OVIPOSITION ECOLOGY OF HOUSE FLIES, *MUSCA DOMESTICA* (DIPTERA: MUSCIDAE): COMPETITION, CHEMICAL CUES, AND BACTERIAL SYMBIONTS.

by

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ABSTRACT

House fly larvae face several challenges during their development to adulthood. They must: (1) condition their nutritional resources while avoiding intraspecific competition; (2) avoid competitive fungi and/or inhibit fungal growth; and (3) obtain sufficient bacteria as food supplements. In my thesis, I show that house fly eggs are provisioned with bacterial symbionts that play a major role in addressing all of these challenges, and that these bacterial symbionts can be vertically transmitted by house flies from one generation to the next. Specifically, I have shown that: (1) gravid female house flies deposit, and respond to, a time-dependent bacterial cue, *Klebsiella oxytoca*, that proliferates over time on the surfaces of deposited eggs, inhibiting further oviposition when a threshold bacterial density is reached. This affords female house flies the resource-conditioning benefits of aggregated oviposition while decreasing the risks of cannibalism by older conspecifics; (2) house fly eggs are associated with several bacterial strains, each with a different spectrum of anti-fungal properties that aid in inhibiting the growth of competitive fungi. Volatile semiochemical cues produced by these fungi inhibit oviposition by gravid female house flies, helping them avoid detrimental competition with them; (3) gravid female house flies deposit bacteria that significantly increase larval survival in resources lacking in appropriate bacterial food, likely through supplementation of larval nutrition.

Using pEGFP-transformed *K. oxytoca*, I demonstrated that *K. oxytoca* introduced onto the surface of house fly eggs is maintained on and in house flies throughout larval, pupal, and adult stages.

Keywords: *Musca domestica*; Diptera; Muscidae; *Klebsiella oxytoca*; communication ecology; microhabitat management; bacterial symbiont, resource competition, vertical transmission.

DEDICATION

To my family:

Mom and Dad, Gyaa and Sis,

Baby and Peeps, Grieslabbers and good friends.

Life is full of joy, thanks to you.

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1.1 General Introduction to Thesis Research

Most vertebrates achieve high fitness by producing high-quality offspring. For example, parents of most mammals and many reptiles produce high-quality offspring by provisioning them with essential prenatal resources, either within eggs or via a placenta (Charnov, 2001; McGraw, 2006; Sibly and Brown, 2009). After birth or hatching of offspring, parents protect them from adverse environmental conditions, pathogens and predators, and they continue to feed and teach them for some time (Sheridan and Ocock, 2008; Fedy and Martin, 2009). All this parental care may come at a cost to the parent, decreasing the time and resources that remain for producing more offspring (Magrath and Komdeur, 2003; Stiver and Alonzo, 2009) and shortening parent longevity (Sparkman and Palacios, 2009). Most insects, in contrast, produce a very large number of offspring that are relatively inexpensive in terms of time and energy investments. Instead of providing direct parental care, insects often locate ephemeral oviposition resources that provide the food, protection, and, in some cases, the host-recognition cues that offspring require to survive and become fully functional, reproductively-successful adults (Sipura *et al.*, 2002; Matsumura, 2004; Lam *et al.*, 2009).

This strategy of using ephemeral ovipositional resources for surrogate parental care may free insects from the energetic costs that constrain the reproductive capacity of mammals and reptiles but it presents unique challenges for the gravid female insect. Firstly, she must locate suitable resources and assess their capacity to support the growth

and development of her offspring. The pertinent attributes and the mechanisms for assessment differ for each type of ephemeral resource. Secondly, she must find a way to avoid or minimize the adverse effects of intraspecific competition while maximizing beneficial Allee effects (when increased conspecific/larval density results in increased fitness/population growth rate). Finally, she must avoid, inhibit, or overcome interspecific competitors, predators, parasites and pathogens, while taking advantage of mutualistic and commensalistic interactions.

In my introductory chapter, I will review how, in general, insects address these challenges.

1.1.1 Suitability of Resource

The quality of ephemeral resources varies and often changes significantly over time. Therefore, a gravid female insect arriving at such a resource must assess its suitability for the development of her offspring and then decide whether to oviposit or seek alternative resources. Each type of ovipositional resource poses different challenges to insects and therefore each is discussed separately.

1.1.1.1 Insect hosts

Preference for host species. For parasitoids, the importance of host choice is reflected in their oviposition preferences for particular species of hosts. When given a choice between pupae of seven fly species, the gregarious ectoparasitoid *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae) parasitizes members of only two families (Rivers and Denlinger, 1995). Likewise, the solitary endoparasitoid *Leptopilina clavipes* (Hartig) (Hymenoptera: Figitidae) prefers particular species of hosts, reflecting the survival

probability of parasitoid offspring in each type of host (Driessen *et al.*, 1991). Female and male larvae of *Spalangia cameroni* Perkins (Hymenoptera: Pteromalidae) develop best in specific hosts, which explains why female parasitoids seek house fly and stable fly host larvae for the development of male and female offspring, respectively. Females of the ectoparasitoid wasp *Agrothereutes lanceolatus* Walker (Hymenoptera: Ichneumonidae) prefer cocooned or paper-concealed hosts over exposed hosts for oviposition; offspring on concealed hosts were shown to experience higher fitness (Ueno, 2000).

Preference for specific age or stage of hosts. Ovipositing female parasitoids also favour the age or developmental stage of a host species that maximize offspring fitness. Females of many parasitoid species prefer to oviposit in younger hosts (Harvey *et al.*, 1999; Hirose *et al.*, 2003; Hegazi and Khafagi, 2005; Greenberg *et al.*, 2008) which can result in reduced superparasitism (Hegazi and Khafagi, 2005), increased suppression of host immune responses (Dover and Vinson, 1990), and better survival, faster development and larger adult size of offspring (Harvey *et al.*, 1999). Conversely, the offspring of the polyphagous larval-pupal endoparasitoid *Aphaereta minuta* Nees (Hymenoptera: Braconidae) develop best in older host larvae (Vet *et al.*, 1993), as does *Encarsia pergandiella* Howard (Hymenoptera: Aphelinidae) (Greenberg *et al.*, 2008). Females of the braconid wasp *Aphidius ervi* Haliday (Hymenoptera: Aphidiidae) optimize fitness by ovipositing in hosts of an intermediate age (Colinet *et al.*, 2005).

Effects of host size. Host size affects both oviposition and larval development. Oviposition in larger hosts may increase host survival (Vet *et al.*, 1993), offspring size (Kouame and Mackauer, 1991; Higaki, 2003; Karsai *et al.*, 2006), the number of

offspring (Karamauna and Copland, 2000), and the proportion of females (Nishimura and Jahn, 1996; Mayhew and Godfray, 1997; Otto and Mackauer, 1998; Pandey and Singh, 1999; Ode and Heinz, 2002; Karsai *et al.*, 2006). Larvae of koinobiont parasitoids develop in hosts that continue to grow for some time after oviposition, which makes both the current size and future growth rates of hosts and their resources important to the parasitoid larvae (Vet *et al.*, 1993; Rivero, 2000). For koinobiont parasitoids, there is not always a linear relationship between host size and parasitoid success; success depends upon dynamic interactions between the growth of both the host and parasitoid(s) (Sequeira and Mackauer, 1992).

Mechanisms for finding suitable hosts. Parasitoids deploy many strategies for locating and recognising suitable hosts. To locate suitable hosts, females exploit host vibrations within fruit (Lawrence, 1981), host odours, kairomones and pheromones (Mossadegh, 1980; Wiskerke *et al.*, 1993; Hedlund *et al.*, 1996; Ohara *et al.*, 1996; Chabi-Olaye *et al.*, 2001; Jumean *et al.*, 2005, 2009) and host food-derived semiochemical cues (Vet, 1983, 1985; Leyva *et al.*, 1991; Wiskerke *et al.*, 1993; Hedlund *et al.*, 1996; Chabi-Olaye, 2001), particularly those released in response to host activity (Reznik and Chernoguz, 1988; Ohara *et al.*, 1996; Wegener *et al.*, 2001; Hoballah *et al.*, 2005). The parasitic wasp *Ixodiphagus hookeri* Howard (Hymenoptera: Encyrtidae) locates its tick host *Ixodes ricinus* L. (Acari: Ixodidae) using tick faeces, as well as odours from the ticks' mammalian hosts (Collatz *et al.*, 2010). Once female wasps have located a host, they assess its suitability by antennating the host cuticle (Chernoguz and Reznik, 1987; Michaud and Mackauer, 1994; Lee and Lee, 1991) or by probing with the ovipositor for internal cues (Chernoguz and Reznik, 1987; Lee and Lee, 1991). While internal probing

requires more time (De Farias and Hopper, 1999), it may provide a fitness benefit through more accurate or thorough host assessment.

Experience and learning. Learning plays an important role during host foraging of parasitoids. Their experience can increase their ability to recognise cues from hosts or their substrates (Vet, 1983; Vet, 1985; Hedlund *et al.*, 1996; Hoballah and Turlings, 2005). For the parasitoid *Trichogramma thalense* Pinto and Oatman (Hymenoptera: Trichogrammatidae), cues from high-quality hosts are more readily learned than those from low-quality hosts (Keasar *et al.*, 2001). For the aphid parasitoid *Lysiphlebia mirzai* Shuja-Uddin (Hymenoptera: Braconidae: Aphidiinae), learning also affects the perception of host quality or size in that it depends upon prior experience with host size (Pandey and Singh, 1999). Ovipositional experience helps the aphid parasitoid *A. ervi* Haliday avoid trails left by *Coccinella septempunctata* L. (Coleopera: Coccinellidae) ladybirds, thus reducing predation on their aphid hosts (Nakashima and Senoo, 2003).

1.1.1.2 Fruit hosts

Type of fruit. Many characteristics of fruit affect the oviposition and offspring development of frugivorous insects. The type of host fruit (Roitberg *et al.*, 1982; Liu and Huang, 1990; Prokopy *et al.*, 1999; Joachim-Bravo *et al.*, 2001), and even the specific host genotype (Rull and Prokopy, 2004), can be critically important. Fruit-derived semiochemicals are often used to locate and distinguish preferred fruits (Prokopy *et al.*, 1991; Eisemann and Rice, 1992; Witzgall *et al.*, 2005; Mendesil *et al.*, 2009). In some cases, such fruit-derived cues are essential promoters of ovarian development, egg maturation (Papaj, 2005) and oviposition (Witzgall *et al.*, 2005). Preference for a particular host plant species can also be enhanced by prior experience of adult insects

with that host, as shown with the apple maggot fly *Rhagoletis pomonella* Walsh (Tephritidiae) (Papaj and Prokopy, 1988).

Ripeness of fruit. Ripeness of fruits is an important oviposition cue for frugivorous insects. Many frugivorous insects prefer ripe over unripe fruits for their offspring (Liu and Huang, 1990; Chu and Tung, 1996; Joachim-Bravo *et al.*, 2001), while others benefit from ovipositing on firm, unripe fruits (Frias, 1995; Diaz-Fleischer and Aluja, 2003, Sidney *et al.*, 2008). Both host fruit firmness and sugar content are important factors assessed by gravid female *Anastrepha ludens* Loew (Diptera: Tephritidae) (Diaz-Fleischer and Aluja, 2003).

Toughness of fruit skin. The type of skin and penetrability of fruits affect oviposition preferences of frugivorous insects. Fruits with a more penetrable skin, or with physical damage caused by biological or physical means, may induce oviposition by insects that take advantage of the easy access to the fruit interior (Liu and Huang, 1990; Chu and Tung, 1996; Delgado *et al.*, 1997; Sidney *et al.* 2008). The fruits' reduced barrier to oviposition may also help explain why some insects prefer ripe fruits (Messina and Jones, 1990; Messina *et al.*, 1991).

Other factors. The size of a fruit affects the number of insect offspring that it can support, in turn affecting the numbers of eggs that female insects oviposit (Aluja *et al.*, 2001). In addition, the presence of a conspecific female can cause other females to more quickly initiate drilling (Prokopy *et al.*, 1999), perhaps because the presence and activity of other females indicate a high-quality host.

1.1.1.3 Dung and manure

Type, age and moisture of dung. The suitability of dung as a larval resource depends upon many factors that affect and modify the oviposition behaviour of the guild of insects that utilizes this type of resource. Firstly, larval development differs in different types or sources of manure, and ovipositing females tend to prefer manure in which their offspring develop best (Amano, 1985; Lumaret and Kirk, 1987; Dougherty and Knapp, 1994). A variety of semiochemicals may attract insects to their preferred types of manure (Campan and Campan, 1979; Reisen and Meyer, 1990; Cosse and Baker, 1996). Secondly, dung beetles, *Aphodius rufipes* L. (Scarabaeidae), and horn flies, *Haematobia irritans* L. (Diptera: Muscidae), discriminate between dung of different ages, often preferring fresher dung pats over older ones (Holter, 1979; Kuramochi, 2000). Thirdly, filth flies (Diptera) prefer dung with moderately high moisture content (Bishop *et al.*, 1996; Kuramochi, 2000) because offspring development, survival and adult size are adversely affected at very low (Fatchurochim *et al.*, 1989; Mullens *et al.*, 2002) or very high (Fatchurochim *et al.*, 1989) moisture levels.

Shape of dung. The shape of dung also affects oviposition behaviour. Flies may use visual (Campbell and Kettle, 1976) and tactile (Bay and Pitts, 1977) cues to orient towards and locate suitable dung patches. Yellow dung flies, *Scathophaga stercoraria* L. (Diptera: Scathophagidae), prefer to oviposit on small hills or rapidly drying microlocations on the dung surface rather than in depressions where rain can collect and drown offspring (Ward *et al.*, 1999). Several species of flies prefer different strata of the dung pile, with offspring developing faster in the upper than lower stratum, but risking desiccation in upper strata (Bishop *et al.*, 1996).

