhopalidae) and *Anasa tristis* (DeGeer) (Coreidae), and two lepidopteran species, *Helicoverpa zea* (Boddie) (Noctuidae) and *Ectomyelois ceratoniae* (Zeller) (Pyralidae) also were tested. All these species live in the geographic region where *O. mirus* would be released as a biocontrol agent. All except *J. haematoloma* and *P. maculiventris* are agricultural pests. *Podisus maculiventris* was chosen because it is a beneficial species that preys on other insects. *Jadera haematoloma* was chosen as a representative native, non-pest species. Unlike all the other species in this study, no parasitoids have been recorded at any stage of the *J. haematoloma* life cycle, noted by by Carroll in 1988 and confirmed by my not finding any records of parasitism in the literature as of 2020. *Anasa tristis* was collected in squash fields grown at the Agricultural Operations Facility (Ag Ops) at UCR. *Chlorochroa ligata, C. uhleri, E. servus, N. viridula* and *T. pallidovirens* were collected from an alfalfa field at Ag Ops. Once established in the insectary, the colonies were occasionally refreshed with wild individuals. *Jadera haematoloma* was collected under trees on the UCR campus and

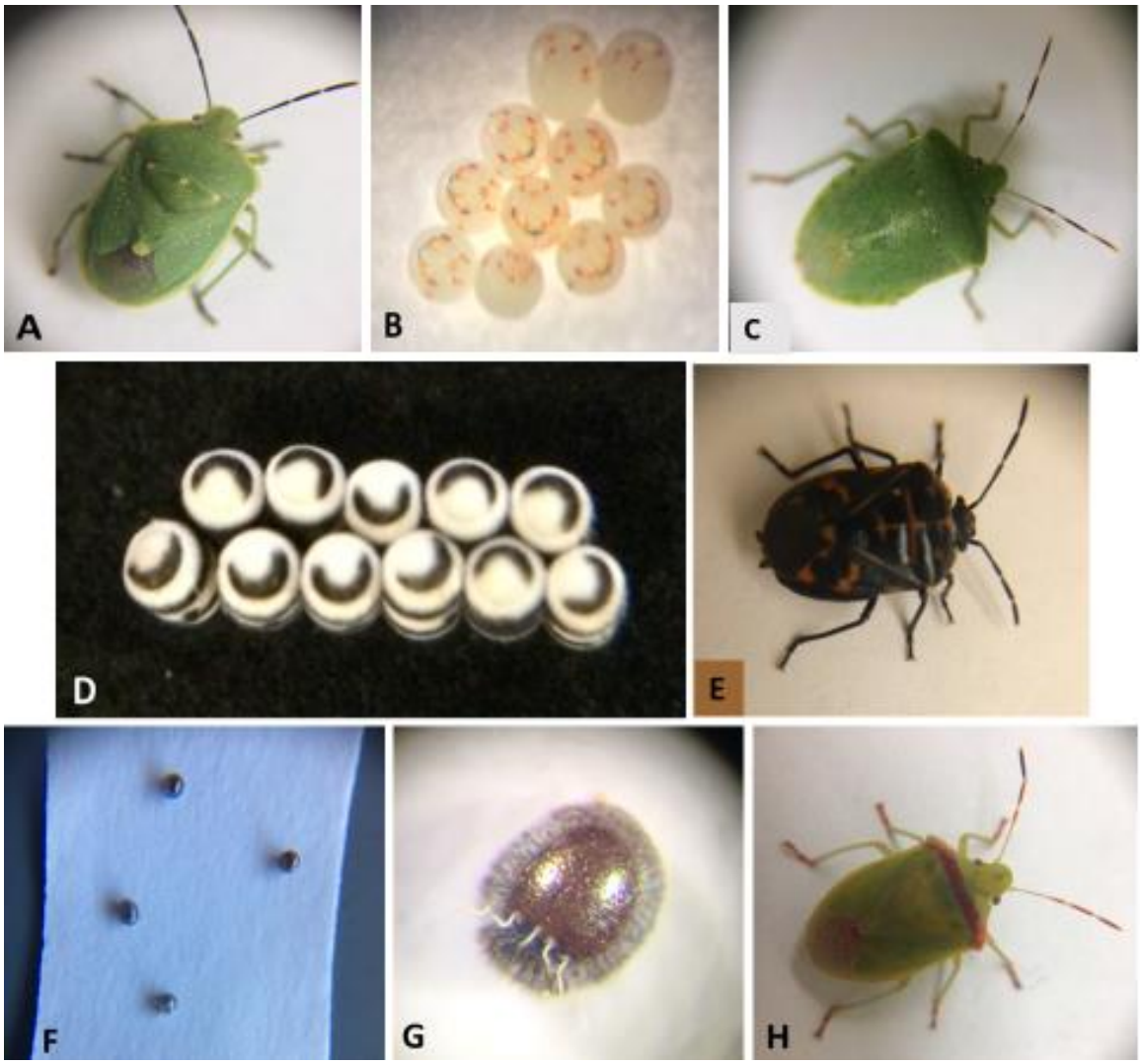

Figure 6-1. Some of the alternate hosts exposed to the parasitoid *Ooencyrtus mirus*. All photos were taken with a macro lens on a cell phone camera or under a stereomicroscope. **A**. *Chlorochroa uhleri* adult. **B**. *Euschistus* sp. eggs. **C**. *Nezara viridula* adult. **D**. *M. histrionica* eggs. **E**. *Murgantia histrionica* adult. **F**. *Podisus maculiventris* eggs glued to a card. **G**. Close-up of a *P. maculiventris* egg. **H**. *Thyanta palllidovirens* adult.

under bladderpod bushes (*Peritoma arborea* (Nuttall): Cleomaceae) in the UCR Botanical Gardens. *Murgantia histrionica* was collected from leaves of those same bushes. *Ectomyelois ceratoniae* were obtained from dates from the Coachella Valley in

Riverside County, Ca. The *E. conspersus* colony was started from eggs sent from Brian Hogg at the USDA-ARS lab in Albany, Ca., and *E. servus* was found in a park near UCR.

Most of the alternate host species were reared in the same insectary room as the *B. hilaris* adults, in the same size Durphy boxes, but with a screened hole in the lid as well as the two holes in the side. In addition to the white paper towel in the bottom, the alternate host adult cages had half of a folded brown paper towel laid on top of the vegetables to absorb waste and provide a hiding place and an ovipositional substrate. *Helicoverpa zea* was reared in the Ring Cardé lab (UCR Entomology Department).

Podisus maculiventris eggs were purchased from Entomology Solutions, LLC, Louisville, Kentucky, U.S., arriving the day after they were laid and used immediately for the host tests. *Anasa tristis* and initially *M. histrionica* were reared in the lab at 23°C instead of in the insectary. The *M. histrionica* colony was transferred to the 30°C insectary part way through the host tests, to see if they would lay more eggs. Enough *J. haematoloma* eggs were obtained from newly collected individuals from the field that no offspring were reared past the egg stage. For all the alternate host species, the cages were checked daily; new eggs were collected and fresh food was supplied as needed. The adult bugs were transferred weekly to clean Durphy boxes with new paper and all new food. *Halyomorpha halys* adults were collected in northern and southern California and transferred into Quarantine at UCR under CDFA (Ca. Dept. of Food and Agriculture) permit #3020. Colonies were established in 61 × 61 × 61 cm ventilated cages. The *H. halys* colonies were founded and reared in quarantine by the Mark Hoddle lab (UCR Entomology Department). The alternate host diets are listed in Table 6-1.

Table 6-1. Alternate host diets.

Alternate host	Colony diet
Anasa tristis	*Cucurbita moschata* Black Futsu squash fruit and fresh squash leaves grown on and near the UCR campus
Chlorochroa ligata *Chlorochroa uhleri*	Green beans, peanuts, sunflower seeds, Russian thistle (*Salsola tragus*) bouquet, alfalfa (*Medicago sativa*) bouquet
Ectomyelois ceratoniae	Lab-prepared mixture of soy meal, sugar and water
Euschistus conspersus	Alfalfa bouquet, green beans, peanuts, sunflower seeds, and pistachios, broccoli floret. The first 5 reps also had a live, potted tomato plant and squash leaf bouquet.
Euschistus servus	Alfalfa bouquet, green beans, peanuts, sunflower seeds
Halyomorha halys	apples, avocados, carrots, grapes, green beans, and fresh cuttings of *Paulownia tomentosa (*empress tree) and *Buddleja davidii* (butterfly bush)
Helicoverpa zea	Lepidoptera Diet – Product "F9772-Tray," Frontier Agricultural Sciences
Jadera haematoloma	*Peritoma arborea* (bladderpod) bouquet
Murgantia histrionica	*Peritoma arborea* (bladderpod) bouquet
Nezara viridula	Fresh organic green beans, shelled peanuts, sunflower seeds
Podisus maculiventris	N/A
Thyanta pallidovirens	Fresh organic green beans, peanuts, sunflower seeds, broccoli floret

"Bouquet" refers to fresh stems with leaves (and for alfalfa, sometimes flowers) in a floral water pick (i.e., tube or vial). The Russian thistle (*Salsola tragus* L.) and alfalfa (*Medicago sativa* L.) were collected from the UCR campus and Ag Ops, respectively. "Peanuts" refers to organic, blanched, shelled, raw peanuts; "sunflower seeds" refers to several organic, raw, hulled sunflower seeds glued to a 2.5 x 2 cm piece of card stock with Elmer's® glue (Elmer's Products, Inc., North Carolina, USA); "pistachios" refers to raw, shelled pistachio nut meats; and "green beans" refers to fresh, organic green beans.

Parasitoid rearing

To provide ovipositional hosts for *O. mirus*, approximately 40 *B. hilaris* eggs were glued onto a 1.27 cm × 4.2 cm piece of card stock; these egg cards were provided to the parasitoids each day. The eggs were placed in a 9.4 cm long × 2.2 cm diameter glass

vial with ten or eleven 3-day-old adult females of *O. mirus* and a cotton plug was inserted in the open end. After 24 hours, the wasps were aspirated out, and the vial of parasitized eggs was placed on a ridged tray (Nordic Ware® 20.3 × 24.7 cm microwave bacon tray) and kept at room temperature (22-23°C) under natural light until the new *O. mirus* adults emerged. Each day, newly-emerged wasps were aspirated into a glass vial streaked with honey and placed in a Percival growth chamber (model I30BLL, Perry, Iowa, USA) at 26 ± 1°C, 50% RH and 14:10 L:D until they were ready to be used for the next generation or for testing at 3 days of age.

Experimental Procedures

Ooencyrtus mirus egg parasitism was compared between *B. hilaris* and each of the alternate hosts in choice and no-choice tests, except for *E. ceratoniae* which was evaluated only in no-choice tests. Each choice test replicate consisted of a card (1.27 × 4.2 cm piece of white card stock) of 10 randomly-placed, 1-day-old *B. hilaris* eggs and a card of 10 clustered, 1-day-old alternate host species eggs. The placement of eggs mimicked how the eggs are laid by each species: for the pentatomid alternate hosts, the eggs were placed in a single-layer cluster, with all eggs touching other eggs. For the other two heteropterans, the eggs were near each other but not every egg was touching other eggs. For *H. zea*, the eggs were left on the paper towel on which they were laid. A line was drawn with pencil around a group of 10 eggs, and the towel was cut along the line. Usually the eggs were near but not touching each other. The *B. hilaris* card and the alternate host card were placed back-to-back in a 9 cm long × 1 cm diameter glass vial and a cotton plug was inserted in the open end (Fig. 6-2 A).

