Reconsideration of the role of yeasts associated with *Chrysoperla* green lacewings

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Abstract

As larvae, lacewings in the genus *Chrysoperla* (Neuroptera: Chrysopidae: Chrysopinae: Chrysopini) are predators of aphids and other soft-bodied insects. Adult *Chrysoperla*, however, are not predacious and feed on pollen, nectar, and honeydew. Earlier studies observed that *Chrysoperla* adults house yeasts in their crops, and based on the results of a sorbic acid yeast suppression experiment, concluded that the yeasts may supplement amino acids missing in the largely carbohydrate diet. In the current study, attempts to cure adult *Chrysoperla comanche* (Banks) and *Chrysoperla carnea* (Stephens) of yeasts using Hagen et al.'s [Bull. Lab. Entomol. Agric. Fil Silv. 28 (1970) 113] protocol, as well as several other fungicides and heat treatment, were unsuccessful, thus calling into question earlier conclusions about the yeasts. Based on our findings, we suggest possible methods for producing yeast-free lacewings so that future studies might determine how yeasts contribute to lacewing fitness. The earlier research also suggested that lacewings eclose without their yeast symbionts and must obtain them from the environment. Our data suggest that yeast may be transmitted vertically, from mother to offspring.
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Keywords: *Chrysoperla comanche*; *Chrysoperla carnea*; *Metschnikowia chrysoperlae*; *Candida picachoensis*; *Candida pimensis*; Yeast symbionts; Vertical transmission

1. Introduction

Symbiotic associations in insects are widespread. Douglas (1989) estimated that 10% of known insect species harbor symbiotic microorganisms, with many of these being fungal associations. Well-known examples include leaf-cutter ants with their fungal gardens (Currie, 2001), and anobiid, platypodid and scolytid beetles with their wood-digesting fungi (Buchner, 1965; Phaff and Starmer, 1987). Currently, however, the ecological roles of many symbioses between insects and their yeasts or yeast-like microorganisms remain largely unexplored.

Plant pathogenic yeasts sometimes rely on insects, e.g., members of Hemiptera, simply as vectors (Phaff and Starmer, 1987). In other examples, the partners are interdependent; typically the insect is a vector for yeasts and the yeasts provide nutrients or vitamins missing from the insect's diet. The distinction between commensal and mutualistic associations is often difficult to discern, as yeasts are widespread and relatively few examples have been the subject of study. Records of yeast–insect associations where the role of the yeasts are not well understood include: green June beetles (Vishniac and Johnson, 1990); nitidulid beetles (Lachance et al., 2003; Lachance and Bowles, 2002); clerid beetles (Lachance et al., 2001); encyrtid parasitoids (Lebeck, 1989); ichneumonid parasitoids (Middeldorf and Ruthmann, 1984); fire ants (Ba and Phillips, 1996); leafcutter bees (Teixeira et al., 2003);

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doi:10.1016/j.biocontrol.2004.06.006

solitary digger bees (Rosa et al., 1999); and vespid wasps, bumblebees (Stratford et al., 2002); honey bees (Spencer and Spencer, 1997), and the subject of the current study, green lacewings in the genus *Chrysoperla* (Hagen et al., 1970).

Earlier studies found that adult *Chrysoperla* (formerly *Chrysopa*) house yeasts in their crop diverticulum, and the authors suggested that yeasts may supplement amino acids missing in the largely carbohydrate diet of adults (Hagen and Tassan, 1972; Hagen et al., 1970). Hagen et al. (1970) also suggested that yeasts were only transmitted horizontally; they proposed that adult lacewings eclose without their yeast symbionts and must obtain them from the environment. In spite of the potential importance of yeasts in the nutritional ecology of *Chrysoperla*, little research has been done on this interaction since Hagen et al.'s influential work in the 1970s (although see Johnson, 1982; Woolfolk and Inglis, 2004), and none has sought to investigate the functional role of the yeasts. This is especially surprising considering the economic importance of this group. *Chrysoperla* species are common natural enemies in agroecosystems and are used widely in augmentation biological control programs (Daane and Yokota, 1997). *Chrysoperla* species used in augmentation programs are mass reared on artificial diets in insectaries over many generations. While insectary diets for adults provide the amino acids purported to be synthesized by the yeasts, one might imagine that adult nutrition after release in the field could be affected by the integrity of the yeast symbiosis.