1.1.1.4 Carrion

Type, age and size of carcasses. The factors affecting oviposition behaviour on dung also affect oviposition on carrion. For example, carrion flies and beetles prefer specific types of carrion (Kneidel, 1984; Smith and Heese, 1993), and make oviposition decisions based on contact semiochemical cues (Holt *et al.*, 1979). The age of carrion also affects oviposition and offspring development, with many species preferring carcasses at an early rather than late state of decay (Fisher *et al.*, 1998; Archer and Elgar, 2003a). Others species, such as blow flies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae), specialise in older carrion (Hall and Doisy, 1993; Fisher *et al.*, 1998; Archer and Elgar, 2003a). Carrion size is another oviposition factor with contrasting effects on carrion flies and burying beetles (Silphidae). To carrion flies, large and small carrion are equally attractive, even though more offspring flies can emerge from the former (Kuusela and Hanski, 1982). Burying beetles reject carcasses below a minimum size that would jeopardize successful brood development (Smith and Heese, 1995). In turn, carcasses above an accepted size threshold are harder to exploit in that they take longer to conceal, are less likely to be rounded into brood balls, and are more often utilized by dipterans (Trumbo, 1992).

Effects of temperature. The temperature of a carcass strongly affects the development of larval offspring. Carrion flies generally develop faster in sunny than in shaded areas (Jenson and Miller, 2001), with temperature modulating the dominant species in that carcass (Hanski and Kuusela, 1977). Burying beetles also prefer warm carcasses in sunny areas over those in shady areas (Smith and Heese, 1995). The burying beetle *Nicrophorus orbicollis* Say requires warm temperatures to find carcasses, whereas *N.*

defodiens Mannerheim does not (Wilson *et al.*, 1984). Carrion-feeding African blowflies (Diptera: Calliphoridae), particularly *Chrysomya marginalis* (Robineau-Desvoidy) with high upper lethal temperature thresholds, can outcompete other blowfly species by using metabolic heat that raises the carcass temperature to levels lethal to other flies (Richards *et al.*, 2009).

1.1.1.5 Water pools

Food, shelter and water. Female mosquitoes (Diptera: Culicidae) oviposit in ephemeral pools of water, with a number of factors affecting their choice of oviposition site. Firstly, they prefer to oviposit in ponds or puddles where there is abundant larval food. To locate them, female *Anopheles* spp. exploit warm water temperature, elevated levels of CO_2 (Rejmankova *et al.*, 1996) and chemical cues produced by cyanobacterial mats (Rejmankova *et al.*, 2000; Rejmankova *et al.*, 2005) that could become food supplements for larvae. The presence of decaying vegetation or manure in the water also induces oviposition (Hoban *et al.*, 1980; Williams *et al.*, 1999; Chua *et al.*, 2004). Secondly, mosquitoes prefer oviposition sites that are sheltered from external environmental influences (Williams *et al.*, 1999; Chua *et al.*, 2004). Finally, larval development is altered by physical and chemical characteristics of the water, including temperature (Ikemoto and Sakaki, 1979), pH (Pillai and Madhukar, 1969; Ikemoto and Sakaki, 1979; Madigosky *et al.*, 1980; Paradise, 1998), quantity of water (Ikemoto and Sakaki, 1979; Paradise, 1998) and concentrations of DO, NH_4-N, free NH_3, organic carbon, and NO_3 (Ikemoto and Sakaki, 1979; Sinha, 1976).

1.1.2 Intraspecific interactions

As ephemeral resources are limited in space and time, interactions between conspecific females and their offspring often occur in or near these resources. Ovipositing female insects have evolved many mechanisms for avoiding adverse interactions with conspecifics while promoting positive interactions.

1.1.2.1 Adverse intraspecific interactions

Adverse effects of intraspecific competition. When ephemeral resources are visited by more than one female for oviposition, intraspecific competition can have adverse effects on their offspring. In parasitoids, superparasitism results in offspring that develop more slowly, suffer greater mortality (Conde and Rabinovich, 1979), attain lighter cocoon weights, and live shorter lives as adults with lower potential fecundity (Sallam *et al.*, 2002). In carrion, intraspecific scramble-type competition between fly larvae results in elevated larval mortality and in small adult offspring with fewer ovarioles (Putman, 1977; Williams and Richardson, 1983; So and Dudgeon, 1989). Similarly, crowding of mosquito larvae *Aedes triseriatus* Say (Diptera: Culicidae) increases mortality and developmental time, and decreases the size of emerging adults (Mahmood *et al.*, 1997). On fruits, multiple egg clutches of Mediterranean fruit flies, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae), translate into lower larval survival rates (Dukas *et al.*, 2001). In the alfalfa blotch leaf miner *Agromyza frontella* Rondani (Diptera: Agromyzidae), both interference (cannibalism) and exploitation (starvation) competition increases with increased larval density (Quiring and McNeil, 1984a). Crowding of dung beetle larvae *Aphodius rufipes* (L.) (Sacarabaeidae) can decrease their growth rate to such

an extent that the dung pat may dry out and kill all larvae before they can complete development (Holter, 1979).

Timing of successive oviposition events affects the outcome of larval competition. The outcome of intraspecific competition depends on the relative timing of oviposition events. In many species of parasitoids, a prolonged time lag between consecutive oviposition events may result in lower offspring survival (Eller *et al.*, 1990; Van Baaren and Nenon, 1996). Young larvae may become subject to physical attacks (Conde and Rabinovich, 1979; Chow and Mackauer, 1986; Lawrence, 1988; Tillman and Powell, 1992; Van Baaren and Nenon, 1996; Wang and Messing, 2003), physiological suppression (Chow and Mackauer, 1986; Hofsvang, 1988; Lawrence, 1988; Tillman and Powell, 1992; Van Baaren and Nenon, 1996; Wang and Messing, 2003) or exploitation competition (Wai and Fujii, 1990) by older larvae, or they may suffer from immune defence reactions of the host (Von Baaren and Nenon, 1996). In other parasitoid species, however, young larvae overcome invaders through interference competition, when the oviposition time-lag ranges between 24 to 48 h (Wai and Fujii, 1990; Tillman and Powell, 1992). In addition, for the aphid parasitoid *Lysiphlebus fabarum* (Hymenoptera: Braconidae: Aphidiinae), the relative success of conspecific parasitoids of different ages within a single *Aphis fabae* Scopoli (Hemiptera: Aphididae) host can vary with host genotype (Vorburger *et al.*, 2010). In Mediterranean fruit flies, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae), a time lag in oviposition events of just 1 day may have adverse effects on younger offspring (Dukas *et al.*, 2001).

Host discrimination by parasitoids. Parasitoids have evolved means to determine whether a host has already been parasitized by a conspecific. Pheromone markers left by

ovipositing conspecifics on host surfaces are common cues that help females avoid competition (Chow and Mackauer, 1986; Hofsvang, 1988; Van Okuda and Yeargan, 1988; Lee and Lee, 1991; Danyk and Mackauer, 1993; Baaren *et al.*, 1994; Gautheir *et al.*, 1996; Van Baaren and Boivin, 1998; Chabi-Olaye *et al.*, 2001; Goubault *et al.*, 2004). In one species of parasitoids, *Ephedrus californicus* Baker (Hymenoptera: Aphidiidae), experience is needed for recognition of chemical markers (Chow and Mackauer, 1986). Parasitoids also probe hosts with their ovipositor to check for internal host markers from conspecifics (Hofsvang, 1988; Lee and Lee, 1991; Gautheir *et al.*, 1996; Van Baaren and Boivin, 1998; Goubault *et al.*, 2004). Generally, external host assessment takes less time but is less accurate than internal host assessment (Goubault *et al.*, 2004). Other cues used for host assessment include chemicals released from conspecific eggs or larvae (Gauthier *et al.*, 1996; Agboka *et al.*, 2002) and changes in host quality (Chow and Backauer, 1986). Some female parasitoids even discern between hosts parasitized by kin and non-kin (Danyk and Mackauer, 1993; Gauthier *et al.*, 1996; Van Baaern and Boivin, 1998; Agboka *et al.*, 2002; Hemerik *et al.*, 2002) or they assess the number of eggs from prior oviposition events (Hemerik *et al.*, 2002).

Host discrimination by other insects. Some female insects recognise the presence of conspecific competitors on ephemeral resources. Female fruit flies (Diptera: Tephritidae), including the cherry fruit fly, *Rhagoletis cerasi* (L.), apple maggot fly, *R. pomonella* Walsh, and the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann, mark the fruit surface after oviposition with oviposition-deterring pheromones (Hurter *et al.*, 1976; Roitberg *et al.*, 1982; Papaj *et al.*, 1989; Mangel and Roitberg, 1989). The presence of conspecific eggs also deters oviposition in the bean weevils *Callosobruchus*

maculates (F.) and *C. chinesis* (L.) (Coleoptera: Bruchidae) (Mitchell, 1991; Chun and Ryoo, 1992), the Pecan weevil *Curculio caryae* Horn (Coleoptera: Curculionidae) (Smith and Mulder, 2009) and in some species of mosquitoes (Kitron *et al.*, 1989; Onyabe and Roitberg, 1997).

Assessing developmental stage and population density of conspecifics. Female insects often must assess not only the presence but also the developmental stage and population density of conspecific competitors. Female parasitoids are more likely to superparasitize hosts that have most recently, rather than some time ago, been parasitized (Tillman and Powell, 1992; Agboka *et al.*, 2002). Similarly, whether female *R. pomonella* oviposit on previously infested fruit depends upon the time lag between first and subsequent oviposition events (Mangel and Roitberg, 1989). Oviposition by dung beetles (Holter, 1979; Edwards, 1986; Yasuda, 1990) and carrion flies (Kuusela, 1984; Trumbo *et al.*, 2001) is dependent upon the population density of conspecifics already present, due to devastating effects of contest competition in crowded resources.

Strategies of early and late arriving females and their offspring. Old larvae typically outcompete young larvae but there are some exceptions. For example, females of the parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) kill conspecific eggs in host nymphs by stabbing them with their ovipositor before they deposit their own eggs (Netting and Hunter, 2000). Or, the endoparasitoid *Anaphes victus* Huber (Hymenoptera: Mymaridae) hyperparasitizes older conspecific offspring (Van Baaren *et al.*, 1995). Therefore, females arriving early or first at a prospective resource benefit from signals or cues such as host-marking pheromones (see above). Burying beetles go one step further by treating and burying carcasses, which helps reduce resource discovery by conspecifics

(Suzuki, 1999). If further oviposition cannot be avoided, other behaviour may decrease the adverse effects of conspecific competition. For example, females of the aphid parasitoid *Praon pequodorum* Viereck (Hymenoptera: Aphidiidae) that expect competition from conspecifics may self-superparasitize, thereby ensuring that one of their offspring will possess the host (Danyk and Mackauer, 1993). Carrion fly maggots move to parts of a carcass where food is still available and competition is less intense (Archer and Elgar, 2003b). *Aedes triseriatus* (Diptera: Culicidae) mosquitoes that oviposit in tree hole pools with stemflow flushing are released from competition due to the frequent input of nutrients and removal of wastes in these micro-habitats (Walker *et al.*, 1991).

Tradeoffs between competition and foraging time. Female insects must balance the benefits of avoiding intraspecific competition with the costs of seeking unutilized resources. *Rhagoletis pomonella* fruit flies spend little time foraging in trees with pheromone-marked fruit (Roitberg *et al.*, 1982), and alter their acceptance of infested fruit according to the frequency with which they encounter uninfested fruit (Mangel and Roitberg, 1989). *Callosobruchus chinensis* bean weevils alter their conspecific-avoidance behaviour according to the density of beans available (Chun and Ryoo, 1992). Female parasitoids *Dinarmus basalis* Rond. (Hymenoptera: Pteromalidae) increase their rate of superparasitism as the availability of unparasitized hosts decreases (Gauthier *et al.*, 1996).

1.1.2.2 Positive intraspecific interactions

Not all intraspecific interactions occurring within ephemeral resources have adverse effects. Many insects benefit from, or even require, the presence of conspecifics.

Benefits of aggregated oviposition. Several mosquito species prefer to oviposit in tree holes or ponds that did or do contain conspecifics (Machado, 1980; Lowenberger and Rau, 1994; Onyabe and Roitberg, 1997; Edgerly *et al.*, 1998; Mokany and Shine, 2003a). Their presence in the oviposition site is indicative of its permanence or productivity, or of larvae benefiting from an aggregation (Edgerly *et al.*, 1998). Aggregated oviposition by *Drosophila subobscura* Collin and *D. melanogaster* Meigen (Diptera: Drosophilidae) may allow larvae to exploit lower quality substrates more efficiently (Wertheim *et al.*, 2002a; Rohlfs and Hoffmeister, 2003). Enhanced activity of adults and a large population density of larvae can decrease fungal growth on fruit substrates (Wertheim *et al.*, 2002; Rohlfs *et al.*, 2005b). Single eggs of the koinobiont parasitoid *Microplitis rufiventris* Kokujev (Hymenoptera: Braconidae) in host larvae do not survive but superparasitism improves the viability of parasitoid larvae (Hegazi and Khafagi, 2005). Sweet potato weevils, *Cylas fromicarius* Fabricius (Coleoptera: Brentidae), benefit from aggregated oviposition because secretions from adults and larvae increase the quality of the host plant (Sakuratani *et al.*, 2001). When there is a risk that a large carcass will be taken over by large heterospecifics or by vertebrates, burying beetles cooperate in concealing and burying a carcass (Wilson and Fudge, 1984, Eggert and Mueller, 1992).