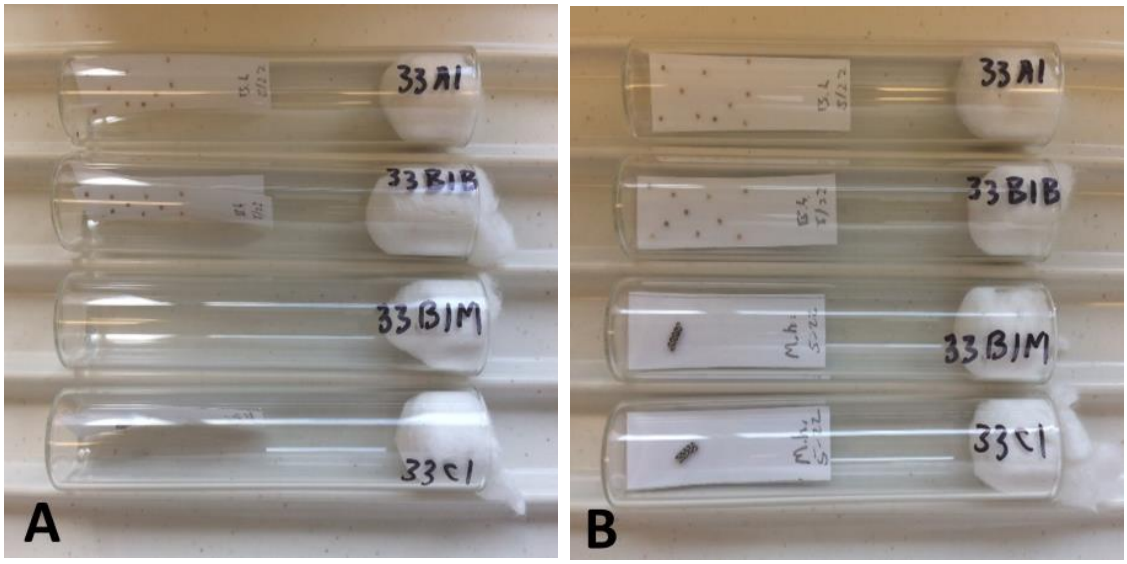

Fig. 6-2. One replicate of choice (middle two vials) and no-choice (top and bottom vials) tests with *M. histrionica*. **A**. Position of the egg cards while an *Ooencyrtus mirus* adult was present. **B**. After the wasp was removed, the choice cards were placed in separate vials. The top two vials each have 10 *Bagrada hilaris* eggs, and the bottom two each have 10 *M. histrionica* eggs.

The choice cards were put in what would become the *B. hilaris* vial in half the replicates and in what would become the alternate host vial in the remaining replicates. A 3-day-old *O. mirus* adult female was added to the vial. The vials were placed horizontally, with the cards standing on their long edge, on ridged trays in the same 26°C Percival chamber mentioned above, with 50% RH and 14:10 L:D. After 24 hours, the wasp was removed from the vial with an aspirator. One of the cards was removed to a separate vial to observe the parasitoid emergence for each species of eggs (Fig. 6-2 B). The vials were checked daily.

At the same time the choice tests were being conducted, no-choice tests were conducted also. The no-choice test was similar to the choice test except that each card of

10 eggs was placed in a separate vial from the beginning, and one 3-day-old *O. mirus* adult female was added to each vial for 24 hours. The vials were kept on trays with the choice vials and stored in the 26°C growth chamber (Fig. 6-3).

Fig. 6-3. One tray with five replicates of both choice and no-choice tests. Each vial has 10 host eggs that were exposed to an *Ooencyrtus mirus* adult female for 24 hours. Each replicate of 4 vials has a different number. The A & C vials are no-choice and the B vials are choice tests. The A and BB vials contain *Bagrada hilaris* eggs and the BJ and CJ vials have *Jadera haematoloma* eggs. The B cards were together in the same vial while the *O. mirus* adult was present.

For *E. ceratoniae* the no-choice tests were conducted by cutting a piece of paper towel on which various numbers of eggs were deposited, and exposing the eggs to a 3-day old *O. mirus* in a 9 × 1 cm glass vial for 24 hours. These vials were held at 22-23°C under natural light and checked daily.

Parasitism in the choice and no-choice tests was measured in a variety of ways. First, we determined if *O. mirus* oviposited on the host eggs by counting the number of pedicels under a stereomicroscope. Typical of encyrtids, each *O. mirus* egg has a pedicel that protrudes from the host egg, serving as a respiratory tube for the developing larva (Fig. 6-4). The pedicels were counted between 6 and 12 days after oviposition, after any surviving *B. hilaris* nymphs emerged but before any wasps emerged. For *P. maculiventris*, the pedicels were counted within a few days, because as they mature, the *P. maculiventris* eggs develop long aero-micropylar processes that hide the pedicels. Pedicel counts allowed us to determine whether differences in wasp emergence between *B. hilaris* and the other hosts was due to *O. mirus* laying fewer eggs, lower survival of the eggs, or both. We also determined the level of *O. mirus* superparasitism (more than one pedicel per egg) on the different host eggs.

Fig. 6-4.
A *Bagrada hilaris* egg with one *Ooencyrtus mirus* pedicel.

Second, all parasitoid-exposed host eggs were checked daily for emergence for 28 days following oviposition. Any host immatures that emerged were removed to prevent them from cannibalizing the remaining eggs. For each emerged parasitoid, the number of days from oviposition to emergence (i.e., developmental time) was recorded. Emerged wasps were removed, sexed, and stored in microcentrifuge tubes of ethanol for measuring their body length (Fig. 6-5).

Fig. 6-5. Microcentrifuge tubes containing *Ooencyrtus mirus* adults emerged from different species of host eggs, in preparation for measuring body lengths.

Third, body lengths of up to 20 *O. mirus* adults from each host species were measured from the anterior end of the head to the posterior end, not including the ovipositor, under a Leica Wild M10 stereoscope. A Bausch and Lomb 0.1 mm and 0.01 mm micrometer were used to calibrate a ruler in one of the eyepiece lenses of the microscope. In addition, 3-10 batches of eggs were weighed of each of the pentatomids *B. hilaris*, *T. pallidovirens*, *N. viridula*, *E. conspersus*, *M. histroinica* and *C. uhleri* to determine if parasitoid body size was related to host egg size.

Finally, we evaluated the reproductive success of parasitoids that emerged from eggs of some of the alternate host species by exposing them to *B. hilaris* eggs. Five wasps from each of five alternate host species were placed into separate 9.4 cm long × 1 cm diameter glass vials streaked with honey and plugged with cotton. After three days, a card of five *B. hilaris* eggs was added to each vial for 24 hours to see if the wasps could reproduce on *B. hilaris* after being reared on the alternate host. These parasitoid-exposed eggs were checked daily and the number of emerged offspring was recorded.

Statistical Analyses

The data were analyzed in R (R Core Team, 2019) and were evaluated for significance at $P < 0.05$. The number of pedicels per 10 host eggs and the number of wasps emerged from those eggs were compared between *B. hilaris* and the alternate hosts. Since these data were discrete, the nonparametric Wilcoxon signed rank test was used for the paired choice data and the Wilcoxon-Mann-Whitney test was used for the no-choice data. Since the data for parasitoid egg success, first day of emergence, and developmental time were continuous, they were tested for normality using Shapiro-Wilk test (sample size < 50) or Jarque-Bera test (sample size > 50) at $P < 0.05$. The data were not normal; therefore, the Wilcoxon-Mann-Whitney test was used for comparing means in each group. Both host egg weight and *O. mirus* body lengths failed the normality test; therefore, each alternate host was compared with *B. hilaris* using a Wilcoxon rank sum test with continuity correction. For all hypothesis tests, the reported *P* values are from the two-sided test, with the alternative hypothesis that the true location shift is not equal to 0. The linear relationship between the mean host egg weight and the mean *O. mirus* body

length was determined in Excel. Too few multiple emergences per host egg occurred for analyzing statistically, but the results are summarized. For the sex ratio, as well, too few males emerged to allow a statistical analysis.

Results

Number of pedicels per host egg

In the choice tests, the mean number of parasitoid eggs (as determined by the number of pedicels) laid in 24 hours was significantly lower ($P \leq 0.05$) for *C. uhleri, E. conspersus, E. servus, M. histrionica, A. tristis* and *H. zea* than for *B. hilaris* (Table 6-2).

Table 6-2. Mean number of eggs (pedicels) laid by *Ooencyrtus mirus* on 10 host eggs in 24 hours. N = number of replicates (1st number for choice test, 2nd number for no-choice test).

			Choice		No-choice	
Host species		**N**	**Mean ± SE**	***P***	**Mean ± SE**	***P***
Pentatomidae	*B. hilaris* *C. uhleri*	19, 18	6.5 ± 0.7 0.3 ± 0.1*	<0.001	6.2 ± 0.6 1.9 ± 0.5	<0.001
	B. hilaris *E. conspersus*	17, 17	7.2 ± 0.7 0.5 ± 0.4	<0.001	7.4 ± 0.4 7.1 ± 0.6	0.294
	B. hilaris *E. servus*	5, 5	6.4 ± 1.1 0.4 ± 0.2	0.021	6.6 ± 1.8 8.2 ± 0.9	0.786
	B. hilaris *H. halys*	15, 15	3.9 ± 1.0 1.5 ± 0.5	0.046	7.6 ± 0.4 2.0 ± 0.5	<0.001
	B. hilaris *M. histrionica*	16, 16	5.3 ± 0.8 1.1 ± 0.4	0.002	7.1 ± 0.5 2.0 ± 0.6	<0.001
	B. hilaris *N. viridula*	17, 17	3.9 ± 0.7 2.3 ± 0.7	0.107	6.5 ± 0.6 5.1 ± 0.7	0.069
	B. hilaris *P. maculiventris*	20, 20	3.9 ± 0.9 3.3 ± 0.7	0.344	7.8 ± 0.6 4.9 ± 0.6	<0.001
	B. hilaris *T. pallidovirens*	13, 13	3.8 ± 1.0 2.2 ± 0.7	0.089	6.8 ± 0.5 7.5 ± 0.4	0.857
Other Heteroptera	*B. hilaris* *A. tristis*	16, 15	4.9 ± 0.9 1.0 ± 0.4	0.004	7.7 ± 0.4 2.3 ± 0.4	<0.001
	B. hilaris *J. haematoloma*	21, 21	3.7 ± 0.8 3.3 ± 0.7	0.255	7.4 ± 0.4 5.0 ± 0.7	0.003
Lepidoptera	*B. hilaris* *H. zea*	15, 15	7.6 ± 0.6 0.8 ± 0.4	<0.001	7.3 ± 0.5 1.2 ± 0.4	<0.001

The number of pedicels did not differ significantly between *B. hilaris* and *H. halys, N. viridula, P. maculiventris, T. pallidovirens* or *J. haematoloma* in the choice tests. In the no-choice tests, the number of parasitoid eggs laid in 24 hours was significantly lower ($P \leq 0.01$) in *C. uhleri, H. halys, M. histrionica, P. maculiventris, A. tristis, J. haematoloma* and *H. zea* (Table 6-2). The number of pedicels did not differ significantly in the no-choice tests between *B. hilaris* and the two *Euschistus* species, *N. viridula* or *T. pallidovirens*. *Ooencyrtus mirus* did not oviposit on *E. ceratoniae* eggs.