The objective of this study was to examine the effects of yeasts on adult fecundity by comparing infected and uninfected *Chrysoperla comanche* (Banks). We reared lacewings in aseptic laboratory conditions, following Hagen et al.'s (1970) and Hagen and Tassan's (1972) assertion that lacewings eclose without their yeasts. These lacewings were then fed protein and/or carbohydrate diets with or without lacewing yeasts from culture to determine the effect of the yeasts on fecundity. This experiment showed that all lacewings were yeast infected regardless of treatment. Therefore, a second experiment followed Hagen et al.'s (1970) protocols in an effort to replicate their earlier work. A final experiment showed the presence of cultivable yeasts on the egg surface, thus suggesting a possible mechanism for vertical transmission in this system.

1.1. Natural history of the lacewings

Adult *Chrysoperla* lacewings produce a series of eggs laid singly, each with a long stalk or pedicel. The larvae hatch 2–3 days later, rest on the chorion, and climb down the pedicel (Canard and Volkovich, 2002). The larvae are voracious predators of aphids and other soft-bodied insects. The three larval instars together last 8–11 days, at which point the third instar larva secretes a silk cocoon and pupates. A sub-imago emerges from the cocoon approximately 8 days later, and the imaginal stage ecloses hours afterwards. There is a preoviposition period of 5–13 days, as the gonads require time for maturation (Canard and Volkovich, 2002). Mating occurs at the end of this maturation period and stimulates oviposition (Sheldon and MacLeod, 1974). As adults, *Chrysoperla* species are non-predacious and feed primarily on honeydew, nectar, and occasionally pollen (Principi and Carnard, 1984). Nectar feeding is considered ancestral in the Chrysopidae, while some genera (e.g., *Chrysopa* and *Atlantochrysa*) have returned to the predacious habit found in more basal neuropteran families (Brooks and Barnard, 1990).

1.2. Natural history of the yeasts residing in Chrysoperla adults

The term yeast is not a taxonomic designation, but refers simply to a growth form. Yeasts are unicellular fungi that reproduce by budding (Alexopoulos et al., 1996). The yeasts in *Chrysoperla* are most abundant in the diverticulum of adults. The diverticulum is a blind pouch, with numerous folds and smaller pockets, connected to the esophagous before the midgut. The diverticulum is supplied oxygen via tracheal trunks and tracheoles. Hagen et al. (1970) noted that non-predacious adults that house yeasts have tracheal trunks that are up to five times larger in diameter than lacewing species where the adults are predacious, presumably to meet the oxygen demand of the resident yeasts. While previous efforts have failed to observe yeasts in larval, pupal or sub-imago stages of *Chrysoperla* lacewings (Hagen et al., 1970, C. Gibson unpublished), Woolfolk and Inglis (2004) recently reported isolating yeasts from the alimentary canals of field-collected *C. rufilabris* larvae. This last finding is consistent with our results, that yeasts are likely present in all life history stages rather than exclusively in adults, and suggests that yeasts are vertically transmitted.

Hagen et al. (1970) identified the yeasts in *Chrysopa carnea* (now likely *Chrysoperla carnea*) as *Torulopsis* sp. (= *Candida*), with 6.8 μm spherical cells. Hagen et al. (1970) also noted that the *Candida* spp. collected from different populations of *C. carnea* appeared to vary among geographic locations. Later, Johnson (1982) identified yeast samples from *Nodita occidentalis* and *Eremochrysa punctinervis* lacewings as *Candida multigemmis* based on morphological characters. This author noted the same yeasts from two populations of *C. carnea*, but owing to differences in some of the fermentation and assimilation tests, was uncertain whether all of these yeasts were conspecific. More recently, Woolfolk and Inglis (2004) consistently isolated *Metschnikowia pulcherrima* (identified from both molecular and morphological data) from two *C. rufilabris* populations in Mississippi.

2. Materials and methods

The lacewing yeasts from *C. comanche* and *carnea* collected in Arizona are readily cultivated on acidified yeast-malt (AYM) agar and have a matte cream color. Cells are round to slightly ovoid and are 3–10 μm in diameter at maturity. The yeasts cultured from *C. comanche* and *C. carnea* adult diverticula were identified as three new species: *Metschnikowia chrysoperlae*, *Candida picachoensis*, and *Candida pimensis* (Suh et al., 2004).