Mechanisms for inducing aggregated oviposition. There are various mechanisms by which insects induce aggregated oviposition on ephemeral resources. In carrion flies, the presence of conspecific eggs (McCall *et al.*, 1994), pheromones (McCall, 1995; McCall *et al.*, 1997a; McCall *et al.*, 1997b) and larval waste products and bacteria (Holt *et al.*, 1979) were all shown to induce further oviposition by conspecifics. Some fruit flies, including *Rhagoletis tomatis* Foote (Diptera: Tephritidae) and *D. melanogaster*, deposit

aggregation pheromones (Frias, 1995; Wertheim *et al.*, 2002a,b; Symonds and Wertheim, 2005) that elicit responses from later-arriving females according to their genotype (Ruiz-Dubreuil *et al.*, 1996), the quality of the fruit (Wertheim *et al.*, 2002a), and the abundance of competitive fungi (Rohlfs *et al.*, 2005). Female fruit flies also have been shown to deposit bacteria that produce an odour attractive to conspecific females (Prokopy *et al.*, 1991). House flies use the pheromone components (*Z*)-9-tricosene and tricosane that attract gravid female conspecifics (Jiang *et al.*, 2002).

Tradeoffs between allee effects and competition. As the population density of conspecifics increases, the benefits obtained from positive intraspecific interactions will eventually be offset by the costs of competition (Rohlfs and Hoffmeister, 2003). This could explain why some materials such as larval waste products and bacteria are attractive at low densities but deterrent at high densities (Holt *et al.*, 1979; Rejmankova *et al.*, 2005).

1.1.3 Interspecific interactions

Each ephemeral resource may be exploited concurrently by a variety of species, most notably insects and microorganisms. Thus, many different positive and negative interspecific interactions can occur.

1.1.3.1 Interactions with heterospecific insects and animals

Asymmetric competition. Insects that oviposit in ephemeral resources often compete with heterospecifics. This type of competition is often very asymmetric, with one species always outcompeting, if not killing, the other, while the winner suffers few or no costs. It occurs in several species of carrion flies (Blackith and Blackith, 1990; Wells and Greenberg, 1992), mosquitoes (Copeland and Craig, 1992; Juliano, 1998), fruit flies

(Rwomushana *et al.*, 2009) and parasitoids (Conde and Rabinovich, 1979). Asymmetric competition is also observed in species of large burying beetles that take large carcasses from smaller burying beetles, kill their offspring and then deposit their own eggs (Trumbo, 1990). The success of the winning competitor may be due to many factors, including the inoculation of a selective pathogen (Copeland and Craig, 1992), inhibition of egg hatching (Edgerly *et al.*, 1993), physical attack (Conde and Rabinovich, 1979), and the ability to develop at higher densities and lower nutrient availability than the competing species (Juliano, 1998).

When interspecific competition is asymmetric, the losing species would benefit from avoiding oviposition in sites already colonized by the winning species. Such avoidance behaviour has been observed in a variety of parasitoids (Chow and Mackauer, 1984; Earl and Graham, 1984; Wang and Messing, 2003), mosquitoes (Mokany and Shine, 2003a), carrion flies (Kneidel, 1984) and fruit flies (Prokopy *et al.*, 1989). Mechanisms that mediate this avoidance behaviour include resource testing with the ovipositor (Van Baaren *et al.*, 1994) and the recognition of fungi associated with the feces of heterospecifics (Mokany and Shine, 2003b). A winning species that is not adversely affected by the presence of heterospecific offspring may ignore their presence (Chow and Mackauer, 1984; Earl and Graham, 1984). If the winning species benefits from the presence of the losing one, “winner” females may deposit more eggs when “loser” offspring are present (Zaviezo and Mills, 2001).

Priority-dependent competition. The outcome of interspecific competition for resources is not consistent and may be modulated by many factors. In many interspecific interactions,

earlier arrivals, or their offspring, outcompete later arrivals. This has been documented for species of competing parasitoids (Leveque *et al.*, 1993; Agboka *et al.*, 2002; Persad and Hoy, 2003), tadpoles and mosquitoes (Caldwell, 1993), carrion flies (Hanski and Kuusela, 1977; Kneidel, 1983) and dragonflies competing for temporary ponds in the desert (Padeffke and Suhling, 2003). Some of these species have means to assess not only the presence but also the age of heterospecific competitors, and they prefer to interact with young rather than old competitors (Agboka *et al.*, 2002; Persad and Hoy, 2003).

Other factors affecting competition. Many other factors contribute to the likelihood and outcome of interspecific competition. The size of a carcass affects the speed with which burying beetles can conceal it, and thus the likelihood and intensity of competition with dipterans (Trumbo, 1992). The body size of competing adult burying beetles affects their ability to occupy and protect a carcass (Suzuki, 2000a). The temperature of a carcass determines the dominant species of carrion flies occupying it (Hanski and Kuusela, 1977). Temperature also affects the ability of the burying beetle *Nicrophorus orbicollis* Say (Coleoptera: Silphidae) to find and utilize carcasses (Wilson *et al.*, 1984). Sunlight exposure alters the distribution of midge species in rain pools (McLachlan, 1988), and moisture content of manure modulates larval development of the dung flies *M. domestica* L., *Muscina staulans* Fallen, *Fannia femoralis* Stein and *Ophyra aenescens* Wiedemann (Fatchurochim *et al.*, 1989).

Ovipositing females themselves may affect future heterospecific competition. For example, burying beetles reduce the attractiveness of a carcass to dipterans by chemically treating and burying it (Suzuki, 2000b). Adult dung beetles kill fly eggs and larvae in

manure (Smith and Matthiessen, 1984; Ridsdill-Smith *et al.*, 1987). Furthermore, partitioning of the common resource may reduce competition (Anderson, 1982) or release a species from density-dependent competition (Walker *et al.*, 1991).

Predation and parasitism. Insects using ephemeral resources face various forms of predation and parasitism. In carrion, flies are subject to size-dependent predation by ants and wasps, with smaller fly species vulnerable to both, and larger ones vulnerable to only wasps (Archer and Elgar, 2003a). In manure, fly eggs and larvae are attacked by mites (Hohmood and Al-Dulaimi, 1986; Borden, 1989), predaceous beetles (Summerlin *et al.*, 1989), dung beetles (Smith and Matthiessen, 1984; Ridsdill-Smith *et al.*, 1987), fire ants (Summerlin *et al.*, 1984) and other predators and parasites (Doube *et al.*, 1988, Failes *et al.*, 1992). These generalist or specialist attackers exploit semiochemical foraging cues from their prey or host or their habitats (Summerlin *et al.*, 1984; Borden, 1989; Hedlund *et al.*, 1996). Interspecific competition can turn into predation when the food supply dwindles (Sowig, 1997) or when one competitor has a sufficient head start on the other (Caldwell, 1993). Predation on larvae can release species from competition, resulting in higher weights of remaining pupal offspring (Peschke *et al.*, 1987; Copeland and Craig, 1992). Burying beetles face brood parasites that oviposit in their carcass (Trumbo, 1994).

Insects respond to the presence of predators and parasites. Mosquitoes avoid ovipositing in ponds containing larval predators (Walton, 2001; Spencer *et al.*, 2002). Fire ants on cattle droppings deter oviposition by flies (Summerlin *et al.*, 1984). Aphid parasitoids and hyperparasitoids avoid trails left by aphid predators (Nakashima and Senoo, 2003), and preferentially (hyper)parasitize those aphids associated with mutualistic ant colonies that protect the hosts of their developing offspring from predators

(Kaneko, 2002; Kaneko, 2003; Kaneko, 2004). Despite better offspring survival in soft ripe fruit, fruit flies prefer to oviposit in firm, unripe fruits, possibly due to higher predation risk within ripe fruit (Diaz-Fleischer and Aluja, 2003). Female burying beetle cannibalize "young" that appear > 20 h before their own eggs are "expected" to hatch, thus eliminating brood parasites (Trumbo, 1994).

Mutualisms and commensalisms. There are also positive interspecific interactions in ephemeral resources. Mutualistic ants that protect aphids from predators indirectly benefit the parasitoids and hyperparasitoids that exploit aphids as hosts (Kaneko, 2002; Kaneko, 2003; Kaneko, 2004). Mosquito larvae that filter-feed decomposing carcass particles within water-filled leaves of carnivorous plants benefit from the presence of midge larvae that eat solid carrion and thus expedite the rate of carrion decomposition (Heard, 1994).

1.1.3.2 Interactions with microorganisms

Effects of competition with microorganisms. For insects ovipositing on ephemeral resources, microorganisms may have adverse effects on larval development, particularly when they are first to colonize the resource. When fungi are established on a resource three days prior to oviposition by female house flies, all of the resulting larvae perish (Zvereva, 1986). Similarly, microorganisms may affect the number of offspring of the burying beetle *Nicrophorus quadripunctatus* Kraatz that complete development (Wilson and Fudge, 1984). Competition with fungal (Fuentes-Contreras *et al.*, 1998, Askary and Brodeur, 1999), viral (Hochberg, 1991) and bacterial (Chilcutt and Tabashnik, 1997) pathogens of the host insect affects ectoparasitoid larvae, and the outcome of this competition depends upon the relative timing of microbial infection.

Mechanisms for avoiding or inhibiting microbial competitors. Insects competing with microorganisms for ephemeral resources have diverse behavioural and chemical adaptations that enhance their competiveness. Most carrion fly species oviposit on carcasses at early, but not late, stages of decay (Hall and Doisy, 1993; Archer and Elgar, 2003a). As decay proceeds, flies select different micro-locations for oviposition, and larval offspring move within the carcass to access food and avoid competition (Archer and Elgar, 2003b). Burying beetles such as *N. quadripunctatus* exploit small carcasses and, while handling them, exude secretions that reduce the growth of competitive mould (Suzuki, 2001). House fly larvae that hatch from aggregated eggs in alkaline media (Zvereva, 1986a) outcompete fungi by releasing allomones that curtail the growth of mycelia and disturb the sporogony process of fungi (Zvereva, 1986b). Similarly, larvae of the fruit fly *D. melanogaster* benefit from aggregated oviposition when competing with fungi (Rohlfs *et al.*, 2005). When larvae of the cabbage white butterfly *Pieris brassicae* (L.) (Lepidoptera: Pieridae) are infected with a granulosis virus, they are short-lived and compromise larval development of the endoparasitoid wasp *Apanteles glomeratus* L. (Hymenoptera: Braconidae), but parent female wasps can maximize competitiveness of their larval offspring by depositing smaller complements of eggs (Hochberg, 1991). The parasitoid wasp *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae) avoids competition with the pathogen *Bacillus thuringiensis* by ovipositing in lepidopteran host larvae that are highly resistant to *B. thuringiensis* (Chilcutt and Tabashnik, 1997).

Effects and responses to microbial pathogens. Female insects ovipositing in ephemeral resources must also avoid, or minimize the effects of, pathogenic or parasitic

microorganisms that may attack them and/or their offspring. Two waterborne bacterial larvicides that kill both mosquito larvae and adults (during oviposition) have been used for many years to control mosquito populations in pools. Recently, however, mosquitoes have been shown to avoid ovipositing in such treated pools (Zahiri and Mulla, 2005). Mosquitoes also avoid ovipositing in ponds that once contained conspecific larvae that were infected with trematode parasites (Lowenberger and Rau, 1994).

Microbial food supplements. Not all microorganisms are harmful. Indeed, many are beneficial or even essential to insects reproducing in ephemeral resources. Microorganisms supplement the diet of many insect larvae. Thus, parent females either seek an oviposition site containing microorganisms or deposit them together with their eggs. Mosquitoes prefer to oviposit in ponds containing cyanobacterial mats and use bacteria-derived semiochemical cues, or pond-derived physical cues (elevated temperature and CO_2 level), to locate them (Rejmankova *et al.*, 1996, 2000). Bacteria found in waters previously inhabited by mosquito larvae induce oviposition by female *Aedes aegypti* L. (Diptera: Culicidae), likely in response to both volatile and contact chemical cues (Benzon and Apperson, 1988). *Drosophila subobscura* Collin (Diptera: Drosophilidae) fruit flies inoculate oviposition sites with yeasts which benefit offspring development (Rohlfs and Hoffmeister, 2005). *Drosophila serido* Vilela and Sene fruit flies, which exploit rotting cacti for larval development, vector beneficial yeast species that serve as food supplement (Morais *et al.*, 1994).

Microbial cues. Microorganisms closely associated with insects and/or their ovipositional resources often affect the ovipositional behaviour of these insects. For example, oviposition by female mosquitoes in waters previously used by conspecific larvae

depends upon the presence of bacteria (Benzon and Apperson, 1988) and fungi (Mokany and Shine, 2003a) closely associated with the larvae. Furthermore, microorganisms associated with eggs of the onion maggot fly, *Delia antiqua* Meigen (Diptera: Anthomyiidae), interact with onion tissue, releasing semiochemicals that induce aggregated oviposition by female flies (Judd and Borden, 1992).

Microbial symbionts. Bacterial symbionts can function as reproductive manipulators, nutritional mutualists, and as defenders of their hosts (Gibson and Hunter, 2010). Many insect parasitoids inject symbiotic microorganisms that improve development of larval offspring into their hosts. Often, successful host parasitism requires the injection of viruses (Dupas *et al.*, 1996; Renault *et al.*, 2002; Kim *et al.*, 2004; Amaya *et al.*, 2005; Lawrence and Matos, 2005) or yeast-like microorganisms (Lebeck, 1989) that suppress the hosts' immune system.

In various species of parasitoids and *Drosophila,* females transmit pathogenic *Wolbachia* bacteria that are lethal to male conspecifics (Schoenmaker *et al.*, 1998; Jaenike *et al.*, 2003; Bouletreau and Fleury, 2005). The bacteria benefit females by decreasing competition between siblings (Jaenike *et al.*, 2003) and by avoiding the adverse effects of inbreeding (Schoenmaker *et al.*, 1998). One species, *Asobara tabida* Nees (Hymenoptera: Braconidae), has even become dependent upon a specific strain of *Wolbachia* for egg production (Bouletreau and Fleury, 2005). By benefitting the female insect hosts, these bacteria increase their own chances for transmission and reproductive success.