Offspring emergence

Surviving host nymphs (Heteroptera) or larvae (Lepidoptera) emerged between days 2-12, only from eggs with no pedicels. In choice tests, successful emergence of parasitoid adults was significantly lower ($P < 0.05$) in *C. uhleri, E. conspersus, E. servus, H. halys, M. histrionica, N. viridula, T. pallidovirens, A. tristis* and *H. zea* than in *B. hilaris* (Table 6-3). The number of emerged adults did not differ significantly between *B. hilaris* and *P. maculiventris* or *J. haematoloma* ($P > 0.05$). In no-choice test, emergence of *O. mirus* adults was significantly higher for *B. hilaris* than for *C. uhleri, H. halys, M. histrionica, P. maculiventris, A. tristis, J. haematoloma* and *H. zea* ($P \leq 0.001$), but emergence from *B. hilaris* did not differ significantly from *E. conspersus, E. servus, N. viridula*, or *T. pallidovirens* ($P > 0.05$) (Table 6-3). The variances were homogeneous between *B. hilaris* and each alternate host, showing that the data had similar distributions. For *Chlorochroa ligata* (Say), eggs were available for only one replication. In the choice test, the *C. ligata* egg card had 3 parasitized eggs, from which 1 wasp emerged, and in the

no choice test, the *C. ligata* egg card had 6 parasitized eggs, from which 5 wasps emerged.

Table 6-3. Mean number of wasps emerged from 10 host eggs exposed to one *Ooencyrtus mirus* adult female for 24 hours. N=number of replicates (1st number is for choice test, 2nd number is for no-choice test).

Host species			Choice			No-choice		
		N	# Wasps emerged	Mean ± SE	*P*	# Wasps emerged	Mean ± SE	*P*
Pentatomidae	*B. hilaris* *C. uhleri*	19 19	110 5	5.8 ± 0.7 0.3 ± 0.1	< 0.001	104 28	5.5 ± 0.5 1.5 ± 0.4	< 0.001
	B. hilaris *E. conspersus*	17 17	111 6	6.5 ± 0.7 0.4 ± 0.3	< 0.001	108 92	6.4 ± 0.5 5.4 ± 0.5	0.251
	B. hilaris *E. servus*	5 5	29 2	5.8 ± 1.0 0.4 ± 0.2	0.043	32 28	6.4 ± 1.7 5.6 ± 0.7	0.325
	B. hilaris *H. halys*	15 15	52 18	3.5 ± 0.8 1.2 ± 0.4	0.040	111 24	7.4 ± 0.4 1.6 ± 0.5	< 0.001
	B. hilaris *M. histrionica*	17 17	87 14	5.1 ± 0.7 0.8 ± 0.4	0.002	115 37	6.8 ± 0.5 2.2 ± 0.6	< 0.001
	B. hilaris *N. viridula*	31 31	110 44	3.5 ± 0.5 1.4 ± 0.3	0.014	171 134	5.5 ± 0.4 4.3 ± 0.4	0.080
	B. hilaris *P. maculiventris*	20 20	73 54	3.7 ± 0.7 2.7 ± 0.6	0.408	136 70	6.8 ± 0.5 3.5 ± 0.5	< 0.001
	B. hilaris *T. pallidovirens*	23 24	91 40	4.0 ± 0.7 1.7 ± 0.5	0.021	138 131	5.8 ± 0.3 5.5 ± 0.6	0.987
Other Heteroptera	*B. hilaris* *A. tristis*	16 15	76 13	4.8 ± 0.9 0.8 ± 0.3	0.004	105 34	7.0 ± 0.3 2.3 ± 0.4	< 0.001
	B. hilaris *J. haematoloma*	21 21	72 32	3.4 ± 0.8 1.5 ± 0.3	0.111	149 60	7.1 ± 0.4 2.9 ± 0.5	< 0.001
Lepidoptera	*B. hilaris* *H. zea*	15 15	104 8	6.9 ± 0.6 0.5 ± 0.2	< 0.001	102 14	6.8 ± 0.5 0.9 ± 0.4	< 0.001

Data from each group were analyzed using two-sided Wilcoxon Signed Rank Test for the choice test, and two-sided Wilcoxon-Mann-Whitney Test for the no-choice test.

Survival (proportion of parasitized eggs that yielded live *O. mirus* offspring)

For most of the alternate host species evaluated, the proportion of successful emergence of *O. mirus* adults from parasitized host eggs (those with one or more pedicels) did not differ significantly between *B. hilaris* and the alternate host in either the choice or no-choice tests ($P > 0.05$). This was true even for *H. zea* eggs, which were much smaller than all the other host eggs (Table 6-4).

Table 6-4. Parasitoid egg survival. Mean proportion of *Ooencyrtus mirus* emerged from host eggs with one or more *O. mirus* pedicels. Proportions greater than 1.00 are due to superparasitized eggs from which more than one wasp emerged. N = number of replicates.

Host species		Choice			No-choice		
		N	Mean ± SE	*P*	N	Mean ± SE	*P*
Pentatomidae	*B. hilaris* *C. uhleri*	17 3	0.98 ± 0.02 1.33 ± 0.33	0.169	16 10	0.92 ± 0.03 1.03 ± 0.17	0.183
	B. hilaris *E. conspersus*	16 2	0.98 ± 0.02 1.00 ± 0.00	0.974	17 17	0.91 ± 0.03 1.01 ± 0.04	0.085
	B. hilaris *E. servus*	5 2	0.94 ± 0.04 1.00 ± 0.00	0.762	4 5	1.00 ± 0.00 0.90 ± 0.06	0.444
	B. hilaris *H. halys*	9 7	0.96 ± 0.02 0.83 ± 0.34	0.475	15 10	0.97 ± 0.02 1.70 ± 0.31	0.001
	B. hilaris *M. histrionica*	14 8	0.95 ± 0.03 0.79 ± 0.18	1.000	17 13	1.00 ± 0.01 1.02 ± 0.02	0.470
	B. hilaris *N. viridula*	13 11	0.93 ± 0.03 0.73 ± 0.10	0.084	17 16	0.94 ± 0.02 0.98 ± 0.06	0.388
	B. hilaris *P. maculiventris*	13 13	0.98 ± 0.02 0.85 ± 0.09	0.225	18 16	0.96 ± 0.02 0.92 ± 0.05	0.803
	B. hilaris *T. pallidovirens*	9 7	0.83 ± 0.11 0.80 ± 0.14	0.945	13 13	0.94 ± 0.02 0.94 ± 0.03	0.852
Other Heteroptera	*B. hilaris* *A. tristis*	12 7	0.99 ± 0.01 1.00 ± 0.11	1.000	16 14	0.98 ± 0.01 1.07 ± 0.07	0.069
	B. hilaris *J. haematoloma*	13 14	0.99 ± 0.01 0.79 ± 0.07	0.047	21 18	0.96 ± 0.02 0.77 ± 0.08	0.039
Lepidoptera	*B. hilaris* *H. zea*	15 5	0.97 ± 0.02 0.80 ± 0.12	0.284	15 9	0.95 ± 0.03 0.83 ± 0.20	0.772

Data from each group were analyzed using a two-sided Wilcoxon-Mann-Whitney Test for both choice and no-choice tests. Note that *C. uhleri*, *E. conspersus* and *E. servus* had ≤ 3 replicates from which adult *O. mirus* emerged in the choice tests.

One exception was that parasitoid survival was significantly higher from *B. hilaris* than from *J. haematoloma* eggs in both the choice ($P = 0.047$) and no-choice ($P = 0.039$) tests. In contrast, wasp emergence per parasitized host egg was lower in *B. hilaris* than in *H. halys* in the no- choice test (means 0.97 and 1.70, respectively; $P = 0.001$) (Table 6-4). This was due to more wasps emerging per host egg in *H. halys*. Because of the more successful superparasitism in *H. halys*, we compared the per pedicel emergence in addition to the per parasitized egg emergence. For the choice tests, N = 9 and 7 for *B. hilaris* and *H. halys*, respectively, and the means were 0.88 ± 0.12 and 0.64 ± 0.18, respectively, with $P = 0.17$. For the no-choice tests, N = 15 and 10 for *B. hilaris* and *H. halys*, respectively, and the means were 0.96 ± 0.02 and 0.73 ± 0.11, respectively, with $P = 0.12$. The per pedicel emergence of *H. halys* thus did not differ significantly from that of *B. hilaris*.

First day of emergence and developmental time

In the choice test, the mean first day of parasitoid emergence was significantly earlier from *B. hilaris* than from the alternate hosts ($P < 0.05$) except for *E. conspersus* and *T. pallidovirens*, for which the first days of wasp emergence did not differ significantly from that of *B. hilaris* (Table 6-5). In the no-choice tests, the mean first day of emergence was significantly earlier from *B. hilaris* than from all the alternate hosts (Table 6-5).

Table 6-5. Mean first day of emergence of *Ooencyrtus mirus* adults from *Bagrada hilaris* vs. alternate hosts in choice and no-choice tests

Host species		Choice			No choice		
		N	**Mean ± SE**	***P***	**N**	**Mean ± SE**	***P***
Pentatomidae	*B. hilaris*	16	14.1 ± 0.1	0.010	17	14.1 ± 0.1	0.006
	C. uhleri	3	15.0 ± 0.0		9	14.8 ± 0.2	
	B. hilaris	16	14.3 ± 0.2	0.098	17	14.2 ± 0.1	< 0.001
	E. conspersus	2	15.0 ± 0.0		17	15.3 ± 0.1	
	B. hilaris	5	14.4 ± 0.2	0.048	4	14.0 ± 0.0	0.008
	E. servus	2	18.5 ± 2.5		5	15.0 ± 0.0	
	B. hilaris	9	14.4 ± 0.2	< 0.001	15	14.3 ± 0.1	< 0.001
	H. halys	5	16.4 ± 0.4		9	16.6 ± 0.4	
	B. hilaris	14	14.1 ± 0.1	< 0.001	17	14.0 ± 0.0	< 0.001
	M. histrionica	5	15.6 ± 0.4		12	15.1 ± 0.2	
	B. hilaris	24	14.3 ± 0.1	< 0.001	31	14.2 ± 0.1	< 0.001
	N. viridula	16	15.4 ± 0.2		28	15.2 ± 0.1	
	B. hilaris	13	14.3 ± 0.2	0.003	18	14.4 ± 0.2	< 0.001
	P. maculiventris	12	15.0 ± 0.1		16	15.1 ± 0.1	
	B. hilaris	17	14.6 ± 0.1	0.362	24	14.3 ± 0.1	0.004
	T. pallidovirens	10	14.9 ± 0.2		22	14.9 ± 0.1	
Other Heteroptera	*B. hilaris*	12	14.3 ± 0.3	0.004	14	14.1 ± 0.1	< 0.001
	A. tristis	7	15.9 ± 0.4		13	15.2 ± 0.3	
	B. hilaris	13	14.6 ± 0.2	< 0.001	21	14.1 ± 0.1	< 0.001
	J. haematoloma	14	15.9 ± 0.2		17	15.8 ± 0.1	
Lepidoptera	*B. hilaris*	15	14.4 ± 0.1	0.029	15	14.4 ± 0.1	< 0.001
	H. zea	5	16.0 ± 0.7		6	16.2 ± 0.4	

N is the number of replicates. Data from each group were analyzed using Exact Wilcoxon-Mann-Whitney Test for both choice and no choice tests ($P < 0.05$).