2.1. Yeast culturing methods

Lacewing yeasts were cultured from sterile dissections of the diverticula. Lacewings were frozen in a sterile petri dish, allowed to thaw approximately 15 min and then rinsed with a 10% bleach (5.25% sodium hypochlorite), 70% ethanol, 20% distilled water solution. Lacewings were dissected in autoclaved insect Ringer's solution, the diverticulum was excised, placed on a glass slide, and a yeast score was determined (see below). In a laminar flow hood, the coverslip was removed, one drop of sterile distilled water was added, and the diverticulum was lacerated into a slurry with flame-sterilized minuten pins. One drop of the slurry was applied to an AYM agar plate, pH 3.5–4.0. Cream-colored colonies appeared after 48 h at room temperature ($\sim$20 °C).

2.2. Yeast augmentation experiment

Using Hagen et al.'s (1970) and Hagen and Tassan's (1972) conclusion that *Chrysoperla* adults eclose without their yeast symbionts as a starting point, we tested whether offering additional lacewing yeasts (from our culture) in diet treatments could increase fecundity in aseptically reared (presumably "symbiont free") individuals. *C. comanche* lacewings used in this experiment were laboratory-cultured, one generation removed from the field. The parents were collected from a pecan orchard (Picacho Pecan, Picacho Peak AZ, USA) on 9 July, and 14 August, 2001. Larvae were reared on cereal aphids (*Schizaphis graminum*) on barley in paper cups throughout their development. Pupae were removed and placed in sterile petri dishes. Male–female pairs were placed in new paper cups upon eclosion. Cups were modified by replacing the top of the lid with organdy fabric for ventilation. The bottom of the cup was lined with sterile filter paper. Eggs were counted each day by placing adults in a sterile petri dish on ice and plucking the eggs from the walls and lid of the cup with forceps.

During the experiment, sterile (millipore filtered) distilled water was provided ad libitum to the lacewings via a 1.2 ml glass vial stopped with cotton. The vial was secured to the organdy on top of the cup with a thin piece of masking tape. Lacewings were held in an environmental chamber at 25 °C and 65% r.h. Treatment diets included Wheast, which is an artificial diet powder composed of yeast hydrolysate and whey (Planet Natural, Bozeman, MT). This powder is mixed with equal parts sugar and water to form a thick paste. In this experiment, 2.3 g Wheast, 4 ml honey, and 4 ml distilled water were used. Specifically, treatment diets were composed of the following: (1) Wheast + honey; (2) Wheast + honey + lacewing yeasts (between 150 and 300 mm^3 of yeast cells from culture); (3) honey; (4) honey + yeasts from culture; and (5) honey + an inoculum of yeasts from culture administered in the first two days after eclosion. Treatment diet (50 μl) was applied to the top of the organdy daily. To ensure that lacewings could not become infected with yeasts from any of the components within the treatment laboratory diets, each component was tested on AYM agar plates. No yeasts were obtained from any of the diet components. Cultivable yeasts were obtained from diets that included lacewing yeasts from culture, indicating that the yeasts were still active in the diet at the end of the experiment.

Females were frozen and their diverticula excised 13 days after the start of oviposition and examined at 100 and 200× with phase contrast optics. Attempts to quantify yeast loads using a hemocytometer yielded such widely variable results that a scoring system was developed that better represented what was observed in vivo. Yeast score was assessed from the density of yeast cells in the diverticulum as well as the surrounding medium. Scores ranged from 1 to 6. On this scale: (1) very light infection, few cells present; (2) cells forming distinct clumps but diverticulum primarily empty; (3) uniform spread of yeast cells where present, but smaller pockets of diverticulum empty; (4) diverticulum uniformly full of yeast cells but not very densely packed (i.e., there was only a single layer of cells); (5) full of yeasts and some parts appearing opaque and cream-colored where multiple layers of cells are present; and (6) diverticulum completely opaque and cream colored.