Microorganisms and Competition. Finally, microorganisms can facilitate the insects' interactions with their competitors. In treehole microcosms, larvae of the mosquito *Aedes hendersoni* Cockerell outcompete those of *A. triseriatus* (Say) because they carry a protozoan pathogen which has little effect on *A. hendersoni* larvae, but strong adverse effects on *A. triseriatus* competitors (Copeland and Craig, 1992). Similarly, two species of tadpoles, *Limnodynastes peronii* Dubéril and Bibron and *Crinia signifera* Girard, use fungi that suppress the rate of survival and pupation in the mosquito larvae *Culex quinquefasciatus* Say and *Ochlerotatus australis* Erichson which compete with them for food (Mokany and Shine, 2003b).

1.2 General Biology of *Musca domestica*

House flies undergo complete metamorphosis, and have distinct egg, larval, pupal and adult stages (Sanchez-Arroyo and Capinera, 2008). Gravid females can lay as many as 500 eggs, in batches of 75 to 150, over a 4-d period. Eggs are about 1.2 mm in length, and hatch eight to 20 h after oviposition. Larvae develop through three instars in four to 13 d at temperatures of 35 to 38°C. They grow from a 3-mm long early-instar to a 12-mm long third-instar. Full-grown larvae crawl to cool, dry places and pupate. The 8-mm long pupae change in colour from yellow to red, brown, and then black as the pupae age.

After two to six days at a temperature of 32-37°C, an adult fly breaks open and ecloses from its pupal case by repeated swelling and shrinking of its ptilinum: a sac found at the front of its head. Adults are six to seven mm long, and males are distinguishable by eyes that almost touch, and an underside that is yellowish; females have a wider space between the eyes and a whitish underside. Newly eclosed adults require food before copulation, and oviposition can commence four to 20 d after compulation. Females

require foods containing protein to produce eggs, and will live considerably longer when suitable food, especially sugar, is available (Lysyk, 1991).

1.3 Focus of Thesis Project

Female house flies face several challenges when they seek ephemeral oviposition sites. They benefit from aggregated oviposition because large numbers of even-aged larvae warm and moisten the organic material (Bryant 1970; Barnard and Geden, 1993) and curtail growth of competitive fungi (Zvereva, 1986b). However, when females oviposit near older conspecific eggs or larvae, their offspring may be cannibalized by these older larvae (Lam, unpubl. data). Therefore, it would be adaptive if house flies were to (*i*) deposit with their eggs cues that first induce and later inhibit oviposition by conspecific females, and (*ii*) avoid oviposition sites already infested with fungi that are harmful to house fly larvae (Zvereva, 1986a,b). It would further be adaptive if ovipositing females were to provision their offspring with bacterial food (Watson *et al.*, 1993) and bacteria that inhibit fungal growth. It would also increase the fitness of house flies if the beneficial bacterial symbionts were transmitted vertically from one generation to the next. The focus of my thesis is to determine how, and why, house flies address all these challenges.

1.4 Overview of Thesis Chapters

My thesis is organized into seven chapters. The introductory chapter sets the stage for five research chapters, and the concluding chapter summarizes my major findings. The research chapters closely resemble manuscripts that are either published or in press (chapters 2-5), or currently in preparation (chapter 6). Each research chapter is presented

as a separate unit, containing an abstract, introduction, methods, results and discussion. Reference lists, tables, figures, and supplementary material are presented at the end of each chapter. The following is a brief outline of each chapter.

In Chapter 1, I describe the many factors affecting insects that oviposit on ephemeral resources.

In Chapter 2, I investigated how fresh house fly eggs first induce, and later inhibit, further oviposition by conspecific females. I demonstrated that a 24-h age-disparity between house fly eggs significantly decreases larval survival, and that ovipositional cues from house fly eggs reverse as eggs age. I then determined that an increasing abundance of microorganisms on the surfaces of house fly eggs is essential for the delayed oviposition-inhibition cue. Finally, I identified *Klebsiella oxytoca* as the key bacterium for oviposition inhibition, and demonstrated that experimental application of *K. oxytoca* to the surface of fresh house fly eggs caused a switch from oviposition-induction to -inhibition.

In Chapter 3, I investigated the potential anti-fungal properties of egg-associated bacteria. First, I demonstrated that microorganisms washed off house fly eggs decrease the rate of fungal growth in chicken manure. I then isolated bacterial strains from house fly eggs, and fungal strains from chicken manure, to determine how each bacterial strain affected each fungal strain. I show that egg-associated bacteria as a group (but no single bacterium) significantly inhibited the growth of all fungal strains tested, and that this inhibition may be due to resource nutrient depletion and/or the release of antifungal chemicals. I also show that prior establishment of fungal strains on larval resources significantly decreases larval survival.

In Chapter 4, I explored how, and why, female house flies avoided oviposition on feces infested with harmful fungi. I identified several fungal strains that inhibited oviposition by house flies on sterilized chicken manure, and I identified the semiochemical cues produced by strains that inhibit oviposition.

In Chapter 5, I investigated whether egg-associated bacteria could supplement the diet of house fly larvae when larval resources are poor in bacterial food. I determined that egg-associated bacteria increase larval survival in bacteria-poor media, and demonstrated that even oviposition-inhibiting bacteria contribute to the survival of larvae.

In Chapter 6, I study the vertical transmission of *K. oxytoca* by house flies. Using pEGFP (an enhanced green fluorescent protein-expressing plasmid) - transformed *K. oxytoca*, I showed that house fly larvae obtain *K. oxytoca* from the egg surface, and retain it internally from the larval to the adult stage. Female house flies then transfer *K. oxytoca* to the surface of their eggs.

In concluding chapter 7, I summarize my major findings, highlight their implications, and suggest directions for future research.

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2: Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies*

2.1 Abstract

Animals commonly leave scent messages by depositing pheromones, faeces, or urine. The intensity of a chemical message may fade over time, but the 'intention' remains the same. We argue that house flies, *Musca domestica* (Diptera: Muscidae), require a message with evolving (sensu changing over time) information content. Gravid females reportedly deploy a pheromone that induces concerted oviposition so that many even-aged larvae ameliorate the resource, such as animal manure. However, continued oviposition by late-arriving females may result in age disparity and cannibalism of larval offspring. Thus, we predicted that house flies have a type of cue that evolves from oviposition induction to inhibition some time after eggs are deposited on a resource. Here we show (1) the existence of such evolving ovipositional cues, (2) the adverse fitness consequences that accrue from ignoring the inhibitory cues and (3) the mechanism by which these cues evolve. The evolving cues depend upon a key bacterial strain, *Klebsiella oxytoca*, which originates with female *M. domestica* and which proliferates over time on the surface of deposited eggs. At a threshold density of this strain, further oviposition is inhibited. By deploying such evolving cues, female *M. domestica* can visit an oviposition site just once and deposit cues that will mediate immediate oviposition

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induction followed by delayed inhibition, thereby ensuring conditions conducive for offspring development.

Keywords: bacterial symbiont; communication ecology; house fly; microhabitat management; *Musca domestica*; reproductive strategy; signalling.

2.2 Introduction

Animals commonly leave scent messages by depositing pheromones, faeces, or urine (Brown & Macdonald 1985; Halpin 1986). Scents can convey diverse information, including sexual receptivity (Ferkin & Li 2005; Nojima *et al.* 2005), occupancy of a territory (Hurst *et al.* 1998), and cues to food and shelter (Taulman 1990; Reinhard *et al.* 2002). While the intensity of chemical messages may fade over time, the 'intention' generally remains fixed. For example, dominant adult male house mice, *Mus domesticus*, mark their territories with scents that deter other males (Hurst *et al.* 1998). Evaporation (Hurst *et al.* 1998) or degradation (Höller *et al.* 1991) of these scents may indicate the time elapsed since placement (Anderson 2002), and may, thus, influence whether recipients respond to the message. However, while old scents may no longer deter other mice, the intended message will probably not change from deterrence to attraction.

Parent insects that use fleeting and resource-limited habitats for offspring development often deploy scent messages that help prevent intraspecific competition. For example, ovipositing cherry fruit flies, *Rhagoletis cerasi* (Diptera: Tephritidae), and apple maggot flies, *Rhagoletis pomonella*, deposit pheromones that are immediately effective in discouraging further oviposition (Averill & Prokopy 1987; Städler *et al.* 1994).

Female house flies, *Musca domestica* (Diptera: Muscidae), also lay their eggs on fleeting organic resources (Keiding 1974). Larvae hatch within 24 h and develop through three instars in 5-7 days. After a 5-day pupal period, adult males and females eclose. Mated females lay eggs in batches of about 120, with as many as six batches per lifetime (Hewitt 1914).

Unlike tephritid fruit flies, female house flies preferentially oviposit near freshly deposited conspecific eggs. The many even-aged larvae warm and moisten the organic material (Bryant 1970; Barnard & Geden 1993), and curtail growth of competitive fungi (Zvereva 1986). Female house flies, however, should avoid oviposition near old eggs or hatching larvae because offspring may then be cannibalized by older offspring from prior oviposition events, as observed among age-disparate larvae of house flies (K. Lam, unpublished data), neotropical mosquitoes, *Tichoprosopon digitatum* (Diptera: Culicidae), and alfalfa blotch leaf miners, *Agromyza frontella* (Diptera: Agromyzidae) (Quiring & McNeil 1984a, b; Sherratt & Church 1994; Anderson 2002).

Oviposition near fresh eggs is stimulated by pheromonal and visual cues from ovipositing adults (Collins & Bell 1996; Jiang *et al.* 2002). However, there is no information on whether, when, or how a shift occurs from oviposition induction to inhibition. A pheromonal message elicits a fixed behavioural response, and therefore is unlikely to mediate a spontaneous shift in oviposition behaviour. In contrast, microorganisms, if consistently associated with house fly eggs, could proliferate over time and thus convey a message with evolving (*sensu strictu* changing over time) information content.

Microorganisms are associated with many insect species. Bark and ambrosia beetles (Coleoptera: Curculionidae) carry symbiotic fungi (Farrell *et al.* 2001) that help curtail host tree defences (Paine *et al.* 1997) or serve as a food source (Francke-Grosmann 1939; Klepzig *et al.* 2001). Microbial symbionts also participate in signalling. For example, attraction of conspecifics to host trees by mountain pine beetles, *Dendroctonus ponderosae* (Colytidae), is inhibited when fungal symbionts oxidize a

component of the beetles' aggregation pheromone to produce an antiaggregation pheromone (Ryker & Yandell 1983). Moreover, onion maggot flies, *Delia antiqua* (Meigen), deploy bacteria that produce oviposition stimulants (Hough *et al.* 1982; Judd & Borden 1992). Conceivably, ovipositing female house flies may deploy microorganisms that multiply and, at some point in time, exceed an abundance threshold above which further oviposition is inhibited.

In this study, we tested the hypotheses that: (1) asynchronous oviposition with ensuing age disparity of larval offspring has adverse fitness consequences for parental females; (2) ovipositing females deploy, together with their eggs, evolving cues that first induce and later discourage oviposition by conspecific flies; (3) egg-associated bacteria are responsible for this shift in oviposition behaviour; and (4) specific bacterial strain(s) increase in abundance over time and thus change oviposition induction to inhibition.

2.3 General Methods

2.3.1 Experimental Insects

Adult house flies were kept in cages at 50-80% relative humidity (RH), 22-30°C and a 16:8 h light:dark regime, and were provided with water, sugar cubes and skim milk powder *ad libitum*. House fly eggs were collected on cotton oviposition sites (OSs; see below) and reared to adult insects on artificial diet prepared from wheat bran (400 g), brewers yeast (15 g), molasses (15 ml) and water (700 ml), with a supplemental protein paste prepared from skim-milk powder and water. The colony had been maintained in the Simon Fraser University (SFU) Insectory for over 30 years and was started with flies captured in British Columbia, Canada.

2.3.2 Design of Oviposition Experiments

In our bioassay procedure for testing the ability of stimuli to induce or inhibit oviposition, each experimental replicate employed a mesh cage (30 × 30 × 45 cm) that contained 50 male and 50 female house flies. Two identical OSs were treated with different stimuli and were randomly assigned to opposite corners of the cage. When oviposition had ceased (3-6 h), the eggs on each OS were removed and weighed or counted (~15 000 eggs/g). Eggs were weighed for all experiments except experiments 2-3 and 22-25; for these six experiments, the egg mass stimuli were given time to hatch, at which time the still-intact eggs deposited by bioassay flies were counted.

2.3.3 Oviposition Sites (OSs)

Manure OSs, used in experiments 2-3 and 22-25, consisted of 2.5 g of fresh (<24 h old) chicken manure packed tightly into the bottom half of petri dishes (35 × 10 mm). A 5-mm diameter well was poked in the centre of the manure to serve as a focal point for oviposition. Distilled water (0.2-0.4 ml) was applied to the manure surface to prevent manure desiccation during bioassays. Manure was collected from free-range, organic chickens in Wind's Reach Farm (Langley, British Columbia, Canada) and stirred thoroughly prior to packing.

Cotton OSs were used as a more convenient and consistent alternative to manure OSs in experiments 4-8, and were also used for the collection of egg masses. Cotton OSs consisted of braided cotton rolls (10 × 150 mm) moistened with skim milk and coiled within petri dishes (35 × 10 mm). Agar OSs, used in experiments 9-20, consisted of petri plates (50 × 15 mm) containing sterile agar media consisting of water (15 ml), nutrient broth powder (0.12 g), skim-milk powder (0.24 g) and agar (0.3 g).