The developmental time (i.e., number of days to emergence) was 14-16 for most of the *O. mirus* immatures, but ranged from 13-24 days, with only one wasp emerging on day 13 and only one on day 24**.** For all of the alternate host species in both the choice and no-choice tests, the mean developmental time at 26°C was longer than in *B. hilaris* ($P \leq 0.02$, with most comparisons significant at $P < 0.001$) (Table 6-6).

Table 6-6. Mean developmental time (egg to adult) of *Ooencyrtus mirus* in choice and no-choice tests.

Host species	Choice			No-choice		
	N	Mean ± SE	*P* value	N	Mean ± SE	*P* value
Pentatomidae						
B. hilaris ***C. uhleri***	110 5	14.3 ± 0.0 15.2 ± 0.2	< 0.001	104 28	14.3 ± 0.1 14.9 ± 0.1	< 0.001
B. hilaris ***E. conspersus***	111 6	14.5 ± 0.1 15.5 ± 0.3	< 0.001	108 92	14.4 ± 0.1 15.7 ± 0.1	< 0.001
B. hilaris ***E. servus***	29 2	14.6 ± 0.1 18.5 ± 2.5	0.004	32 28	14.4 ± 0.1 15.5 ± 0.3	< 0.001
B. hilaris ***H. halys***	52 18	15.0 ± 0.1 16.1 ± 0.1	< 0.001	111 24	14.5 ± 0.1 16.0 ± 0.2	< 0.001
B. hilaris ***M. histrionica***	87 8	14.3 ± 0.1 15.4 ± 0.3	< 0.001	115 31	14.3 ± 0.1 15.4 ± 0.1	< 0.001
B. hilaris ***N. viridula***	110 44	14.7 ± 0.1 15.7 ± 0.2	< 0.001	171 134	14.6 ± 0.1 15.4± 0.1	< 0.001
B. hilaris ***P. maculiventris***	73 54	14.3 ± 0.1 15.3 ± 0.1	< 0.001	136 70	14.6 ± 0.1 15.5 ± 0.1	< 0.001
B. hilaris ***T. pallidovirens***	91 40	14.9 ± 0.1 15.1± 0.1	0.020	138 131	14.9 ± 0.1 15.2 ± 0.1	< 0.001
Other Heteroptera						
B. hilaris ***A. tristis***	76 13	14.4 ± 0.1 15.8 ± 0.3	< 0.001	105 34	14.3 ± 0.0 15.2 ± 0.2	< 0.001
B. hilaris ***J. haematoloma***	72 32	14.4 ± 0.1 16.2 ± 0.1	< 0.001	149 60	14.4 ± 0.1 16.2 ± 0.1	< 0.001
Lepidoptera						
B. hilaris ***H. zea***	104 8	14.9 ± 0.1 16.1 ± 0.4	0.001	102 14	14.8 ± 0.1 16.6 ± 0.3	< 0.001

N is the number of replicates. SE = standard error.

The five *O. mirus* adult females from each of five alternate host species that were subsequently exposed to ten *B. hilaris* eggs for 24 hours successfully produced offspring on those eggs as follows: 25 total offspring from the 5 adults reared on each of *E. servus* and *J. haematoloma* eggs, 22 from adults reared on each of *E. conspersus* and *P. maculiventris* eggs, and 8 offspring from *H. zea*-reared adults.

Superparasitism

"Superparasitism occurs with more than one oviposition by one or more individuals of the same parasitoid species into the same host individual" (Mills, 2009). By this definition, *O. mirus* superparasitized some of the host eggs in our studies. *Halyomorpha halys*, with the largest eggs of all host species tested, had the highest rate of superparasitism per parasitized egg in both the choice and no-choice experiments (Table 6-7). No *H. zea* eggs, the smallest eggs tested in which *O. mirus* could develop, were superparasitized in either test. Eggs of all the other species (except *C. ligata*, which had very small sample sizes), had some superparasitized eggs in the choice and/or no-choice tests. The superparasitized eggs yielded different numbers of adult parasitoids. From *Halyomorpha halys* eggs, 3 wasps emerged from the same egg, with one instance in the choice test and three in the no-choice test. The other hosts had at most two parasitoids emerge from the same egg. *Chlorochroa uhleri* eggs in the choice test and *A. tristis* and *M. histrionica* eggs in the no-choice test produced two *O. mirus* adults from 100% of the superparasitized eggs. In the choice test, *H. halys*, *N. viridula* and *B. hilaris* had 75, 50 and 11%, respectively, of the superparasitized eggs yield two or more wasps. In the no-choice test, *H. halys* had 63.6% of superparasitized eggs give rise to two or more wasps (Table 6-7).

Table 6-7. Superparasitism of *Ooencyrtus mirus* on eggs of different host species (listed in alphabetical order) in choice tests and no-choice tests

Test	Host species	# parasitized eggs	Proportion of superparasitized eggs per parasitized egg	Proportion of superparasitized eggs from which >1 wasp emerged
Choice	*Anasa tristis*	13	0.15	0.00
	Bagrada hilaris	867	0.52	0.11
	Chlorochroa ligata	3	0.00	N/A
	Chlorochroa uhleri	4	0.25*	1.00
	Euschistus conspersus	4	0.50	0.00
	Euschistus servus	2	0.00	N/A
	Halyomorpha halys	14	0.57	0.75
	Helicoverpa zea	12	0.00	N/A
	Jadera haematoloma	48	0.42	0.05
	Murgantia histrionica	17	0.00	N/A
	Nezara viridula	37	0.54	0.50
	Podisus maculiventris	45	0.22	0.00
	Thyanta pallidovirens	24	0.17	N/A
No-choice	*Anasa tristis*	32	0.31	1.00
	Bagrada hilaris	915	0.38	0.43
	Chlorochroa ligata	6	0.00	N/A
	Chlorochroa uhleri	27	0.26	0.57
	Euschistus conspersus	98	0.21	0.29
	Euschistus servus	32	0.28	0.00
	Halyomorpha halys	13	0.85	0.64
	Helicoverpa zea	18	0.00	N/A
	Jadera haematoloma	78	0.35	0.37
	Murgantia histrionica	36	0.28	1.00
	Nezara viridula	82	0.78	0.57
	Podisus maculiventris	74	0.16	0.83
	Thyanta pallidovirens	94	0.21	0.00

These data were not analyzed statistically because of the low numbers of eggs from which >1 wasp emerged. The *B. hilaris* numbers are high because they include the *B. hilaris* controls for all the alternate hosts. For all the alternate hosts except *H. halys*, no more than two wasps emerged from one host egg. For *H. halys*, 3 wasps emerged from one egg in the choice test and from three eggs in the no-choice test. N/A indicates no superparasitized eggs. *The number of pedicels for *C. uhleri* may be underestimated. For this species, the black base of the pedicels, which distinguishes the pedicels from host micropylar processes, was buried in the host chorion and not visible.

Chlorochroa uhleri and *N. viridula* each had 57.1% of superparasitized eggs produce two wasps. *Euschistus conspersus* had 28.6%, *P. maculiventris* had 83%, *B. hilaris* had 43% and *J. haematoloma* had 37% of superparasitized eggs yield two wasps.

Host egg weight and *O. mirus* body length

Egg weights (an indication of size) of the various stink bug hosts differed significantly. *Bagrada hilaris* eggs weighed significantly less than those of all the alternate host species whose eggs were weighed (*T. pallidovirens, N. viridula, E. conspersus, E. servus, M. histrionica, C. uhleri and A. tristis*) ($P < 0.05$) (Figure 6-6).

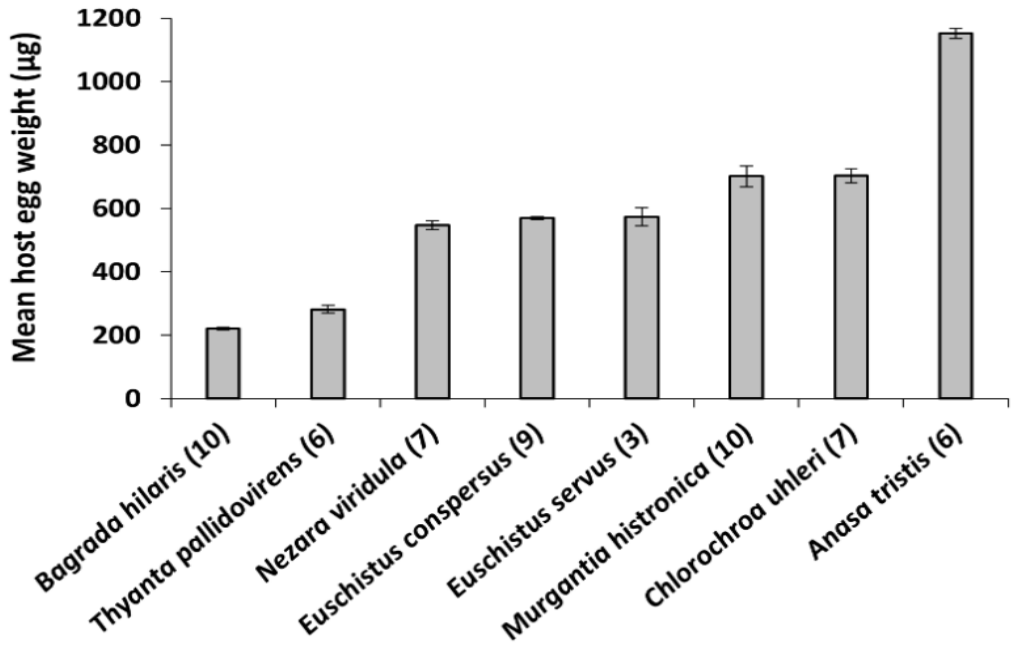

Fig. 6-6. Comparison of mean egg weight with standard error (SE) bars among different pentatomid hosts. Number of replicates is given in parentheses. Egg weights of the alternate hosts differ significantly from those of *B. hilaris* according to 2-sample Wilcoxon rank sum tests with continuity correction ($P < 0.02$).

Adult *O. mirus* females reared on *B. hilaris* also differed significantly in body length compared to all the alternate hosts ($P < 0.02$) (Fig. 6-7). The *O. mirus* adults that emerged from *B. hilaris* were larger than those from *H. zea* but smaller than those from all the other alternate hosts ($P < 0.02$). A linear regression analysis of mean egg weight per host species vs. *O. mirus* adult female body length produced the following regression equation with an adjusted R^2 of 0.90: Mean *O. mirus* adult female body length = (0.000391*Mean host egg weight) + 0.8129 ($P = 0.0007$) (Fig. 6-7). Regarding sex ratio of the emerged adults, four *O. mirus* males emerged from *E. conspersus* eggs and no males emerged from the other hosts.