2.3. Modified Hagen et al. (1970) experiment

Chrysoperla carnea lacewings in this experiment were purchased from Rincon-Vitova Insectaries (Ventura, CA 93002-1555) in the late third instar larval or pupal stage. Upon eclosion one male and female pair was placed in a sterile plastic petri dish. Diet was offered to the lacewings in small cups fashioned from aluminum foil. Sterile distilled water was offered ad libitum in a 1.2 ml glass vial stopped with cotton and held in place with removable adhesive putty (UHU tac, ME 04364). Eggs were counted every third day by chilling adults in a refrigerator (4 °C) and transferring them to clean petri dishes with fresh food and water. Eggs were then counted in the vacated petri dishes. Lacewings were held in an environmental chamber at 25 °C and 65% r.h. Females were dissected at the end of 30 days and a score determined for

yeasts in the diverticulum as in the previous experiment. Diverticula of adults that died during the experiment were often too desiccated to determine yeast infection; these individuals were excluded from the yeast score analysis. Lacewings were weighed as pupae and the diverticula were measured.

This experiment was designed to reproduce four of Hagen et al.'s (1970) diet treatments that were most critical in suggesting a connection between yeast infection and lacewing nutrition. Hagen et al. (1970) used sorbic acid, a fungistat, to suppress yeast infections in lacewings. In a treatment with nine amino acids, where valine was excluded and sorbic acid added, Hagen et al. (1970) noted much lower fecundities than those in which valine and sorbic acid were both omitted. This led Hagen et al. (1970) to conclude that yeasts supplied the missing amino acid. We replicated these treatments, as well as treatments with 10 amino acids with sorbic acid and nine amino acids without sorbic acid. In fermentation and assimilation tests of the lacewing yeasts reported elsewhere (Suh et al., 2004), weak growth was noted in the presence of 0.01% cycloheximide. Therefore, we included a fifth diet treatment with 0.01% cycloheximide in an attempt to cure lacewings. Diet treatments included: (1) 10 amino acids as in the Hagen et al. (1970) experiment (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) in the concentrations specified in Hagen et al. (1970); (2) 10 amino acids + sorbic acid (50 mg added to total diet); (3) nine amino acids (valine omitted); (4) nine amino acids (valine omitted) + sorbic acid (50 mg added to total diet); (5) 10 amino acids +0.01% cycloheximide. In addition, all diets included fructose, B vitamins, Wesson salt mixture and cholesterol as specified by Hagen et al. (1970). Due to extremely high mortalities (up to 58%) in the nine amino acid treatments, on day 15 of the experiment one randomly selected female from each treatment was sacrificed to get an approximate measure of yeast infection. After this time point, between 150 and 300 mm^3 of yeast cells cultured from *C. carnea* adults collected in Tucson, AZ were added to all diet treatments. At the end of the experiment, a 250 µl sample from each treatment diet was cultured on an AYM agar plate, showing active lacewing yeasts in all but the diet that included cycloheximide. Finally, unlike Hagen et al. (1970), all lacewings were dissected at the end of the experiment to determine yeast infection status.

2.4. Yeast transmission experiment

Gravid *Chrysoperla comanche* and *carnea* females were collected (6–12 May, 2002) in Tucson, AZ, USA, held in paper cups and fed Wheast artificial diet mixed with honey. Eggs were collected after 3 days of oviposition. Egg collections from each cup represented the offspring of up to 10 females, and eggs were randomly

distributed among treatments (see below). Forceps were flame-sterilized and eggs were handled carefully to prevent contamination. Eggs were rinsed in a treatment solution in a 2 ml Eppendorf tube, and the solution was subsequently wicked off with filter paper. Treatments included: 50% bleach (5.25% sodium hypochlorite), amphotericin B fungicide, sterile water, or no rinse. Preliminary trials showed that with less than 30 min exposure, the chorion does not rupture in 50% bleach solution. Therefore, 10 min in 50% bleach solution was considered a surface sterilization of the egg. Eggs were homogenized in 750 µl sterile distilled water with a flame-sterilized probe. Homogenate (375 µl) was then applied to an AYM agar plate and incubated.

2.5. Analysis

Kruskal–Wallis rank sum and Student–Newman–Keuls non-parametric tests were used to analyze fecundity data in the yeast augmentation and modified Hagen et al. (1970) experiment, as some females laid many times fewer eggs than other females, and therefore, the data were not normally distributed (Zar, 1996). Yeast scores were normally distributed and ANOVAs and linear contrasts were used for these analyses.