2.3.4 Statistical Analyses

For experiment 1, a two-tailed two-sample *t* test was conducted to compare the mean numbers of offspring flies that completed development when rearing medium was inoculated with age-disparate eggs. For experiment 21, a two-tailed matched-pairs *t* test was conducted to determine whether the abundance of *Klebsiella oxytoca* (strain SFU-1) on house fly eggs increased between 0 and 24 h postoviposition.

For experiments 2-20 and 22-25, weights or counts of eggs deposited on treatment and control oviposition sites within each replicate pair were converted to two separate percentages adding up to 100% of total eggs deposited. This approach was necessary because of variation in the total amount of eggs deposited by the flies in each replicate. For experiments 2-8 and 22-25, two-tailed one-sample bootstrap tests on the means were conducted to determine whether the mean percentages of eggs deposited on treatment OSs were significantly different from 50%. Furthermore, for paired experiments 2 and 3, 22 and 23, and 24 and 25, two-tailed matched-pairs bootstrap tests on the means were conducted to compare the mean percentages of eggs deposited on treatment OSs of each experiment. For each bootstrap test, 1000 bootstrap samples were randomly generated with replacement, and each sample contained the same number of replicates as the original data set. The mean percentage of the original data set was then compared with the mean percentages of its bootstrap samples. All of the above data analyses were conducted with SAS (ver. 9.1; SAS Institute, Cary, North Carolina, U.S.A.) with $\alpha = 0.05$.

For experiments 9-20, the percentages of eggs deposited on treatment OSs were converted to binomial data, with percentages above and below 50% indicating a

preference for oviposition on treatment and control OS, respectively. A logistic regression was then conducted to determine whether the preference changed significantly in relation to bacterial dose, and to generate a graph showing the predicted preference at any particular dose. For each experiment that showed no significant effect of dose on preference, the data from all doses were pooled and a two-tailed binomial *t* test was conducted to determine whether there was an overall preference for either treatment or control OSs. Neither logistic regressions nor binomial tests were possible when the binomial data showed 100% preference for a particular treatment. To summarize the percentages observed at each dose for each experiment, replicates with doses within each order of magnitude were grouped, and means and standard error bars for each group were included in each figure. Data analyses of experiments 9-20 were conducted using JMP IN (version 5.1; SAS Institute), with $\alpha = 0.05$.

2.4 Experiment 1: 24-h Age Disparity in Deposited Eggs Reduces Survival of Offspring

2.4.1 Methods

To assess the fitness consequences of ignoring the oviposition-inhibition signals of 24-h-old eggs, 800 eggs aged for 0 or 24 h were reared (see Experimental Insects) together with 800 additional 0-h-old eggs, so that the age disparity of eggs, but not their total number, differed. To increase competition between larvae, only 25% of the larval media described in 'Experimental Insects' was used for each replicate. The number of adult flies eclosing from each treatment was counted.

2.4.2 Results

Significantly fewer offspring survived to adulthood when 0-h-old eggs were reared with 24-h-old eggs (*t* test: $t_{39.43} = 2.05$, $P = 0.0473$; Fig. 2.1), indicating that a 24-h age disparity of eggs reduced total offspring survival.

2.5 Experiments 2–3: Evidence for Evolving Cue(s) on Ageing Eggs

2.5.1 Methods

A fresh egg mass (0.4-0.5 g) was obtained from thousands of house flies ovipositing on a cotton OS for 3 h. Eggs (0.2 g) were put onto each of two treatment manure OSs, and no eggs were put on two control manure OSs. One set of treatment and control OSs was immediately bioassayed in an oviposition experiment (see General Methods; experiment 2). The other set of treatment and control OSs was incubated for 24 h at 24°C and 85% RH and then bioassayed (experiment 3).

2.5.2 Results

The bioassay flies oviposited significantly more eggs on OSs with fresh egg masses than on egg-free control OSs (experiment 2; bootstrap test: raw mean = 81.40%, $P < 0.0001$; Fig. 2.2), with 81% of these eggs deposited near fresh egg masses, and only 19% deposited on egg-free control OSs. However, when the eggs were aged for 24 h, bioassay flies deposited only 11% of their eggs on OSs with aged eggs (and some hatched larvae), which was significantly less than the 89% of eggs deposited on egg-free control OSs (experiment 3; bootstrap test: raw mean = 10.55%, $P < 0.0001$; Fig. 2.2). The egg mass' effect on oviposition changed significantly over 24 h (matched-pairs bootstrap

test: raw mean difference = 70.85%, $P < 0.0001$; Fig. 2.2), with fresh (0 h) eggs inducing oviposition and old (24 h) eggs inhibiting oviposition.

2.6 Experiments 4–7: Washing Cue(s) from Eggs of Various Ages

2.6.1 Methods

To begin isolating the ovipositional cues present on house fly eggs, and to further test their activity over time, fresh egg masses (0.4-0.5 g) were collected on cotton OSs and kept at 23-25°C and 50-80% RH for 0 h (experiment 4), 8 h (experiment 5), 16 h (experiment 6), or 24 h (experiment 7). Each egg mass was then washed with distilled water (2 ml); water was used because preliminary experiments with other polar and nonpolar solvents suggested that water produced the most bioactive washes. Each egg wash was poured through glass wool to remove eggs, and a 0.2-ml aliquot was applied to a treatment cotton OS. Taking into account that cues from the cotton OS supporting the egg mass during ageing may have entered the egg wash, a 2-cm segment of a correspondingly aged, egg-free cotton OS was also washed with water, and 0.2-ml aliquots were applied to both the treatment and control OSs. Treatment and control OSs were bioassayed as previously described.

2.6.2 Results

Oviposition sites (OSs) treated with washes of 0-h-old eggs received significantly more eggs than did control OSs (experiment 4; bootstrap test: raw mean = 83.31%, $P = 0.0102$; Fig. 2.3). No significant effect was observed when OSs were treated with washes of 8-h-old eggs (experiment 5; bootstrap test: raw mean = 77.63%, $P = 0.0607$; Fig. 2.3) or 16-h-old eggs (experiment 6; bootstrap test: raw mean = 58.15%, $P = 0.741$; Fig. 2.3).

In contrast, OSs treated with washes of 24-h-old eggs received almost no eggs (experiment 7; bootstrap test: raw mean = 0.844%, $P = 0.0001$; Fig. 2.3). These results suggest that the oviposition cues change from inducing to inhibiting over time, and that these cues can be washed from the eggs.

2.7 Experiment 8: Egg-associated Microorganisms Are Required for Oviposition Inhibition

2.7.1 Methods

To determine whether microorganisms are associated with house fly eggs, and whether these microorganisms inhibit oviposition after 24 h, treatment cotton OSs received a 0.2-ml aliquot of a wash of fresh eggs and were incubated (24°C, 85% RH) for 24 h before being tested in oviposition experiments. Control OSs were treated identically, except the wash was first filtered (0.22 μm MillexGP Filter unit, Millipore S.A.S., 67120 Molsheim, France) to remove all microorganisms.

2.7.2 Results

Flies oviposited significantly fewer eggs on OSs aged with egg-associated microorganisms than on OSs aged without egg-associated microorganisms (bootstrap test: raw mean = 21.04%, $P < 0.0001$; Fig. 2.4), indicating that the inhibition cue found on 24-h-old eggs requires the presence of these egg-associated microorganisms or the semiochemicals (message-bearing chemicals) that they produce.

2.8 Experiments 9–10: Sufficient Abundance of Egg-associated Microorganisms Required for Oviposition Inhibition

2.8.1 Methods

To determine whether ovipositional preference changed with increasing abundance of egg-associated microorganisms, treatment agar OSs were inoculated with various doses of washes of fresh eggs, incubated (24°C, 85% RH) for 24 h, and bioassayed (experiment 9). Control OSs were treated identically, except the wash was first filtered to remove all microorganisms. The number of colony forming units (CFU) in each initial treatment inoculum was determined by counting the number of colonies per plate after incubation; doses of more than 300 CFU were extrapolated from plated dilutions of the initial inoculum.

An analogous experiment (experiment 10) was conducted with microorganisms washed from cotton OSs so that their effect could be compared with that of house fly-specific microorganisms.

2.8.2 Results

When various doses of egg-associated microorganisms were bioassayed, ovipositional preference for treatment OSs decreased significantly with increased bacterial dose (experiment 9; logistic regression: $\chi_1^2 = 10.97$, $P = 0.0009$; Fig. 2.5). No such effect was evident with microorganisms washed from cotton OSs (experiment 10; logistic regression: $\chi_1^2 = 2.88$, $P = 0.0897$; Fig. 2.5), and these microorganisms did not have any overall effect on ovipositional preference (binomial test: $\chi_1^2 = 0.00$, $P = 1.0$; Fig. 2.5). This result suggests that oviposition decisions depend on the presence and quantity of specific egg-associated microorganisms.

2.9 Experiment 11: Isolation of Egg-associated Microorganisms

2.9.1 Methods

Egg wash microorganisms were isolated by restreaking single-colony isolates on nutrient agar that was supplemented with 16 g of skim-milk powder/litre of agar. Each strain was then cultured in vitro on nutrient agar. All isolated strains were combined in a nutrient broth suspension and bioassayed at various doses (see Experiment 9, Methods). The washing procedure and agar media may not yield isolation and growth, respectively, of every egg-associated microorganism, but experiment 11 determined whether the strains responsible for inhibition of oviposition were among those isolated.

2.9.2 Results

All egg wash microorganisms isolated were bacteria, and 19 different strains were found. When all 19 strains were bioassayed together, preference for treatment OSs again decreased significantly with increased bacterial dose (logistic regression: $\chi_1^2 = 28.93$, $P < 0.0001$; Fig. 2.6). This result suggests that the behaviour-modifying bacterial strains were among these 19 isolates.

2.10 Experiments 12–20: Identification of Behaviour-modifying Bacteria

2.10.1 Methods

To identify the specific behaviour-modifying strains, the 19 isolates were divided into two groups by Gram staining, and each group was tested at various chosen doses (see General Methods). The inhibiting group from the preceding division was further subdivided by oxidase enzyme activity, and then into individual strains. Inhibitory strains

were identified to genus or species level using Biolog GN2 Microplates (Biolog Inc., Hayward, California, U.S.A.) and 16S rRNA sequencing (see Supplementary Methods and Results for details).

2.10.2 Results

Among the 19 isolated bacterial strains, the five Gram-negative strains prompted a dose-dependent decrease in preference for treatment OSs (experiment 12; logistic regression: $\chi_1^2 = 12.74$, $P = 0.0004$; Fig. 2.6). In contrast, the effect of the remaining 14 Gram-positive strains on ovipositional preference did not change with dose (experiment 13; logistic regression: $\chi_1^2 = 1.039$, $P = 0.308$; Fig. 2.6). Instead, these strains increased ovipositional preference for treatment OSs (binomial test: $\chi_1^2 = 15.11$, $P = 0.0001$). These results indicated that the inhibitory strain(s) were within the Gram-negative group (experiment 12), and not within the Gram-positive group (experiment 13; Fig. 2.6). The results also suggest that, when all 19 strains are together at above-threshold doses (experiment 11), the oviposition-inhibition effect of the Gram-negative strains overrides the oviposition-inducing effect of the Gram-positive strains.

The five Gram-negative strains were further divided by oxidase enzyme activity. The three oxidase-positive strains together decreased ovipositional preference for treatment OSs with increased dose (experiment 14; logistic regression: $\chi_1^2 = 6.58$, $P = 0.0103$; Fig. 2.7). However, when any one oxidase-positive strain was omitted, no dose-dependent effects on preference were observed (experiment 15: logistic regression: $\chi_1^2 = 0.335$, $P = 0.563$; experiment 16: logistic regression: $\chi_1^2 = 2.75$, $P = 0.0975$; experiment 17: logistic regression: $\chi_1^2 = 1.31$, $P = 0.252$; Fig. 2.7), and no overall effect on ovipositional preference was found (experiment 15: binomial test: $\chi_1^2 = 0.79$, $P = 0.374$;

experiment 16: binomial test: $\chi_1^2 = 0.00$, $P = 1.0$; experiment 17: binomial test: $\chi_1^2 = 1.17$, $P = 0.280$; Fig. 2.7). Therefore, all three oxidase-positive strains had to be present to provoke a dose-dependent oviposition inhibition.

The remaining two oxidase-negative strains together did not provoke a dose-dependent effect on ovipositional preference (experiment 18; logistic regression: $\chi_1^2 = 1.19$, $P = 0.275$; Fig. 2.8). However, these strains caused significant oviposition inhibition (binomial test: $\chi_1^2 = 6.63$, $P = 0.010$). Oxidase-negative strain 1 alone was inhibitory at all doses tested (experiment 19; logistic regressions and binomial tests could not be conducted on binomial data with 100% preference for control OSs; Fig. 2.8), whereas oxidase-negative strain 2 alone had no dose-dependent or overall effects on ovipositional preference (experiment 20; logistic regression: $\chi_1^2 = 0.0841$, $P = 0.772$; binomial test: $\chi_1^2 = 0.050$, $P = 0.819$; Fig. 2.8).

Oxidase-negative strain 1 and the three oxidase-positive strains were identified as *Klebsiella oxytoca* (strain SFU-1), *Pseudomonas fulva*, *Chryseobacterium gleum/indologenes*, and *Comamonas* sp., respectively (see Supplementary Methods and Results for detailed data). Because *K. oxytoca* alone strongly inhibited oviposition, we hypothesized that it may be solely responsible for indicating the age of deposited house fly eggs to gravid female house flies.

2.11 Experiment 21: Abundance of *K. oxytoca* Increases with Egg Age

2.11.1 Methods

To determine whether the abundance of *K. oxytoca* on house fly eggs increases over time, we washed 0-h-old eggs and 24-h-old eggs (with some hatched larvae) with

water and plated serial dilutions of the wash on nutrient agar for counting. Eggs were collected and aged upon fresh chicken manure (10 g) that was autoclaved in petri plates (50 × 15 mm) at 121°C for 25 min and cooled to room temperature.