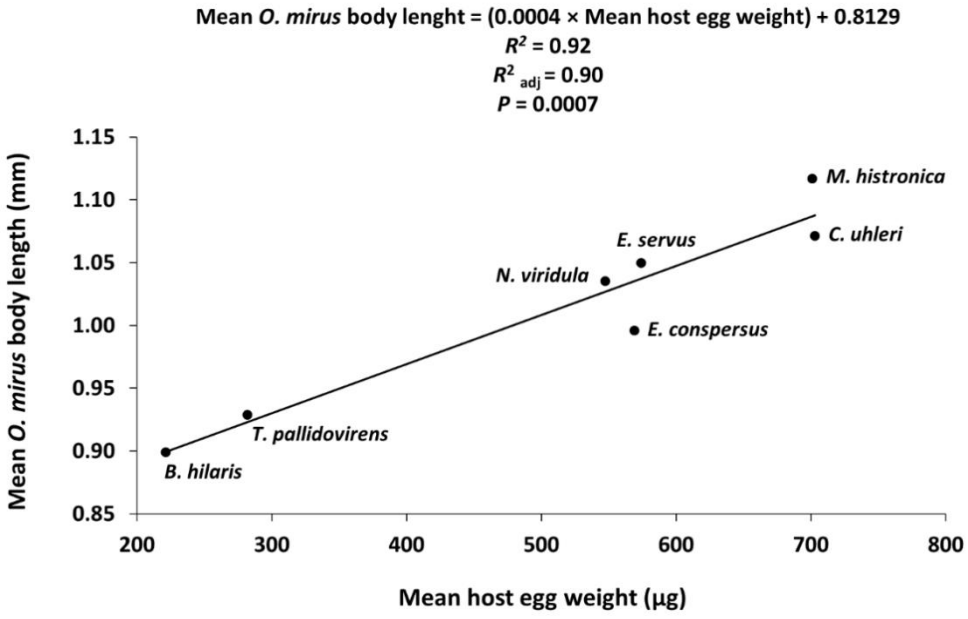

Fig. 6-7. Linear relationship between mean *Ooencyrtus mirus* body length and respective mean host egg weight. Parasitoid body length increases with host egg weight.

Discussion

The increase in parasitoid size with increasing host size also occurs in another stink bug solitary egg parasitoid, *Trissolcus mitsukurii* (Hymenoptera: Scelionidae) (Arakawa et al. 2004) and in *Edovum puttleri* on *Leptinotarsa* species (Coleoptera: Chrysomelidae) (Corrigan & Lashomb 1990). Other authors have also noted a positive correlation between parasitoid size and host size (Jackson 1966; Lewis et al. 1976; Mendel 1986; Smith & Pimentel 1969; Wylie 1966). On *H. halys*, the largest host tested herein, emerged *O. mirus* adults appeared larger than normal if one or two emerged per host egg, but normal size if three wasps emerged per host egg. Survival per parasitized *H. halys* egg was significantly higher because often 2-3 wasps emerged from each host egg, rather than the usual one wasp from other host species eggs. In most cases where emergence was lower in the alternate host than in *B. hilaris*, the difference was due to fewer pedicels being laid by *O. mirus* (i.e., lower host acceptance - Table 6-2) rather than to lower survival per parasitized egg on the alternate host (i.e., lower host suitability - Table 6-4). One exception to this was that both fewer pedicels and lower survival contributed to low emergence in *J. haematoloma* in the no-choice test.

The results of this study can be divided into four groups, described below in order from the most to the least suitable alternate host species for *O. mirus*. First, on *N. viridula* and *T. pallidovirens*, the number of pedicels did not differ from *B. hilaris* regardless of whether the parasitoids had a choice or not. Emergence was significantly higher in *B. hilaris* in the choice tests but it was the same as these alternate hosts in the no-choice tests. The survival per parasitized *T. pallidovirens* egg was the same as for *B.*

hilaris ($P > 0.85$) in both the choice and no-choice tests (Table 6-4). Although the developmental time was longer in *T. pallidovirens* than in *B. hilaris*, the first day of emergence (Table 6-5) and mean developmental times (Table 6-6) were among the lowest of all the alternate hosts tested. Combined, these results suggest that *T. pallidovirens* is the most suitable host for *O. mirus* after *B. hilaris*, even though *O. mirus* and *T. pallidovirens* have never existed in sympatry; *T. pallidovirens* is native to North America whereas *O. mirus* is from Africa and Asia. We found *Bagrada hilaris* to be easier to rear long-term, and thus still the best rearing host for *O. mirus*.

Second, given a choice between *B. hilaris* and either *Euschistus* species, *O. mirus* laid more eggs on *B. hilaris*, but in no-choice tests they laid a similar number of eggs on the *Euschistus* spp. eggs as on *B. hilaris* eggs. Thus *O. mirus* prefers *B. hilaris* to *Euschistus* spp. but will accept them as a host in the absence of *B. hilaris*.

Third, when given a choice between *B. hilaris* and *P. maculiventris* or *B. hilaris* and *J. haematoloma*, *O. mirus* females laid a similar number of eggs as on *B. hilaris*. But given no choice, they laid significantly fewer eggs on the alternate host than on *B. hilaris*. Perhaps some factor in the *B. hilaris* eggs stimulated the wasps to oviposit and once stimulated in the choice test, the parasitoids oviposited regardless of host species. Since *B. hilaris* was the parasitoid rearing host, it is reasonable that they may be stimulated to oviposit in the presence of *B. hilaris* eggs. For this third group, the explanation(s) might be elucidated by chemical ecology studies followed by behavioral studies involving chemical components of the host eggs. Also, to be noted is that *O. mirus* could reproduce on *J. haematoloma* even though this host has no recorded parasitoids (Carroll 1988 and a

current literature search). The fact that this was the only host tested on which the survival was lower than on *B. hilaris*, though, may signal that *J. haematoloma* was still resisting parasitism.

Fourth, *O. mirus* laid significantly more eggs in *B. hilaris* than in *C. uhleri, H. halys, M. histrionica, A. tristis* and *H. zea* in both the choice and no-choice tests (Table 6-2). Likewise, significantly more wasps emerged from *B. hilaris* eggs than from these alternate host eggs in both the choice and no-choice tests (Table 6-3). *Bagrada hilaris* is thus a more suitable host than the alternate species in this fourth group. One of the species in this group, *Halyomorpha halys*, had the largest eggs evaluated. While *O. mirus* adult females typically laid only one egg per host egg on the other hosts, they laid 2-3 eggs per *H. halys* egg, with the highest incidence of superparasitism of all the alternate hosts (Table 6-7)**.** On most of the other hosts, the infrequent number of eggs with two pedicels usually produced only one *O. mirus* adult, but on *H. halys*, superparasitized eggs typically produced 2-3 *O. mirus* adults.

The fact that *O. mirus* superparasitized the highest percentage of eggs on *H. halys*, the largest eggs, and none on *H. zea*, the smallest eggs, along with intermediate percentages on the intermediate size eggs, suggests that the female *O. mirus* adults can discern the host egg size and choose the number of eggs to lay on a given host egg based in part on host egg size. *Ooencyrtus mirus* thus can be considered a gregarious parasitoid on *H. halys*, whereas it is mostly a solitary parasitoid on the other species tested.

Conclusions

These physiological host range tests indicate that *O. mirus* is a generalist but has an innate host preference for, and greater success on, *B. hilaris.* This would likely reduce parasitism on non-target heteropterans, as Botch and Delfosse (2018) suggested would be the case for the parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), when it was being evaluated for controlling *H. halys*.

Parasitoids of the Pentatomidae commonly use multiple stink bug host species, or a combination of pentatomid and lepidopteran hosts. For example, Talamas et al. (2015) report *T. basalis* emerging from 30 different pentatomid host species, *Trissolcus cultratus* (Mayr) from three Pentatomoidea species, *Trissolcus cosmopeplae* (Gahan) from four pentatomids, *Trissolcus edessae* Fouts from four pentatomoids, *Trissolcus euschisti* (Ashmead) from seven pentatomids, *Trissolcus hullensis* (Harrington) and *Trissolcus thyantae* Ashmead each from two pentatomids and a lepidopteran species, *Trissolcus japonicus* (Ashmead) and *Trissolcus solocis* Johnson each from two pentatomids, *Trissolcus occiduus* Johnson from three pentatomids, and *Trissolcus utahensis* (Ashmead) from five pentatomid species. Likewise, the parasitoid *Trissolcus brochymenae* Ashmead (syn. *Trissolcus murgantiae*) is associated with at least 11 pentatomid species occurring in the New World (Salerno 2000). Samra et al. (2016) found that *Ooencyrtus pityocampae* Mercet, an egg parasitoid of *Thaumetopooea wilkinsoni* Tams (Lepidoptera: Notodontidae) can also parasitize the pentatomid *Stenozygum coloratum* (Klug).

While many parasitoids of stink bugs use multiple hosts, it is also known that *B. hilaris* is attacked by multiple parasitoids. Ganjisaffar et al. (2018) found *Trissolcus hyalinipennis* Rajmohana & Narendran and *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae), a parasitoid released in Ca. in 1987 to control *N. viridula*, parasitizing *B. hilaris* eggs in the field. Further research in our group has identified another *Ooencyrtus* species that has recently been described as *Ooencyrtus lucidus* Triapitsyn & Ganjisaffar (Triapitsyn et al. 2020) attacking *B. hilaris* eggs in the field.

Ooencyrtus mirus has other traits that characterize an effective biocontrol agent. In the current study, no parasitized host eggs survived; i.e., no egg with a pedicel produced a host offspring. Such parasitoid-induced host egg mortality may add to the biological control impact of the parasitoid (Abram et al. 2016). It also may signal that oviposition by *O. mirus* is accompanied by venom or substantial physical damage to the host. In addition, *O. mirus* does not need to diapause, but it can go into an arrested developmental state in the larval stage at 14° and 16°C and then revives in warmer temperatures (Chapter 4). This could be used in mass-rearing by cold-storing parasitized host eggs when adults are not needed for release. Furthermore, *O. mirus* has a short life cycle, which can be as low as 10 days at 32°C, the highest constant temperature that still produces mostly females in the F_2 generation (Chapters 4 & 5).

Combined with the short life cycle, *O. mirus* is parthenogenetic, predominantly producing females that can lay eggs without needing to find a mate. Parasitized *B. hilaris* eggs show high emergence rates of 80-100% in the lab (Table 6-3) and in the 4 years we have had the insect in colony, it has not experienced any diseases, hyperparasites or other

limits to its population growth. In addition, *O. mirus* can be raised on alternate hosts. We have maintained a colony of *O. mirus* on *N. viridula* eggs for more than three years, on *T. pallidovirens* eggs for more than one year, and on *C. uhleri* eggs for several months. These colonies survived until no more host eggs were added. These long-term alternate host colonies demonstrate that *O. mirus* can sustain a population without needing *B. hilaris* as even an intermittent host.