3. Results

3.1. Yeast augmentation experiment

The experiment was run in two blocks, but the absence of any statistical significance attributed to block allowed the analysis of the data as a single data set. Females fed artificial Wheast diets laid significantly more eggs per day than females in the honey diet treatments ($\chi^2 = 35.6$, $df = 4$, $P < 0.0001$; Fig. 1A). There was also a significant treatment effect with respect to yeast score ($F_{4,49} = 5.9$, $P = 0.0006$), however linear contrasts showed that the females fed the Wheast only diet had the highest yeast score, while yeast scores in all other diet treatments were indistinguishable (Fig. 1B). In particular, yeast score was not influenced by the presence of lacewing yeasts in the diet.

3.2. Modified Hagen et al. (1970) experiment

Before addition of yeasts in the diets, there was a significant difference ($\chi^2 = 30.84$, $df = 4$, $P < 0.001$) between the female fecundities in the 10 amino acid treatments and the nine amino acid treatments; with no effect of sorbic acid (Fig. 2). On day 15, the dissections from one female in each treatment showed that all of the females were yeast infected except the female in the nine amino acid + sorbic acid treatment. After addition of yeasts in diets, there was no significant difference with respect to
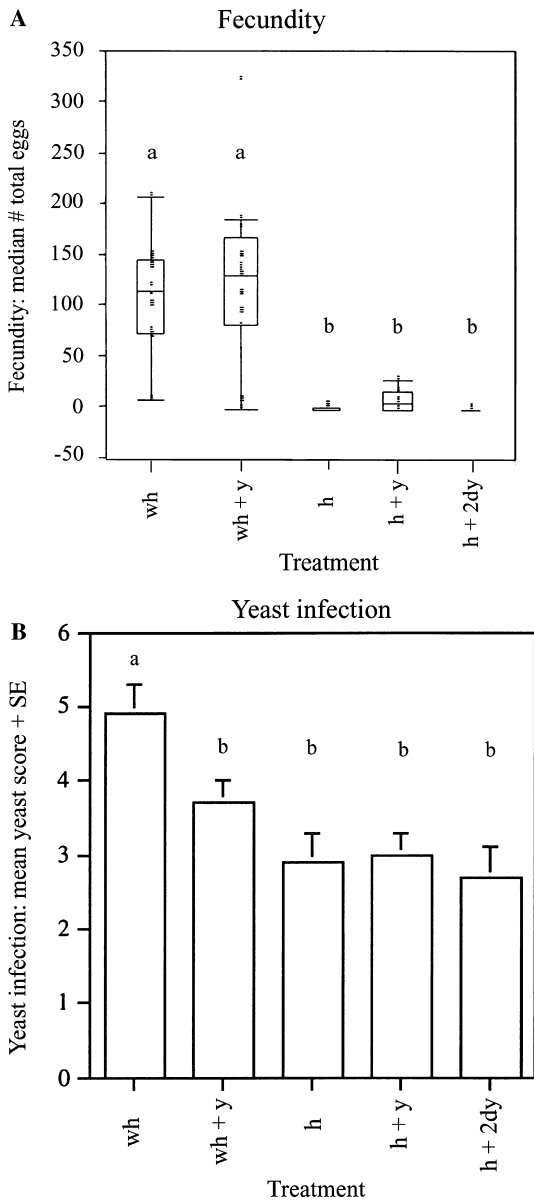
A

B

Fig. 1. Fecundities and yeast infection scores in the yeast augmentation experiment. Treatments and sample sizes are as follows: wh, Wheast ($n = 10$); wh + y, Wheast + yeasts from culture ($n = 12$); h, honey ($n = 10$); h + y, honey + yeasts from culture ($n = 11$); h + 2dy, honey + yeasts from culture for the first two days of the experiment only ($n = 17$). Bars with different letters are statistically significantly different from one another ($P < 0.05$).