2.11.2 Results

The total abundance of *K. oxytoca* washed from eggs increased from a mean of 3.78×10^6 CFU per 0.2 g of 0-h-old eggs to a mean of 1.50×10^8 CFU per 0.2 g of 24-h-old eggs (matched-pairs *t* test: $t_3 = 1.93$, $P = 0.0745$). This constitutes a mean arithmetic increase of 1.46×10^8 CFU.

2.12 Experiments 22–25: Experimentally Increasing Apparent Egg Age

2.12.1 Methods

To determine whether experimentally increasing the abundance of *K. oxytoca* on fresh eggs increases the apparent egg age and results in oviposition inhibition, we conducted a modified version of experiments 2 and 3. Experiment 22 was the same as experiment 2, except that 0.2 ml of dH_20 was applied to the centre of both the treatment and control OSs as a control solution before starting the bioassay. In experiment 23, instead of ageing the eggs as we did in experiment 3, we applied approximately 1.46×10^8 CFU of *K. oxytoca* in 0.2 ml of dH_20 to the treatment OS, and 0.2 ml of dH_20 to the control OS, and then immediately bioassayed these OSs.

To ensure that the apparent age shift of eggs could not be provoked with the addition of just any egg-associated bacteria, the above methods were also used to bioassay a suspension of equal amounts of all 14 Gram-positive strains (total dose ~1.46×10^8 CFU), instead of *K. oxytoca* (experiments 24 and 25).

2.12.2 Results

The OSs carrying fresh eggs treated with water received significantly more eggs than did the control OSs (experiment 22; bootstrap test: raw mean = 73.92%, $P < 0.0001$; Fig. 2.9), but OSs carrying fresh eggs treated with *K. oxytoca* suspension received significantly fewer eggs than did the control OSs (experiment 23; bootstrap test: raw mean = 13.03%, $P < 0.0001$; Fig. 2.9). There was a significant change in oviposition on treatment sites between experiments 22 and 23 (experiments 22-23; matched-pairs bootstrap test: raw mean difference = 60.90%, $P < 0.0001$; Fig. 2.9): experimentally increasing the abundance of *K. oxytoca* on fresh eggs to the levels found on aged eggs caused gravid female house flies to respond to these fresh eggs as if they were 24-h-old eggs (experiment 3; Fig. 2.2).

In contrast, the addition of Gram-positive strains did not alter the oviposition-inducing effect of fresh eggs (experiments 24-25; matched-pairs bootstrap test: raw mean difference = 9.23%, $P = 0.58$; Fig. 2.9).

2.13 Experiment 26: *Klebsiella oxytoca* Found in Other House Fly Populations

2.13.1 Methods

To determine whether *K. oxytoca* is associated with eggs from house flies outside SFU's laboratory colony, we obtained adult house flies from two separate laboratory colonies that are maintained at the Universities of Minnesota and Florida, respectively (see Supplementary Methods and Results for details). Eggs from both colonies were collected on autoclaved manure and washed. Egg wash bacterial isolates with morphologies resembling *K. oxytoca* were identified via 16S rRNA sequencing.

2.13.2 Results

Klebsiella oxytoca was found on eggs from both colonies. The colony at the University of Minnesota had only recently (August 2005) been established, suggesting that *K. oxytoca* is associated with eggs of feral house flies. That *K. oxytoca* is also present on eggs of colonies that have been reared for more than 30 years (SFU) and more than 38 years (University of Florida) further suggests that *K. oxytoca* is transmitted from generation to generation.

2.14 Discussion

The results support our hypotheses that: (1) age disparity among house fly offspring, resulting from asynchronous oviposition, decreases fitness for parental females; (2) ovipositing females deploy evolving (sensu changing over time) cues that first induce and later inhibit oviposition by conspecifics; (3) egg-associated bacteria are responsible for this shift in oviposition behaviour; and (4) specific bacterial strains increase in abundance over time and thus change oviposition induction to inhibition. Among the bacteria isolated from fresh eggs, *K. oxytoca* plays a key role in reversing the ovipositional response of gravid female house flies. It proliferates over time and, above a threshold abundance, most strongly inhibits oviposition. Its presence in three discrete house fly colonies in North America underlines its close association with, and importance for, house flies.

To be an evolutionarily stable communication system, oviposition signals must benefit both the emitter (Hoffmeister & Roitberg 2002) and the receiver. With the evolving cues, or possibly signals, described herein for house flies, the oviposition-induction cues benefit both, because induced synchronous aggregation and offspring

deposition result in optimal colonization densities of even-aged larvae (Bryant 1970; Zvereva 1986). The inhibition cues also benefit both. Later-arriving females that receive and respond to inhibitory cues ensure that their offspring are not cannibalized or outcompeted by older larvae (experiment 1; Fig. 2.1) (Quiring & McNeil 1984a, b; Sherratt & Church 1994; K. Lam, unpublished data). The same benefits accrue for females that inadvertently revisit the same oviposition site, and thus receive their own inhibitory cues (Hoffmeister & Roitberg 2002). To verify that these cues are indeed signals in an evolutionarily stable signalling system, the fitness benefits that they confer need to be clearly demonstrated. The specificity of oviposition decisions by female house flies in response to *K. oxytoca*, along with the widespread and stable association between *K. oxytoca* and house fly eggs, certainly suggest that egg-associated bacteria are part of a true signalling system.

This work also demonstrates that an increasing abundance of *K. oxytoca* on house fly eggs causes, or contributes to, a shift from oviposition induction to oviposition inhibition. Augmenting the abundance of *K. oxytoca* on fresh house fly eggs to levels present on 24-h-old eggs strongly inhibited oviposition (experiments 22-23; Fig. 2.9), with *K. oxytoca* or its products overriding the effect of oviposition-inducing bacteria (experiment 13; Fig. 2.6) and pheromone (Jiang *et al.* 2002). These results underline the key role that *K. oxytoca* plays in oviposition decisions by house flies.

Based on our research, we propose the following scenario. Female house flies are attracted to, and stimulated to oviposit near, fresh house fly eggs by complex stimuli, including resource-derived semiochemicals (Cosse´ & Baker 1996), egg-associated pheromone (Jiang *et al.* 2002) and bacterial strains (experiment 13; Fig. 2.6), and by

visual cues from ovipositing conspecifics (Collins & Bell 1996). When *K. oxytoca* has proliferated beyond a threshold abundance (experiment 21), it inhibits oviposition, overriding any oviposition-inducing cues at that site (experiment 23; Fig. 2.9).

House flies must carry 'their' bacterial symbionts from one resource, and from one generation, to the next (Kellner 2002). Some beetles and wood wasp species, *Sirex* sp. (Hymenoptera: Siricidae), carry specific fungi essential for killing and exploiting host plants in specialized organs known as mycangia (Francke-Grosmann 1939; Whitney & Farris 1970; Farrell *et al.* 2001). Inoculated into host trees by parent insects, these microorganisms are reacquired by next-generation adults that carry them to new hosts (Francke-Grosmann 1939; Raffa 1988; Whitney & Farris 1970; Farrell *et al.* 2001). Similarly, females of some cockroaches, aphids and leafhoppers transmit symbiotic microorganisms transovarially (Kellner 2002). House flies may use similar structures and strategies for perpetuating their association with the bacterial symbionts found in this study. The presence of *K. oxytoca* in fresh egg masses from three discrete laboratory colonies of house flies (one recently established with feral flies and two reared for decades) suggests that *K. oxytoca* is closely associated with several different house fly populations and is transferred from generation to generation. The mode of transfer, however, is yet to be determined.

Klebsiella oxytoca is associated with other insect species. The leek moth parasitoid, *Diadromus pulchellus* (Hymenoptera: Ichneumonidae), is attracted to volatile alkyl disulphides produced by *K. oxytoca* in the frass of host larvae (Thibout *et al.* 1995). *Klebsiella oxytoca* also resides in the crop and stomach, or gut, of several tephritid fruit flies (Diptera: Tephritidae) in the genera *Dacus*, *Ceratitis* and *Rhagoletis* (Howard *et al.*

1985; Jang & Nishijima 1990; Marchini *et al.* 2002; Raghu *et al.* 2002). The biological function of *K. oxytoca* in most of these flies is still to be investigated.

In conclusion, ovipositing female house flies use evolving bacterial cues for optimal exploitation of an ephemeral larval resource. The egg-associated bacterium *K. oxytoca* (strain SFU-1) proliferates over time and, at a threshold density, inhibits further oviposition by late-arriving gravid females. These cues help parental female flies avoid the adverse fitness consequences that accrue from intraspecific competition of age-disparate larval offspring developing in a finite resource.

2.15 References

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2.16 Figures

Fig. 2.1 Effect of age disparity of larvae on the number of house fly larvae completing development in experiment 1. Eight-hundred house fly eggs aged for 0 or 24 h were reared together with 800 additional 0-h-old eggs, and the numbers of offspring flies completing development were counted.

Fig. 2.2 Effect of egg mass age on house fly oviposition. Treatment oviposition sites with a 0.2-g egg mass stimulus (■) and egg-free control (□) oviposition sites were aged for 0 h (experiment 2) and 24 h (experiment 3) before exposure to house flies. Numbers of eggs deposited by bioassay flies on treatment and control oviposition sites within each replicate pair were converted to two separate percentages adding up to 100% of total eggs deposited.

Fig. 2.3 Effect of washes of egg masses aged 0 h (experiment 4), 8 h (experiment 5), 16 h (experiment 6), or 24 h (experiment 7) on house fly oviposition. A wash of an egg-free oviposition site was applied to both treatment (■) and control (□) oviposition sites to account for possible changes in the substrate upon which egg masses were aged before washing. Washes of aged egg masses were applied only to treatment oviposition sites. Weights of eggs deposited on treatment and control oviposition sites within each replicate pair were converted to two separate percentages adding up to 100% of total eggs deposited.

Fig 2.4 Effect of egg-associated microorganisms on oviposition by house flies. Filter-sterilized (microorganism-free control) and unfiltered (microorganism-containing treatment) washes of 0-h-old eggs were applied to oviposition sites and exposed to house flies to determine oviposition preference. Weights of eggs deposited on treatment (■) and control (□) oviposition sites within each replicate pair were converted to two separate percentages adding up to 100% of total eggs deposited.

Fig. 2.5 Dose-response experiments with microorganisms washed from 0-h-old house fly eggs (experiment 9) and from oviposition sites (experiment 10), incubated on agar oviposition sites, and exposed to house flies to determine

oviposition preference. Control agar plates in both experiments received filter-sterilized (microorganism-free) egg washes. Sample sizes for each dose varied because counts of bacterial colonies were performed post-incubation. Weights of eggs deposited on treatment (■) and control (□) oviposition sites within each replicate pair were converted to two separate percentages adding up to 100% of total eggs deposited. Vertical bars present the means and standard errors of these percentages at each dose. The logistic regression line in each experiment indicates the probability that flies deposit more eggs on treatment oviposition sites than on control sites. Ovipositional preference changes significantly with changes in dose when $P < 0.05$. CFU = colony forming units of microorganisms/bacteria applied in the inoculum.

Fig. 2.6 Dose-response experiments with suspensions of bacterial strains isolated from house fly eggs. All 19 bacterial isolates were bioassayed in experiment 11. The 19 strains were divided into two groups by Gram staining, and the group of five Gram-negative strains (experiment 12) and the group of 14 Gram-positive strains (experiment 13) were bioassayed. All other details as given in Fig. 2.5 legend.

Fig. 2.7 Dose-response experiments with suspensions of Gram-negative, oxidase-positive bacterial strains isolated from house fly eggs. The three strains were bioassayed in ternary combination (experiment 14) and in all possible binary combinations (experiments 15-17). All other details as given in Fig. 2.5 legend.

Fig. 2.8 Dose-response experiments with suspensions of Gram-negative, oxidase-negative bacterial strains isolated from house fly eggs. The two strains were bioassayed in combination (experiment 18) and singly (experiments 19-20). All other details as given in Fig. 2.5 legend.