If *O. mirus* could find and use alternate hosts in the field, this could help its populations subsist even when *B. hilaris* individuals are scarce. Most of the alternate hosts tested in the present study, whether native or not, are agricultural pests themselves, so *O. mirus* potentially could assist in managing multiple pest insects if introduced in North America. However, the fact that it also parasitizes *P. maculiventris*, a beneficial species, could be problematic in areas where *P. maculiventris* is an important natural enemy.

The approved release of natural enemies whose physiological host range includes native, non-target hosts has occurred in the past. For example, the USDA APHIS granted permission to release the egg-parasitic, parthenogenetic encyrtid *Oobius agrili* Zhang and Huang against the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) in Michigan. No-choice tests showed it could attack native *Agrilus* beetle species, but choice tests showed that it greatly preferred to oviposit on the target host on ash trees (*Fraxinus* spp.) than on non-target hosts on their respective host plants (Bauer et al. 2008). The same biological control effort also received APHIS approval for release of a braconid larval parasite of *A. planipennis*, *Spathius agrili* Yang. Like *O. agrili*, this

species showed a physiological host range overlap with native *Agrilus* species. However, Y-tube olfactometer tests showed that *S. agrili* was only attracted to three tree species, making the parasitoid unlikely to encounter non-target hosts in the wild. In addition, field-collected larvae of 6 other *Agrilus* species in the native range of *S. agrili* in China produced no *S. agrili*, suggesting a narrow host range in the field. Furthermore, the success rate was lower in non-target hosts in no-choice tests in the lab (Bauer et al. 2008). Similarly, future choice tests that include the hosts' respective host plants, olfactometer studies of *O. mirus'* searching behavior, and investigation of its actual host range in the field in its native region, could help assess the impact *O. mirus* might have on non-target species. Such studies could further inform its suitability as a potential biological control agent for *B. hilaris* in North America.

Chapter 7

Ability of *Ooencyrtus mirus* to find *Bagrada hilaris* eggs in soil and on broccoli plants

Abstract

One aspect of evaluating *Ooencyrtus mirus* as a potential biological control agent of *Bagrada hilaris* is testing how well *O. mirus* can find *B. hilaris* eggs in soil and on host plants. *Bagrada hilaris* is unusual among pentatomids in laying eggs singly or in small, loose groups instead of in large batches of contiguous eggs, and often in soil rather than on plants (Taylor et al. 2014). Two preliminary tests were conducted by exposing *O. mirus* females to *B. hilaris* eggs on broccoli seedlings and in soil. Among the eggs on plants, one *O. mirus* egg was laid on a single host egg out of 80. In the soil, 6 of the 40 Petri dishes had *O. mirus* emerge, and all six had two or more wasps emerge. Further lab and field testing are needed to determine how *O. mirus* searches for hosts, because the low search success in this study raises the question of how the population succeeds in the field. Low searching success would argue against deploying *O. mirus* as a biocontrol agent. However, their long lifespan (Chapter 3) may provide sufficient time to find host eggs.

Introduction

Parasitoid females detect their hosts long range mainly by olfaction (Vinson 1976), using olfactory sensillae on the antennae. They often find the host habitat first, followed by finding the host, and then accepting it (Doutt 1959). For egg parasitoids, the host eggs do not tend to release long-range volatile compounds that could be used for

detection, so the parasitoid first may search for the host adult or its host plant. Insect host cues (kairomones) that have been reported to elicit responses by parasitoids include: sex, aggregation and epidietic (regulating population density) pheromones; salivary chemicals; body and scale odors; webbing; honeydew; frass; and defensive compounds (reviewed by Whitmann 1988; Mattiacci et al. 1993; Agelopoulos et al. 1995). Plant volatiles, sometimes those induced by insects feeding on the plant, are another source of location information for parasitoids (Blaakmeer et al. 1994; Powell et al. 1998). The plant can be very species-specific to which host is feeding on it and which volatiles it releases to attract parasitoids. For example, cabbage plants (*Brassica oleracea* L.) fed on by *Pieris rapae* L. emit a volatile that attracts *Cotesia rubecula* L., but cabbage plants fed on by *Pieris brassicae* L. emit a volatile that attracts *Cotesia glomerata* L. (Blaakmeer et al. 1994). The two *Cotesia* spp. are specific to their respective *Pieris* hosts. Once the parasitoid finds the adult or plant, it may use visual, olfactory, mechanosensory, sound and/or gustatory (contact chemical) cues to find and accept the host (Godfray 1994). Gustatory cues are generally perceived by the labellum and maxillary palp mouthparts and/or the tarsi.

Usually in parasitoids, immediate host information such as host pheromone is innate, and thus an unconditioned stimulus. However environmental cues around the host may be learned by association as conditioned stimuli and used as cues in future searching (Turlings et al. 1993). The learned response is stronger and lasts longer if the parasitoid actually oviposits on the host, and even more so if it oviposits repeatedly. The olfactory information can be integrated with other sensory information as well. Compared to other

insects, parasitoids are particularly adept at incorporating olfactory and visual cues in learning. In some species, even the immatures are capable of learning (Turlings et al. 1993).

Ooencyrtus telenomicida (Vassiliev), the species most closely related to *Ooencyrtus mirus*, responds to only the sex pheromone of the host *Nezara viridula* (L.). It does not respond to other odors from this host or to odors of tomato plants, *Lycopersicum esculentum* L., a host of *N. viridula* (Peri et al. 2011). A competitor species of *Ooencyrtus telenomicida*, *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae), responds to a kairomone used by adult male *N. viridula* to attract second instar nymphs from a non-host plant to a host plant (Leal et al. 1995). *Trissolcus basalis* can also find injured host plants (Leal et al. 1995). Once it is within 1-2 mm of the host eggs, it can detect the adhesive secreted by the female *N. viridula* to attach her eggs to the substrate (Bin et al. 1993).

Stink bugs have a dorsal abdominal gland and a metathoracic gland that produce their various odors. *Trissolcus basalis* is attracted to one of these *N. viridula* kairomones (Mattiacci et al. 1993). *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae) is also attracted to pentatomid methathoracic defense secretions (Laumann et al. 2009). However, another parasitoid species, *Ooencyrtus nezarae* Ishii, and *O. telenomicida* do not respond to these chemicals. *Ooencyrtus nezarae* does respond to both the sex pheromone and one of three aggregation pheromone compounds produced by males of the host bug *Riptortus clavatus* (Thumberg) (Leal et al. 1995; Numata et al. 1986).

Bagrada hilaris males and females produce nonanal, decanal and (E)-2-octenyl acetate. The males produce much more of the latter, which is likely the male sex pheromone (Guarino et al. 2008). Female *B. hilaris* are attracted to it, but neither males nor females are attracted to any odor produced by the female. *Bagrada hilaris* also seems to have a lipophilic contact pheromone in the cuticle (Guarino et al. 2008). *Bagrada hilaris* is unusual among pentatomids in laying its eggs singly or in small, loose groups rather than large bunches, and not only on plants, but also in soil (Taylor et al. 2014). This ovipositional behavior and the lack of attraction to female odors may make the eggs less susceptible to predation and parasitism than those of other pentatomid species.

I have observed that once an *O. mirus* female finds a host egg, she antennates the egg for at least an hour before attempting to oviposit. This raises many questions. How does *O. mirus* find its hosts in the field? Like *B. hilaris*, *O. mirus* crawls more than it flies; is that how it behaves in the field? Does it use gustatory cues? Can it learn host associations? How does it determine if a host egg is acceptable? These questions have led me to the current study to see how well *O. mirus* can find *B. hilaris* eggs laid in soil and on plants.

Materials & Methods

Host finding on plants

Bagrada hilaris and *O. mirus* were reared according to the protocols in Chapter 2. Broccoli seedlings were grown from seed, one plant per 5 x 5 x 9 cm pot or three plants per 10 x 10 x 9 cm plastic pots filled with potting soil. The plants were transferred to

Quarantine. To prevent *B. hilaris* from ovipositing in the soil, the top of each pot was covered with plastic wrap held in place with transparent tape on the sides, except for where the plant stem protruded through (Fig. 7-1 A, B, C). Six seedlings at a time were placed inside a 30 x 30 x 30 cm white plastic BugDorm®-1 (MegaView Science Co., Taiwan) (Figure 7-1 C). Thirty mating pairs of *B. hilaris* were added to the BugDorm. After 72 hours, the bugs were brushed off the plants gently and the plants were removed from the BugDorm. The location of *B. hilaris* eggs on the plant leaves (no eggs were found on the stems) was marked by drawing a circle around one or more eggs with a Sharpie. Additional eggs were glued (Elmer's water-based glue, Newell Rubbermaid Inc., Atlanta, GA, U.S.) onto the leaves to make the total number of eggs a multiple of 4. The number of eggs was recorded. A second BugDorm was prepared by taping row cover fabric over the outside to prevent *O. mirus* from escaping (Figure 7-1 D). The plants were transferred to this second cage. One 3-day-old adult female *O. mirus* was added for every 4 *B. hilaris* eggs. As a control, an egg card was made with the same number of *B. hilaris* eggs as the total on the seedlings in the cage. The egg card was placed in a 9.4 cm long x 2.2 cm diameter glass vial plugged with cotton. The same number of *O. mirus* females was added to the vial as to the BugDorm. Both the BugDorm and the control vial were placed in a growth chamber set at 26°C, 50% RH, and 14:10 L:D.

Fig. 7-1. *Ooencyrtus mirus* host-finding on plants experimental setup. **A**. Broccoli plants in 5 x 5 cm pots with soil covered with plastic wrap, before exposure to *Bagrada hilaris*. **B**. Close-up of top of broccoli pot. **C**. Broccoli plants after exposure to *B. hilaris*. **D**. Reinforced BugDorm® cage for *O. mirus* adults to be exposed to plants bearing *B. hilaris* eggs.

The wasps were removed after 24 hours and small pieces of leaves bearing *B. hilaris* eggs were cut out from the plant. The eggs were examined for pedicels under a stereomicroscope. The leaf pieces then were placed in the same type of vial as the egg card, labeled with the date and number of eggs. Both vials were placed on a ridged, plastic tray (Nordic Ware® compact microwave bacon tray) in a growth chamber at the same conditions as above and checked daily. The number and day of emergence of *B. hilaris* nymphs and *O. mirus* adults were recorded along with the sex of each *O. mirus* adult. A total of 40 *B. hilaris* eggs each in the plant group and the control group was tested this way. Another 40 were tested with two mating pairs of *B. hilaris* added to the

second BugDorm in case the parasitoid females need chemical cues from the adult bugs for finding eggs, as occurs in some other species. A third set of 40 eggs was tested without wasps as a control for the number of *B. hilaris* nymphs emerging.

The *B. hilaris* females did not oviposit much on the broccoli seedlings, so I also tried a canola, *Brassica napus* L. The bugs did not oviposit on these either, so the canola plants were excluded from the study.