fecundity among any of the treatments. Females fed the nine amino acid diet had a significantly higher yeast score than females in the nine amino acid + sorbic acid or ten amino acid + cycloheximide treatments, but not significantly different from the ten amino acid + sorbic acid treatment (Fig. 3). There was no significant relationship between female pupal weight and diverticulum length or between these variables and total fecundity or yeast score (data not shown). There was a significant positive relationship between yeast score and total fecundity ($F_{1,67} = 6.22$, $P = 0.02$).
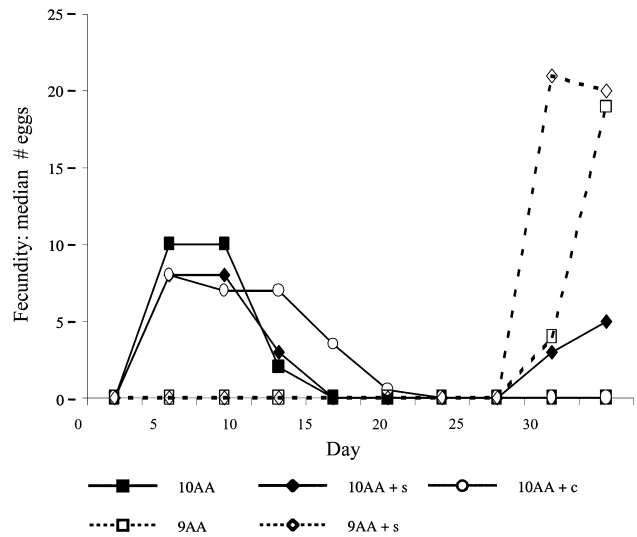

Fig. 2. Fecundities over time in the modified Hagen et al. (1970) experiment. Treatments and sample sizes are as follows: 10 AA, 10 amino acids ($n = 19$); 10 AA + s = 10 amino acids + sorbic acid ($n = 18$); 10 AA + c = 10 amino acids + cycloheximide ($n = 13$); 9 AA, nine amino acids (valine omitted) ($n = 19$); and 9 AA + s, nine amino acids (valine omitted + sorbic acid) ($n = 19$). Lacewings were fed yeasts from culture starting on day 15.
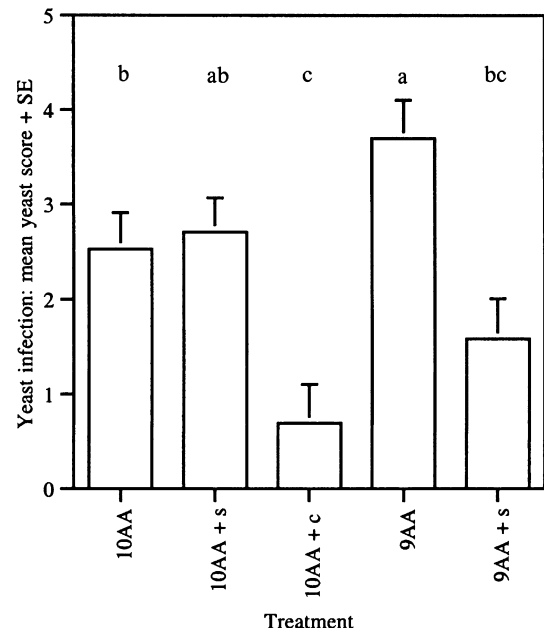

Fig. 3. Yeast infection scores in the modified Hagen et al. (1970) experiment. Treatments and sample sizes are as follows: 10 AA, 10 amino acids ($n = 15$); 10 AA + s, 10 amino acids + sorbic acid ($n = 17$); 10 AA + c, 10 amino acids + cycloheximide ($n = 13$); 9 AA, nine amino acids (valine omitted) ($n = 13$); and 9 AA + s, nine amino acids (valine omitted + sorbic acid) ($n = 12$). Bars with different letters are statistically significantly different from one another ($P < 0.05$).

3.3. Yeast transmission experiment

Lacewing egg homogenate from eggs rinsed in sterile distilled water or from those not rinsed produced a

Table 1
Results of the vertical transmission experiment

Trials	No. of eggs per trial	Treatment	Duration (min)	AYM agar culture plate results
n = 3	60	Amphotericin B	40	Other fungus (1), bacteria (1), nothing (1)
n = 3	60	Sterile distilled water	10	Lacewing yeasts (1), nothing (2)
n = 3	60	Bleach	10	Nothing (3)
n = 5	60 (3), 200 (2)	No solution	na	Lacewing yeasts (5) + other fungus (1)

Numbers in brackets refer to replicates of each event. (see Section 2 for further explanation).

culture of lacewing yeasts on agar plates (Table 1). In contrast, homogenate from eggs rinsed in amphotericin B fungicide (0.1 mg/ml) resulted in the growth of only bacteria and another fungus (possibly *Cladosporium* sp.) on agar plates. Homogenate from eggs rinsed in a 50% bleach solution resulted in no microorganisms growing on the agar plates.