Fig. 2.9 Effect of adding *Klebsiella oxytoca* (experiment 23), or an equal total dose of all 14 Gram-positive house fly egg-associated bacterial strains (experiment 25), to a 0-h-old egg mass on ovipositional preference of female house flies for that egg mass. For each experiment (experiments 22-25), a 0-h-old egg mass was placed on each treatment (■) oviposition site, whereas control (□) oviposition sites received neither egg masses nor bacteria. Control experiments (experiments 22 and 24) were conducted

concurrently, with no bacteria added, for comparative purposes. Numbers of eggs deposited by bioassay flies on treatment and control oviposition sites within each replicate pair were converted to two separate percentages adding up to 100% of total eggs deposited. CFU = colony forming units of bacteria applied in the inoculum

Fig. 2.1

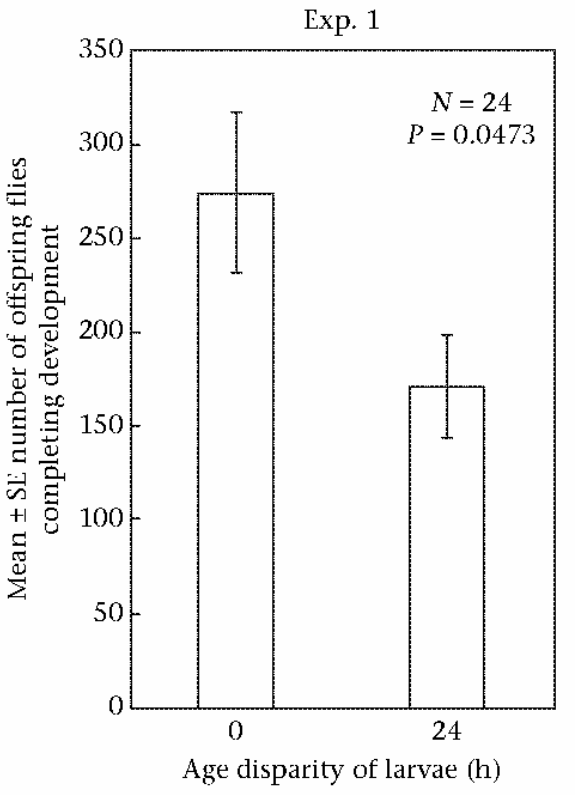

Exp. 1
N = 24
P = 0.0473
Mean ± SE number of offspring flies completing development
350
300
250
200
150
100
50
0
0
24
Age disparity of larvae (h)

Fig. 2.2

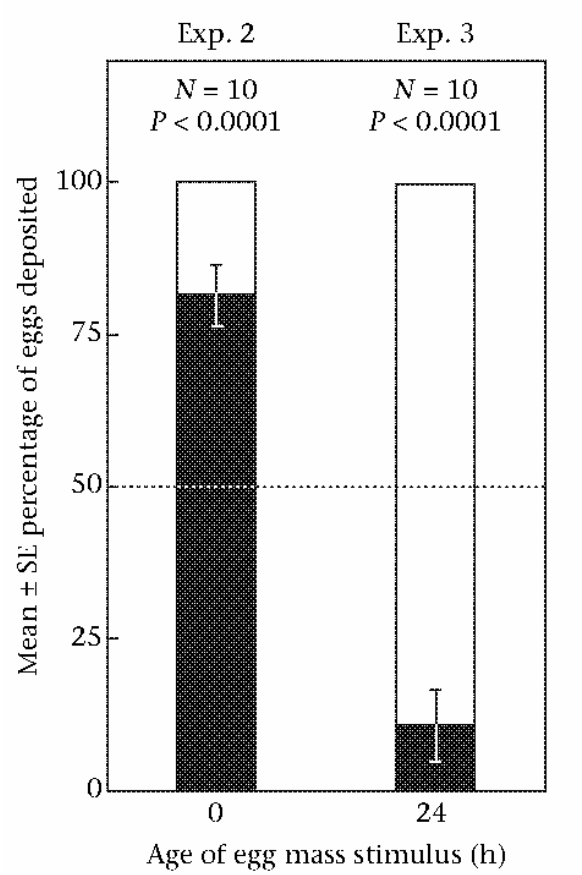

Exp. 2
Exp. 3
N = 10
P < 0.0001
N = 10
P < 0.0001
100
75
50
25
0
Mean ± SE percentage of eggs deposited
0
24
Age of egg mass stimulus (h)

Fig. 2.3

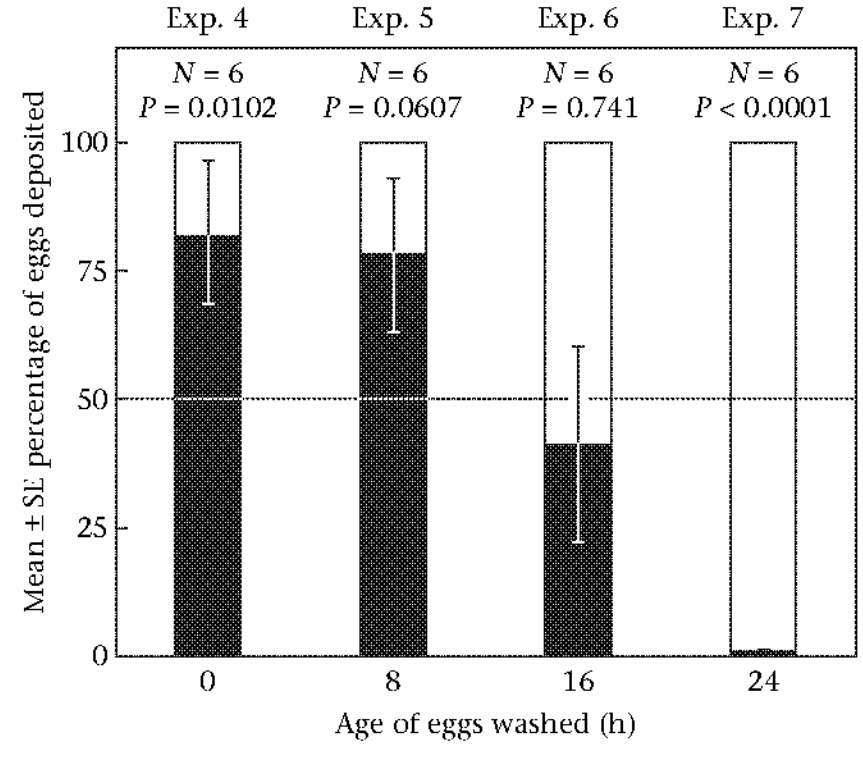

Exp. 4
Exp. 5
Exp. 6
Exp. 7
N = 6
P = 0.0102
N = 6
P = 0.0607
N = 6
P = 0.741
N = 6
P < 0.0001
Mean ± SE percentage of eggs deposited
100
75
50
25
0
0
8
16
24
Age of eggs washed (h)

Fig. 2.4

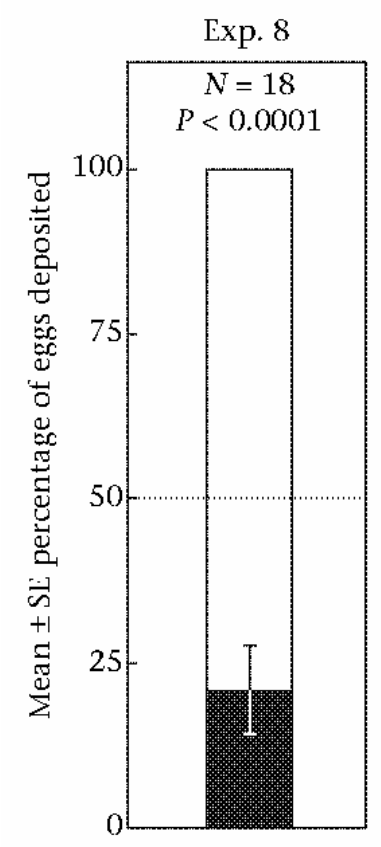

Exp. 8
N = 18
P < 0.0001
Mean ± SE percentage of eggs deposited
100
75
50
25
0

Fig. 2.5

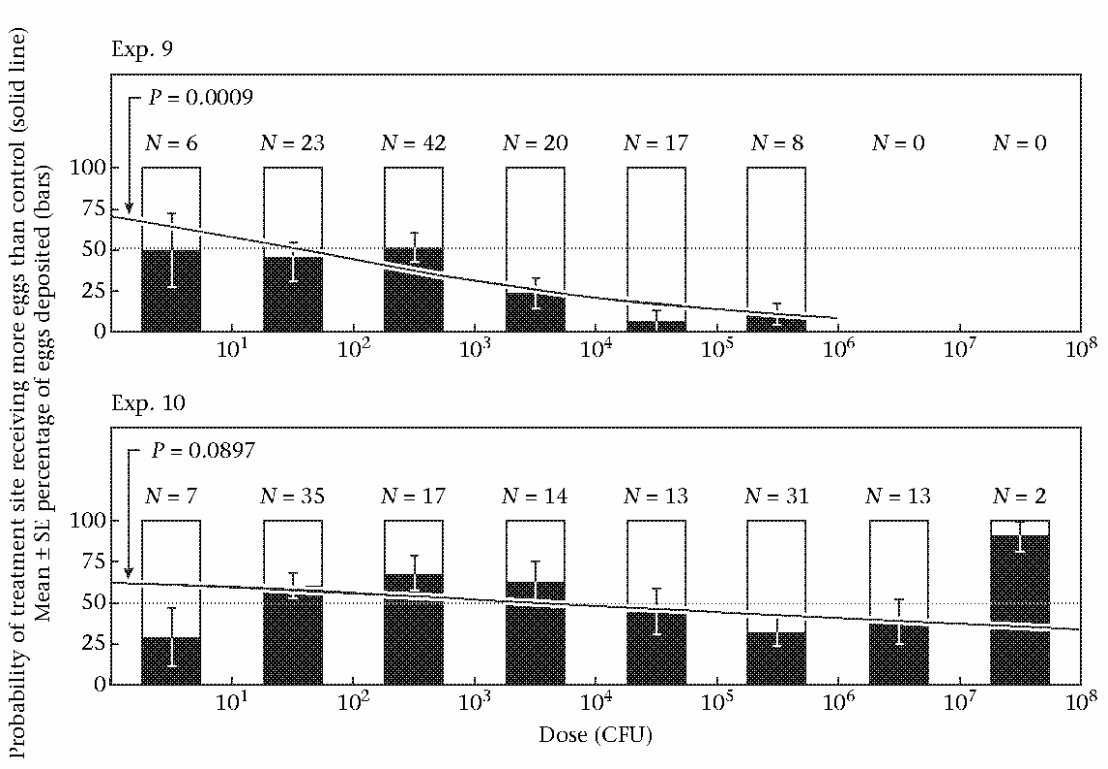

Exp. 9
P = 0.0009
N = 6
N = 23
N = 42
N = 20
N = 17
N = 8
N = 0
N = 0
Exp. 10
P = 0.0897
N = 7
N = 35
N = 17
N = 14
N = 13
N = 31
N = 13
N = 2
Probability of treatment site receiving more eggs than control (solid line)
Mean ± SE percentage of eggs deposited (bars)
Dose (CFU)

Fig. 2.6

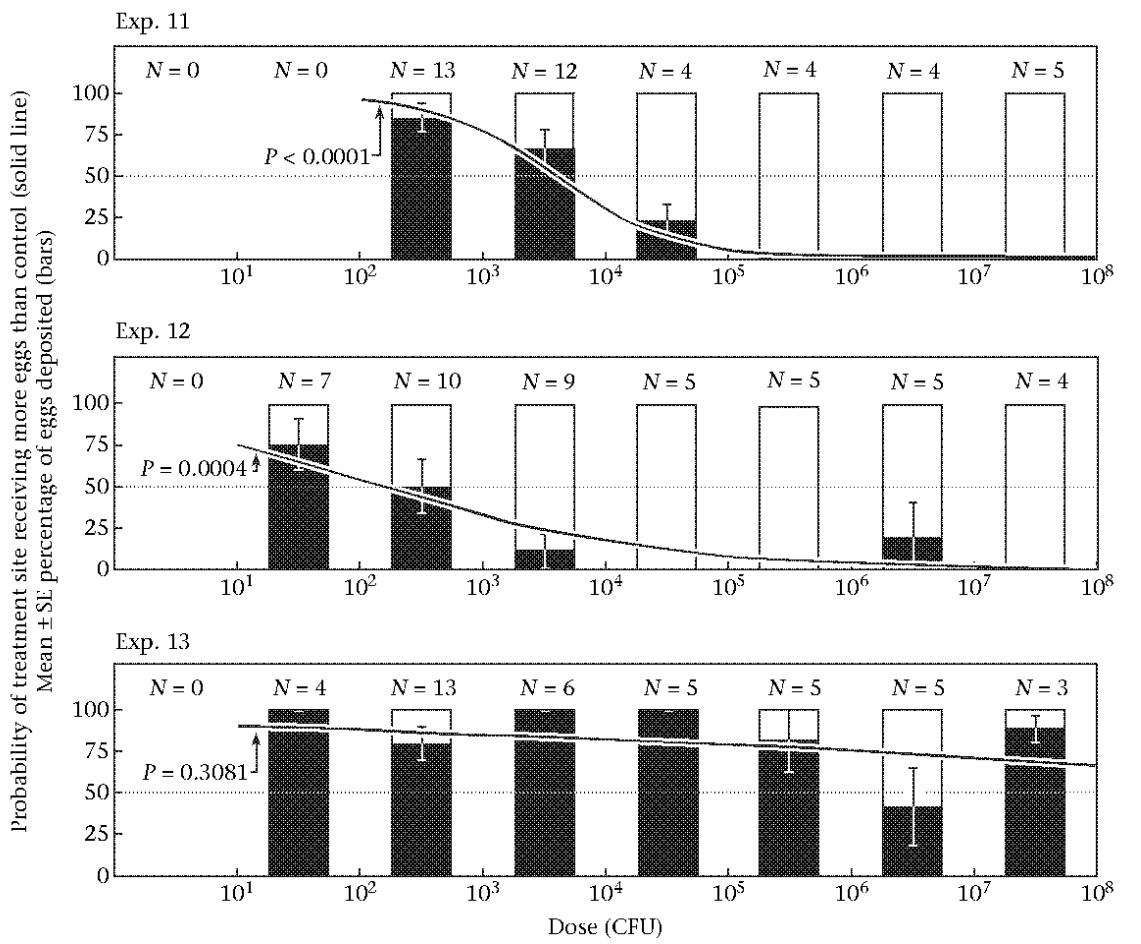

Exp. 11
N = 0
N = 0
N = 13
N = 12
N = 4
N = 4
N = 4
N = 5
P < 0.0001
Exp. 12
N = 0
N = 7
N = 10
N = 9
N = 5
N = 5
N = 5
N = 4
P = 0.0004
Exp. 13
N = 0
N = 4
N = 13
N = 6
N = 5
N = 5
N = 5
N = 3
P = 0.3081
Probability of treatment site receiving more eggs than control (solid line)
Mean ± SE percentage of eggs deposited (bars)
Dose (CFU)