Host finding in soil

Fifth (final) instar *B. hilaris* nymphs were placed in a round, plastic Durphy box (Warminster, PA, U.S.) with two screened holes in the side. Every day, newly eclosed adults were aspirated into a separate Durphy box and labeled with the date. All bugs from each 2-day period were combined into a single Durphy box and checked each day. Each pair found in the act of mating was placed into a 3.4 cm diam. x 0.9 cm deep Petri dish filled with soil to about half the depth. Two to three *B. hilaris* mating pairs were added to each dish. Two dishes had commercial orchid mix (very coarse; mostly wood chips ranging from 0.5 – 2 cm long), to which a few drops of water were added, and the remainder had dried, sandy loam soil from a residential back yard in Riverside (Fig. 7-2). After two days, the bugs were removed and the Petri dishes containing soil and any *B. hilaris* eggs were transferred to Quarantine. Without disturbing the soil, they were checked under a microscope to see if any eggs were visible. The bottom of the dish was checked using 3.25x reading glasses.

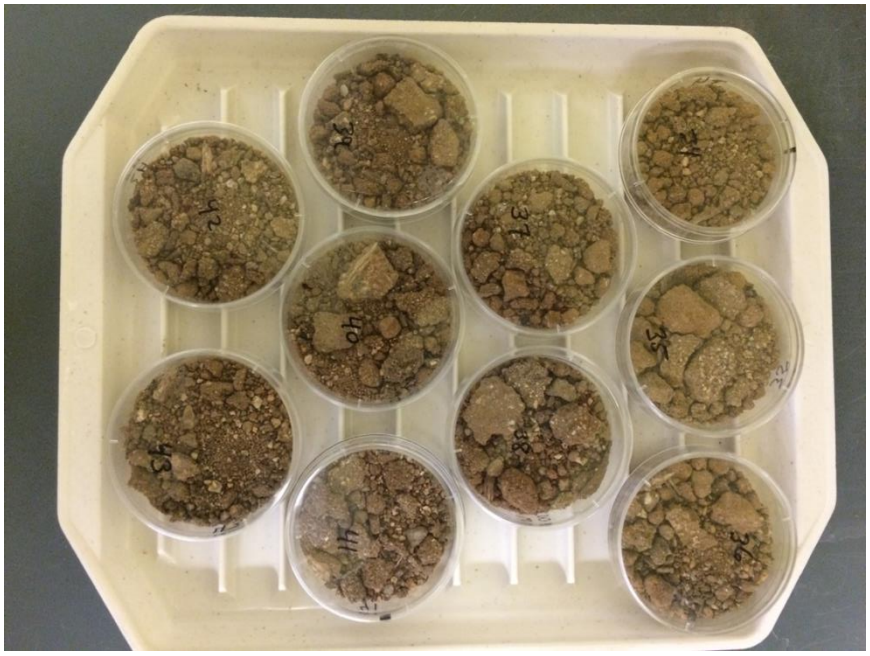

Fig. 7-2. Petri dishes of soil + *Bagrada hilaris* eggs used to test whether *Oooencyrtus mirus* females can find host eggs in soil.

A reusable honey applicator was prepared by attaching the thicker end of a cat whisker to the end of a wooden dowel, ~ 14.5 cm long x 0.2 cm diameter, with Gorilla Glue (Sharonville, OH, U.S.). After using the applicator to streak honey inside the lid of the Petri dish, two *O. mirus* adult females were added. The dish was checked again under the microscope to make sure the wasps were in the dish, as they are difficult to see against the dark soil. The dish was kept at room temperature (22-23°C) and natural light from a north-facing (indirect light) window. After 24 hours, the wasps were aspirated out and the Petri dishes were placed in a growth chamber at 26°C, 14:10 L:D and 50% RH. The dishes were checked daily for 23 days and emerged bugs and wasps were recorded and removed.

Due to a lack of visible eggs or bugs emerging, after the first two replicates, the bugs were left in for 3-4 days instead of 2 days. A small piece of organic broccoli floret was added to each Petri dish daily to keep the bugs alive for the longer time period. In total, 2 orchid mix and 40 sandy loam soil Petri dishes were prepared.

Results

The *B. hilaris* females laid few eggs on the plants. They laid more eggs on the sides of the cage and on the paper towel on the bottom of the cage. When they did lay eggs on plants, they oviposited on leaves, not stems. They usually laid more than one egg, with the eggs placed near the edge of the underside of a leaf. The eggs were not touching each other.

In the "*B. hilaris* eggs + *O. mirus*" treatment, 25 *B. hilaris* nymphs emerged from the eggs on plants and 3 emerged in the control vials (Table 7-1). In the "*B. hilaris* eggs + *O. mirus* + *B. hilaris* adults*"* treatment, 4 *B. hilaris* nymphs emerged from eggs on plants and 3 in the control vials. In the control vial with no wasps added, 13 nymphs emerged from eggs on plants and 19 in the control vials. Of the 80 *B. hilaris* eggs on plant leaves exposed to *O. mirus* females in the first two treatments combined, only one egg was parasitized. It produced one *O. mirus* offspring. Meanwhile, the control groups in glass vials produced 73 *O. mirus* offspring in the two groups combined (Table 7-1).

Table 7-1. Number of *Bagrada hilaris* nymphs and *Ooencyrtus mirus* adults emerging from 3 treatments in the plant study. For each treatment, N=40 eggs.

	Number of *B. hilaris* nymphs emerged		**Number of *O. mirus* adults emerged**	
Treatment	**On plants**	**In vials (control)**	**On plants**	**In vials (control)**
B. hilaris eggs + *O. mirus*	25	3	0	31
B. hilaris eggs + *O. mirus* *B. hilaris* adults	4	3	1	42
B. hilaris eggs only; no *O. mirus*	13	19	N/A	N/A

No *B. hilaris* or *O. mirus* emerged from the two Petri dishes with orchid mix. From the 40 dishes with sandy soil, 233 *B. hilaris* and 19 *O. mirus* emerged from 22 Petri dishes. Using the number of emerged bugs plus emerged wasps (233 + 19 = 252) as a minimum number of host eggs that were available to the wasps, the mean proportion of emergence was 19/252 = 0.075, with standard error of 0.033. The standard error was calculated from the proportion of eggs (i.e., emerged wasps/(emerged bugs + emerged wasps)) yielding wasps in each of the 22 dishes from which bugs and/or wasps emerged. The proportions were 0.00, 0.07, 0.00, 0.08, 0.00, 0.00, 0.40, 0.00, 0.00, 0.00, 0.00, 0.00, 0.40, 0.50, 0.30, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00 and 0.00. *Ooencyrtus mirus* adults emerged in 6 of the 40 dishes with sandy soil. The dishes from which *O. mirus* emerged had a minimum of 2 wasps emerge, with a range of 2-6 wasps per dish. The actual number of wasps emerged per dish was 0, 2, 0, 2, 0, 0, 4, 0, 0, 0, 0, 0, 2, 6, 3, 0, 0, 0, 0, 0, 0, 0 for the 22 dishes that had any insect emergence. The remaining 18 dishes of sandy soil had no *B. hilaris* or *O. mirus* emergence.

Discussion

Despite the fact that *B. hilaris* is the main host for *O. mirus* (Chapter 6), this study showed that it was ineffective at finding *B. hilaris* eggs in soil (19 out of at least 252 host eggs, or 7.5%). Furthermore, it was even less effective at finding host eggs on plants (1/80 host eggs, or 1.2%). Since all *B. hilaris* eggs parasitized by *O. mirus* are killed whether or not a wasp emerges (Chapters 2 - 6), the high emergence of *B. hilaris* nymphs in the soil test indicates that the *O. mirus* females either did not find the host eggs or did not oviposit on them. This is confirmed in the plant study in which only one *O. mirus* pedicel was found among 80 host eggs exposed to *O. mirus*.

When the source insects for our *O. mirus* colony were recovered in Pakistan, they were found on canola plant debris; none were found on mustard debris (*Brassica juncea* (L.)) or on host eggs on sentinel cards (Mahmood et al. 2015). Perhaps *O. mirus* specializes on finding insect hosts on dead host plants using olfactory cues from decaying brassicas. Alternatively, perhaps some other cue is missing in the simple system I set up in the lab. However, each of the cues mentioned in the introduction (adult-emitted pheromones; salivary chemicals; body and scale odors; webbing; honeydew; frass; and defensive compounds) were either present or irrelevant in the current study.

The fact that more than one wasp emerged from each Petri dish that had any wasp emerge raises the possibility that learning took place; i.e., once the *O. mirus* found and oviposited on one egg, they looked for more in the soil. Such learning has been demonstrated in other parasitoids (Turlings et al. 1993). The fact that in 34 of the 40 dishes neither of the two wasps added produced offspring may suggest that the colony

wasps have lost some of their host finding ability after 3^{+} years of having eggs supplied to them without needing to search.

If the results of the present study were validated in field studies, it would lower the usefulness of *O. mirus* as a biocontrol agent. However, their long lifespan (Chapter 3) gives them time to find host eggs. Furthermore as *B. hilaris* eggs mature, they become less suitable hosts for *O. mirus* (Chapter 2). Therefore, *O. mirus* must find the host eggs within the first few days of being laid. Additional research on searching success should use *O. mirus* newly collected from the field. In addition, experiments could be conducted with *B. hilaris* eggs on dead host plants (similar to those on which the original *O. mirus* were collected in Pakistan) and on different plant species.

Chapter 8

General Conclusions of the Dissertation

Reproductive biology of *Ooencyrtus mirus*

The research showed *Ooencyrtus mirus* to be a solitary egg parasitoid on *B. hilaris* and most of the other host species tested, but gregarious on *H. halys* eggs, which are much larger (Chapter 6). When it superparasitized *B. hilaris* eggs, typically only one *O. mirus* adult emerged, but when it laid multiple eggs on one *H. halys* egg, typically 2 or 3 adults emerged.

Compared to *B. hilaris*, *O. mirus* reproduces faster and lives longer, showing that is has the physiological capacity to manage *B. hilaris* populations (Chapter 3). It is active at similar temperatures to *B. hilaris* (Chapter 4).

The fecundity, temperature and *Wolbachia* studies (Chapters 3-5) combine to show that *Ooencyrtus mirus* is a synovigenic species, maturing eggs both before and during adult life. As the parent wasp increased in age with a steady, daily supply of host eggs, both oviposition and offspring emergence occurred in two distinct waves that paralleled each other, since egg survival stayed fairly constant with maternal age. The first wave of oogenesis occurred while the female parent was in the immature stage, and the second wave while it was an adult. A lull in oviposition occurred in between these two waves; at 26°C, the lull was on days 10-12 of the adult's life. The sex ratio (M:F) of the first wave was determined by the temperature experienced during the final two thirds of the parent's immature development, not by the rearing temperature of the offspring, since immatures reared at high temperature were still mostly female (Chapter 4). The sex

ratio of the second wave was determined by the conditions that the parent experienced as an adult. The conditions that led to higher proportions of male offspring were a continuous supply of eggs for the parents (Chapter 3) and high temperatures (Chapter 5). In both cases, the higher proportion of males could be explained by a decline in *Wolbachia*. With less *Wolbachia* to make haploid eggs become female, *O. mirus* reverted to its ancestral arrhenotoky in which haploid eggs became male offspring. These results enable us to predict that the sex ratio likely would be high (i.e., more males) in the second wave if the adult parents were exposed to antibiotics. Indeed, a preliminary test of feeding parent *O. mirus* 0.5% Rifampicin in honey showed this to be the case, with 100% male offspring in all four vials in the F_2 generation.