4. Discussion

In our first experiment, where we sought to identify the influence of lacewing yeasts on adult nutrition, we were unable to produce uninfected lacewings, despite aseptic rearing conditions. Lacewings reared without a source of yeasts had no lower levels of infection than lacewings reared with yeasts (Fig. 1B). In this and in other curing experiments not reported here, fewer than 15 of approximately 500 lacewings dissected were ever found to be free of yeasts (interestingly, these 15 were all insectary-reared *C. carnea* females). In general, adults dissected less than 24 h post-eclosion generally had extremely light infections (often consisting of very few cells) but serial dissections of lacewings from the same cohort showed that infections in adults dissected 72 h post-eclosion were well established. While we cannot rule out the possibility that an unknown source of contamination caused yeast infection of the laboratory-reared lacewings in this study, these results support the hypothesis that lacewing yeasts are transmitted from mother to offspring (vertical transmission). This hypothesis contrasts with that of Hagen et al. (1970) who proposed that yeasts are only transmitted horizontally. Our other observations also appear more consistent with vertical transmission. For instance, the yeast transmission experiment results (Table 1) support this hypothesis by providing a mechanism by which adults may pass the infection to larvae. The surface of *Chrysoperla* eggs were found to yield active yeasts. Lacewing larvae likely ingest yeasts as they chew their way out of the egg chorion (Buchner, 1965; Fukatsu and Hosokawa, 2002). Also supportive of the vertical transmission hypothesis are the observations of yeasts in larval *C. rufilabris* (Woolfolk and Inglis, 2004). Lastly, the recent identification of *Chrysoperla* sp. yeasts as new species (Suh et al., 2004), readily cultured but never before identified in environmental sampling efforts, undermines the idea that these yeasts are common contaminants in the laboratory environment. Suh et al. (2004) also showed that *M. chrysoperlae* can be recovered from adults as well as eggs. More definitive support of the vertical transmission hypothesis would be provided by consistent detection of yeasts in larvae. Amplification of lacewing yeast-specific gene sequences from larval stages via PCR may be a way to detect low density yeast infections that are difficult to discern from dissections alone. It is important to note that these findings do not exclude the possibility that the yeasts may also be transmitted horizontally, as suggested by Hagen et al. (1970). We observed the mating trophallaxis mentioned by Hagen et al. (1970) and Hagen and Tassan (1972). The droplets of regurgitate exchanged in mating trophollaxis are similarly exuded when lacewings are alarmed (for instance, if lacewings are manipulated with forceps). We found these droplets yield cultivable yeasts, which suggests that yeasts are also likely to be exchanged during mating trophallaxis.

The results of our experiments failed to confirm the finding of Hagen et al. (1970) and Hagen and Tassan (1972) that yeasts supply missing amino acids and hence are able to augment fecundity. The authors of these studies based their conclusions on the difference in fecundity between the diets with and without sorbic acid, inferring that sorbic acid suppressed yeast growth. Our results showed no reduction in fecundity attributable to sorbic acid. In fact, we found that the dose of sorbic acid used in this experiment and specified by Hagen et al. (1970) had no effect on the level of yeast infection observed. Hagen et al. (1970) did not systematically assay lacewings for yeasts following experimental treatments, and thus there is no record of the effect of sorbic acid on the yeast infections of the females from their study. Our observations suggest it is very difficult to cure lacewings of their yeast infection. In other experiments, we attempted to cure lacewings by a variety of methods: concentrations of sorbic acid 2.5 times the concentration of Hagen et al. (1970), two generations of amphotericin B, a fungicide used for human systemic yeast infections (0.25 mg/ml), and two generations of heat treatment (four days after eclosion at 35 °C). None of these treatments influenced yeast densities (data not shown). Cycloheximide alone appears to be effective at lowering yeast infection levels (Fig. 3).

The results of the modified Hagen et al. protocol reported here showed that the addition of lacewing

yeasts from culture may "rescue" females on a diet without valine (Fig. 2). Before addition of yeasts, these females had a high rate of mortality and laid very few eggs, but following the addition of yeasts, the nine amino acid treatments were statistically indistinguishable from the others. However, it is not possible to determine from these data whether the nutritional benefit of the yeasts came from an infection first establishing and then producing valine, or whether the lacewings were simply digesting the yeasts as a source of protein as they would Wheast powder. It is doubtful that the former explanation is correct, as most of the lacewings dissected prior to the introduction of yeasts into the diets were already infected with yeasts. The results of the yeast augmentation experiment, also indicate that supplemental yeasts are not effective in restoring egg production when lacewings are on strictly carbohydrate diets. This suggests that lacewings are unable to receive full nutritional benefits from relying on the yeasts alone as a protein source. Resident yeasts may require some minimum quality of diet to become established, and only then can provide benefits to the adult lacewing.