For some reason yet to be discovered, the female parent laid few or no eggs upon emergence, but rather built up to the maximum oviposition rate by her third day as an adult at 26°C. Even though the females laid few eggs the first few days, the survival of those eggs was similar to that of eggs laid later. The age at which the adult female starts synthesizing the second wave of eggs, while beyond the scope of this dissertation, could be determined by raising the temperature or administering antibiotics on successive days in different sets of adult *O. mirus* females and recording the sex ratio of the offspring.

Variability among *O. mirus* individuals

Ooencyrtus mirus is parthenogenetic, and despite being reared under exactly the same conditions of temperature, light, exposure to host eggs and access to honey, the life history parameters varied considerably among *O. mirus* individuals. My studies showed differences in fecundity, fertility, longevity (Chapter 3), developmental time (especially

at lower temperatures) (Chapter 4), day of first oviposition (Chapters 2, 3 & 4), host choices (Chapter 6), ability to find host eggs in soil (Chapter 7), and response to temperatures of 16-18°C (Chapter 4). Some or all of the variation could be due to genetic variation in the host eggs, since *B. hilaris* produce sexually. On the other hand, *O. mirus* may have a high rate of transposon activity or a low rate of transposon silencing that leads to genetic variation among individuals in the population.

Ecology and potential of *Ooencyrtus mirus* as a biological control agent

My research revealed that *Ooencyrtus mirus* has a number of traits that characterize an effective biocontrol agent. For example, in the current studies, no parasitized host eggs survived; i.e., no *B. hilaris* or alternate host species egg with an *O. mirus* pedicel produced a host offspring in any of the studies (Chapters 2-7). This egg mortality adds to the biological control impact of a parasitoid (Abram et al., 2016). In addition, *O. mirus* does not need to diapause, but it can undergo quiescence in the larval stage at 14° and 16°C and then revive in warmer temperatures (Chapter 4). Parasitized host eggs could thus be cold-store when adults are not needed for release, saving on labor. It also suggests that an introduced population could survive seasonal changes in weather in a Mediterranean climate. *Ooencyrtus mirus* also has a short life cycle of only 10 days at 32°C, the highest constant temperature that yields mostly females in the F_2 generation (Chapters 4 & 5). Furthermore, *O. mirus* has high fecundity, fertility and longevity (Chapter 3); higher than that of *O. telenomicida* which is being considered as an augmentative biocontrol agent for other pentatomids such as *Halyomorpha halys* (Roversi 2018).

In addition to the short life cycle, *O. mirus* was found to be parthenogenetic, producing females that can lay eggs without spending energy on producing males and finding mates. Parasitized *B. hilaris* eggs show high emergence rates of 80-100% in the lab. In the 4.5 years we have had the insect in colony at UCR it has not experienced any diseases, hyperparasites or other limits to its population growth. In addition, *O. mirus* was raised successfully on alternate hosts. We maintained a colony of *O. mirus* on *N. viridula* eggs for more 3+ years, another on *T. pallidovirens* eggs for 1+ years, and a third on *C. uhleri* eggs for months. These colonies all survived until we discontinued them by not adding host eggs, demonstrating that *O. mirus* can sustain a population without needing *B. hilaris* even as an intermittent host. Furthermore, after being reared on *N. viridula* or *T. pallidovirens*, the offspring still preferred *B. hilaris* as their host (F. Ganjisaffar, unpublished data).

If *O. mirus* could similarly parasitize alternate hosts in the field, this could assure its survival even when *B. hilaris* populations are low. In addition, most of the alternate hosts tested herein are agricultural pests themselves, so *O. mirus* could potentially assist in managing more than one pest at a time if introduced in North America. The fact that it parasitized *P. maculiventris*, however, argues against the release of *O. mirus* where *P. maculiventris* is a significant natural enemy.

While generalist species are usually considered ineligible for release, the USDA APHIS has approved the release of natural enemies whose physiological host range includes non-target, native hosts. As noted in Chapter 6, it permitted the release of another egg-parasitic, thelytokus encyrtid, *Oobius agrili*, against the emerald ash borer,

Agrilus planipennis, a severe, invasive forest pest in Michigan, U.S. Although it attacked native *Agrilus* beetle species in the lab, choice tests showed that it greatly preferred to ovipositing on *A. planipennis* on ash trees than on native hosts on their respective host plants (Bauer et al. 2008). As part of the same project, APHIS also approved the release of *Spathius agrili* Yang, a braconid parasite of larval *A. planipennis*. This species, like *O. agrili*, parasitized native *Agrilus* species in the lab, but in Y-tube olfactometer tests showed attraction to only three tree species, making it unlikely to find non-target hosts in a forest. Furthermore, larvae of six other *Agrilus* species collected in China in the native range of *S. agrili* yielded no *S. agrili*, implying a narrow host range in the field. Even in the lab, it was less successful in non-target hosts than on *A. planipennis* in no-choice tests (Bauer et al. 2008). Similarly, further research on *O. mirus* host finding and its host range in its native region could help predict its potential impact on non-target species in North America.

While many parasitoids of stink bugs use multiple host species, it is also known that *B. hilaris* is attacked by multiple parasitoids. Preliminary genetic screening indicated that all the *B. hilaris* now in North America are descended from Pakistani populations (Sforza et al. 2017). In Pakistan, at the same time that *O. mirus* was collected, two other egg parasitoids were recovered from *B. hilaris* eggs: *Trissolcus hyalinipennis* Rajmohana & Narendran and *Gryon gonikopalense* Sharma (Mohammad et al. 2015; Sforza et al. 2017; Martel et al. 2019). Two tachinid parasitoids also have been reported attacking *B. hilaris* in Pakistan (Cheema et al. 1973). In southern California, Ganjisaffar et al. (2018) found *T. hyalinipennis* and *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae)

attacking *B. hilaris* eggs on sentinel cards. That was the first record of *T. hyalinipennis* in North America. This species may have arrived with *B. hilaris*, since it is a known parasitoid of *B. hilaris* in the latter's native range. *Trissolcus basalis* was released in California in 1987 to control *N. viridula*. Further research in our group identified another *Ooencyrtus* species, recently described as *Ooencyrtus lucidus* Triapitsyn & Ganjisaffar, attacking *B. hilaris* eggs in the field in southern California (Triapitsyn et al. 2020). Since it is a newly described species, whether it is native to California or arrived with *B. hilaris* is not known. Likewise, in Mexico, the new species *Idris elba* Talamas (Hymenoptera: Scelionidae) was found parasitizing *B. hilaris* eggs (Lomeli-Flores et al. 2019).

These findings are in contrast to the Enemy Release Hypothesis, which purports that invasive species thrive because they are released from the natural enemies that control their populations in their native region (Keane & Crawley 2002). In North America, at least, the native parasitoids (assuming the two new species, *O. lucidus* and *I. elba* are native), combined with the adventive *T. hyalinipennis* and the previously introduced *T. basalis*, may be able to keep *B. hilaris* populations at low levels. Parasitoids are especially helpful for controlling insects like *B. hilaris* that build their populations on unmanaged weeds near crop land and then invade the crops (Abram et al. 2020); in this case moving from invasive mustard weeds to brassica crops that are planted in late summer or early fall (Reed et al. 2013).

Although egg parasitoids are considered particularly beneficial because they attack the pest before it has time to cause damage, they do not always control the pest population effectively, especially if pest nymph mortality is density-dependent (Abram et

al. 2020). Indeed, *B. hilaris* is a pest in its native range, even where the native natural enemies are present (Howard 1907; Husain 1924). A stage-structured matrix model of the effect of an introduced parasitoid on stink bug populations showed that a combination of egg parasitoids with some type of management of the reproductive adult stage is more likely to be effective than an egg parasitoid alone (Abram et al. 2020). As such, if *O. mirus* were deployed in the field, it likely would be used as one aspect of an overall Integrated Pest Management program of *B. hilaris*. For conventional farms, the impact of insecticides on *O. mirus* would need to be evaluated to determine the timing and type of insecticides, if any, to use for *B. hilaris* and other insect pests so as to minimize the negative impact on resident or introduced parasitoid populations.

Whereas Chapter 7 revealed poor searching ability of *O. mirus* for *B. hilaris* eggs on plants and in soil, one of the other two species originally collected by Mahmood et al. (2015) is proving to be adept at finding *B. hilaris* eggs in soil and to have a narrower host range than *O. mirus* (Hogg 2019). This species, *Gryon gonikopalense* Sharma (identified by Martel et al. 2019) may be a first choice of classical biocontrol agent in regions where *B. hilaris* is still a major pest. *Ooencyrtus mirus* may be a complementary biocontrol agent, especially if it exploits a different niche than *G. gonikopalense*. For example, the cottony cushion scale was controlled effectively by the introduction of two biocontrol agents, one that preferred the hot, dry inland climate and the other that preferred the cooler, more humid coastal climate of California (Quezada & DeBach 1973). Even where their geographic ranges overlapped, the two species were abundant in different seasons (Quezada & DeBach 1973). Perhaps *O. mirus*' niche is finding host eggs on

post-crop plant debris. *Ooencyrtus mirus* could also be considered for augmentative use on a variety of pentatomid hosts, including in its native range.

In summary, the current study provides information for rearing *O. mirus* such as optimum age of the wasp (3-10 days) and its host eggs (0-1 days, Chapter 2), temperature (32°C, Chapters 4 & 5), cold storage at 14-16°C (Chapter 4), timing of host egg availability (not a constant supply, Chapter 3), and ratio of wasp:host eggs (1:4 for 24-hour exposure, Chapters 2 & 3). The host range study showed *O. mirus* to be a generalist with a preference for *B. hilaris*. The fecundity, fertility, life cycle and longevity of *O. mirus* are comparable to those of other species used for biological control, and they indicate a capacity for managing *B. hilaris*. The host-finding ability of *O. mirus* seems poor, warranting further research on how it finds host eggs in the field. These studies describe the biological characteristics of *O. mirus* that suggest it could be an effective biological control agent. Future research should extend the results herein to greenhouse and field studies to further determine if *O. mirus* could contribute to the integrated pest management of *B. hilaris* and other herbivorous insect pests.

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Appendix A

Contingency tables for the fresh vs. frozen host egg study (Chapter 2)

Table 2-8. Numbers and proportions of *Bagrada hilaris* eggs parasitized by *Ooencyrtus mirus* out of 40, including two different ages of fresh eggs as well as eggs frozen for 24 hours.

	Parasitized egg			
group	**yes**	**no**	**proportion parasitized**	***P***
0-day-old	37	3	0.925	1
frozen	36	4	0.900	
1-day-old	34	6	0.850	0.737
frozen	36	4	0.900	
0-day-old	37	3	0.925	0.481
1-day-old	34	6	0.850	

Table 2-9. Numbers and proportions of *Bagrada hilaris* eggs out of 40 from which *Ooencyrtus mirus* emerged, comparing two different ages of fresh eggs as well as eggs frozen for 24 hours.

	Emerged *O. mirus* adult			
group	**yes**	**no**	**proportion emerged**	***P***
0-day-old	33	7	0.825	0.531
frozen	35	5	0.875	
1-day-old	29	6	0.829	0.571
frozen	35	5	0.875	
0-day-old	33	7	0.825	0.968
1-day-old	29	6	0.829	