Why were the results of this study so different from those of Hagen et al. (1970)? Some aspects of the studies were different, and some details of the original study conditions were not described in detail. The data reported in both Hagen et al. (1970) and Hagen and Tassan (1972) came from a single data set. Lacewings used in that study were laboratory-reared F_3 to F_{30} from the field (Kerman, California), but it is unclear whether special efforts were made to reduce fungal contamination in the laboratory or whether the lacewings were reared separately or in groups. In addition, there were only 10 females in each treatment, with mortalities as high as 90%, and no clear records of which females were yeast infected at the end of their experiment. With this limited data set, the difference between treatments may simply have been due to chance. We suggest that these authors conducted their experiments on lacewing yeasts as incidental to a larger study on lacewing nutrition, and would likely have been surprised at how uncritically the interpretation of their results has been repeated in the 30 years since.

In the absence of convincing evidence that resident yeast infections provide nutritional benefits to lacewings, it may be more conservative to make a starting assumption that the yeasts are largely neutral for the lacewing partner. Some *Drosophila* and *Apis* species feed on nectar that also includes *Metschnikowia* spp., and the yeasts are then concentrated in their crops or diverticula (Lachance et al., 1998; Spencer and Spencer, 1997). It is possible that the diverticulum may simply be a structure easily colonized and/or usurped by yeasts and tracheal trunks might grow larger in response to oxygen demand regardless of whether yeasts are mutualists, commensals or parasites. Our results do not rule out the notion that lacewings are simply vectors for yeasts. Based on Martin's (1992) distillation of Taylor's (1983) research, five stages are proposed for the pathway to obligate mutualism: (1) consistent and extended contact; (2) avoidance of lethal harm during contact; (3) coadaptation, leading to increased tolerance; (4) further coadaption, leading to dependence and/or interdependence; and (5) still further coadaptation leading to permanent association. *Chrysoperla* lacewings and their yeasts have consistent and extended contact with no evidence of harm to either partner. However, the ease with which the yeasts can be cultured suggests these two organisms are not interdependent obligate mutualists as in the case of other intracellular bacterial symbionts (Douglas, 1998).

The current study raises serious doubts about the two central conclusions of the earlier Hagen et al. (1970) work as it pertained to lacewing yeasts: That lacewings are exclusively horizontally transmitted, and that yeasts supply essential amino acids to the lacewings. A reproduction of the protocols with a larger sample size, and a more rigorous examination of infection status of the treated lacewings failed to reproduce the results of the earlier study. It is important to note, however, that we were not able to definitively demonstrate vertical transmission nor rule out the possibility of a nutritional role of the yeasts. These ideas must be tested by effective curing of the lacewings. In our experiments, cycloheximide reduced yeast levels, and we suggest that several generations of treatment at low doses might yield a yeast-free line. Furthermore, experiments with bleach sterilized eggs and careful attempts to rear the larvae may yield yeast-free larvae and hence adults. Finally, we did not exhaust the possible fungicides that may have activity against lacewing yeasts, for example nystatin may be effective (Chen et al., 1981).

The results of this study underline the dangers of uncritical repetition of findings from the literature. Two of the findings of the Hagen et al. (1970) research (i.e., that yeasts are only transmitted horizontally and that the yeasts supplements amino acids in lacewing diets) have been cited repeatedly (e.g., Canard, 2002; Spencer and Spencer, 1997; Woolfolk and Inglis, 2004). Nutritional roles for symbionts are often anticipated, likely because of the tremendous influence of Buchner's (1965) work. These ideas have been rigorously supported in other symbioses e.g., bacteriocyte symbionts in many Hemiptera (Moran and Telang, 1998) and yeast-like symbionts in *Nilaparvata lugens* (Wilkinson and Ishikawa, 2001). However, until a reliable method of producing yeast-free lacewings is available, these ideas cannot be adequately tested in this system.

Acknowledgments

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