Fig. 2.7

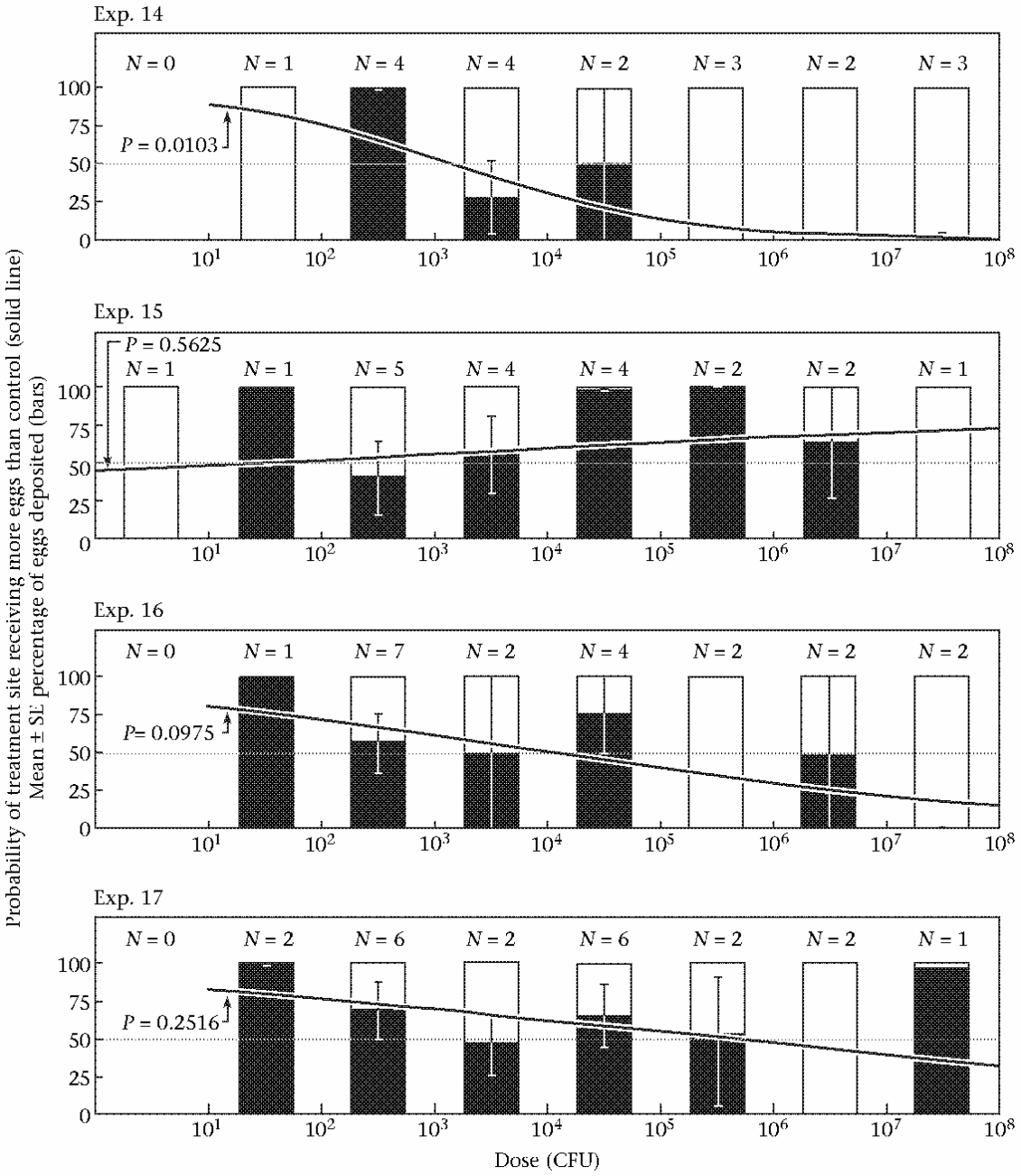

Exp. 14
N = 0
N = 1
N = 4
N = 4
N = 2
N = 3
N = 2
N = 3
P = 0.0103
Exp. 15
P = 0.5625
N = 1
N = 1
N = 5
N = 4
N = 4
N = 2
N = 2
N = 1
Exp. 16
N = 0
N = 1
N = 7
N = 2
N = 4
N = 2
N = 2
N = 2
P= 0.0975
Exp. 17
N = 0
N = 2
N = 6
N = 2
N = 6
N = 2
N = 2
N = 1
P = 0.2516
100
75
50
25
0
10¹
10²
10³
10⁴
10⁵
10⁶
10⁷
10⁸
Probability of treatment site receiving more eggs than control (solid line)
Mean ± SE percentage of eggs deposited (bars)
Dose (CFU)

Fig. 2.8

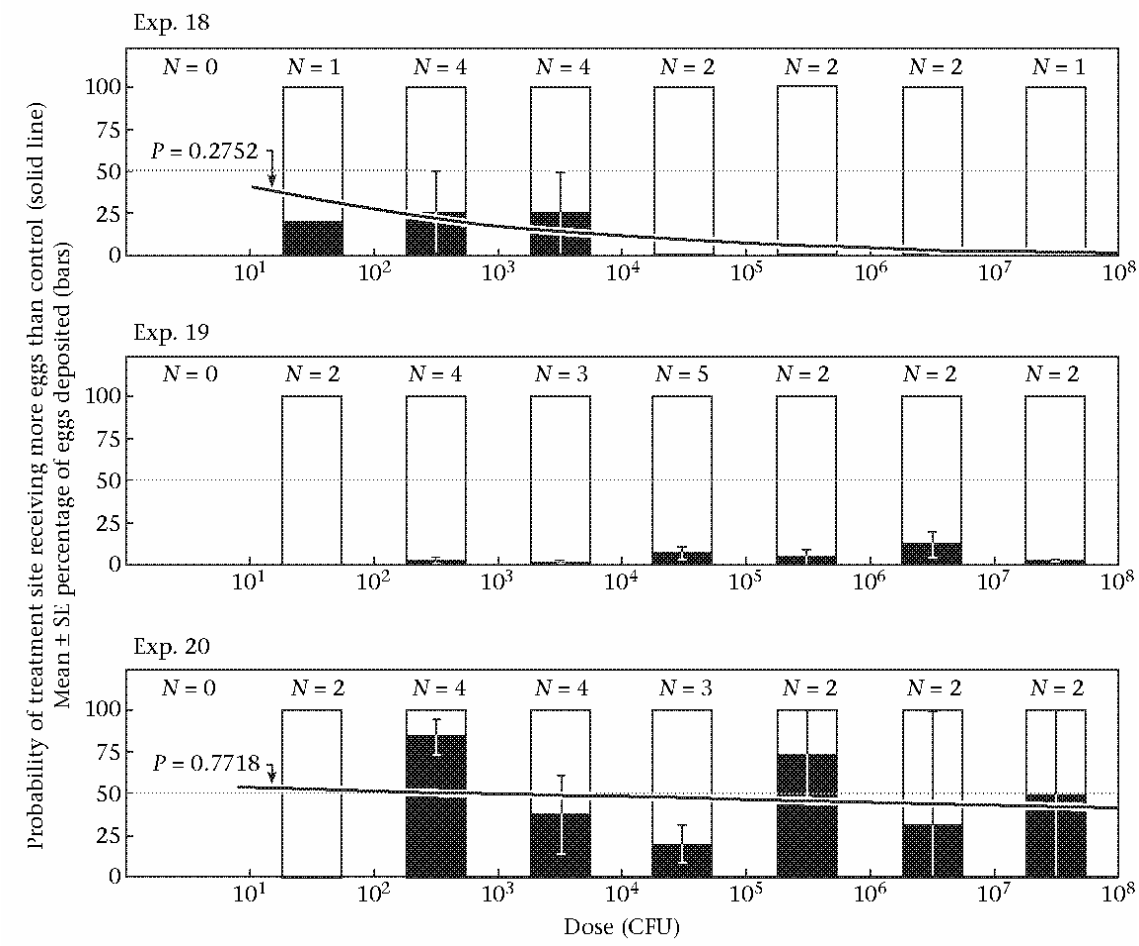
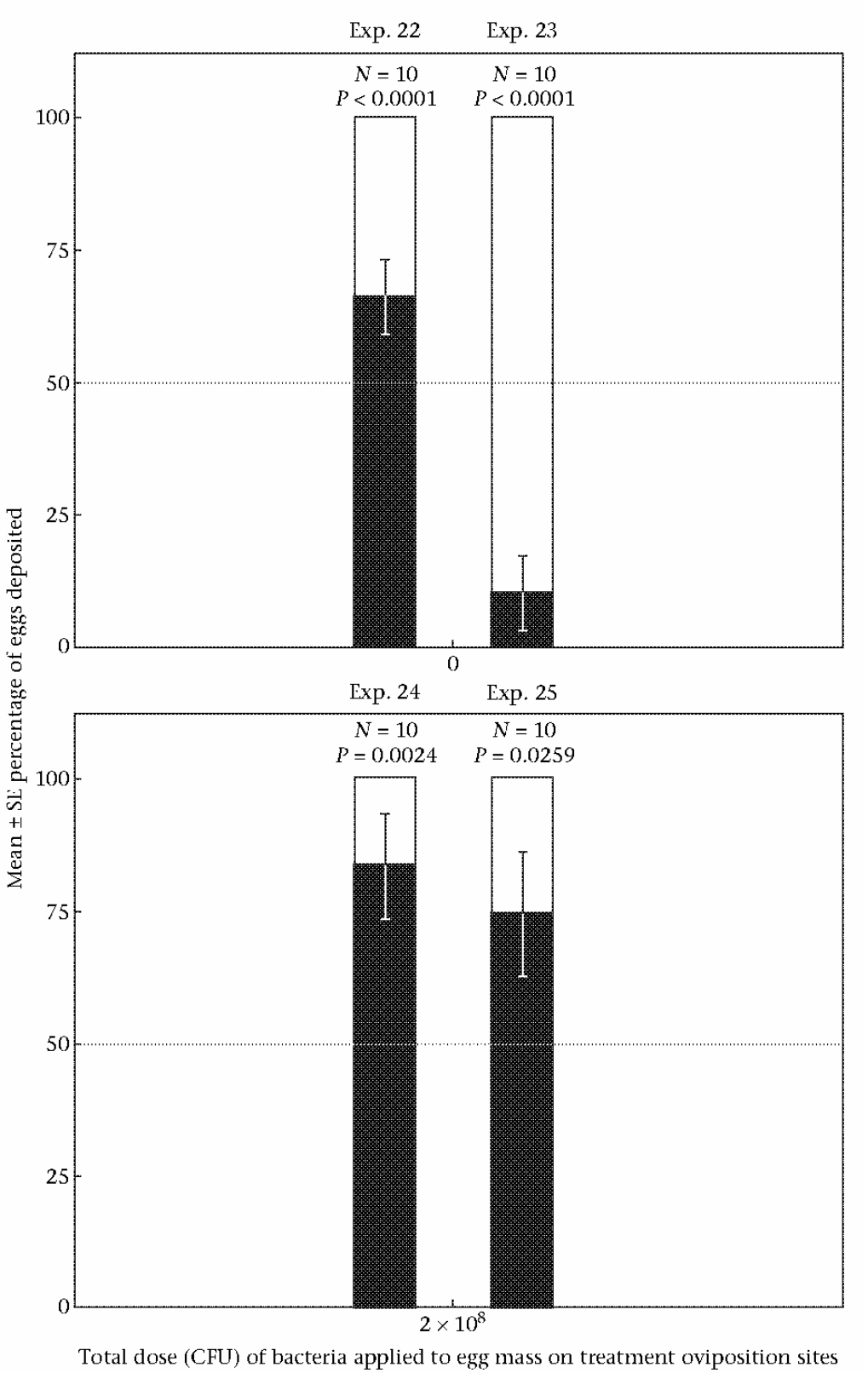

Exp. 18
N = 0
N = 1
N = 4
N = 4
N = 2
N = 2
N = 2
N = 1
P = 0.2752
Exp. 19
N = 0
N = 2
N = 4
N = 3
N = 5
N = 2
N = 2
N = 2
Exp. 20
N = 0
N = 2
N = 4
N = 4
N = 3
N = 2
N = 2
N = 2
P = 0.7718
100
75
50
25
0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
10^8
Probability of treatment site receiving more eggs than control (solid line)
Mean ± SE percentage of eggs deposited (bars)
Dose (CFU)

Fig. 2.9


Exp. 22
Exp. 23
N = 10
P < 0.0001
N = 10
P < 0.0001
100
75
50
25
0
0
Exp. 24
Exp. 25
N = 10
P = 0.0024
N = 10
P = 0.0259
100
75
50
25
0
2 × 10^8
Mean ± SE percentage of eggs deposited
Total dose (CFU) of bacteria applied to egg mass on treatment oviposition sites

2.17 Supplementary Methods and Results

2.17.1 Identification of Bacterial Strains

To identify the four oviposition-inhibiting bacterial strains, two identification assays were used: Biolog GN2 Microplates (Biolog Inc., Hayward, California, U.S.A.) and 16S rRNA sequencing.

For Biolog testing of each strain, a cell suspension was transferred into each of 96 separate wells of a GN2 microplate, which was then incubated (30°C, 85% RH) for 16–24 h. A different test was performed in each well, and the resulting reaction pattern (or 'metabolic fingerprint') was compared with those found in the MicroLog3 4.20 database. Oxidase-positive strains 1 and 2 were identified as *Pseudomonas fulva* and *Chryseobacterium gleum/indologenes*, respectively (Supplementary Fig. S1). The fingerprints of oxidase-positive strain 3 and oxidase-negative strain 1 did not match those found in the database.

16S rRNA sequences for each bacterial isolate were obtained using 27f and 519r primer sets (Lane, 1991). A BLAST search of the 16S rRNA sequences of oxidase positive strains 1 and 2 (Supplementary Figs S2, S3) against the nucleotide-nucleotide BLAST database of the National Centre for Biotechnology Information confirmed the above taxonomic assignments. The sequence of oxidase positive strain 3 matched that of *Comamonas* sp. (Supplementary Fig. S4), and the sequence for oxidase negative strain 1 (strain SFU-1) matched that of *Klebsiella oxytoca*. For this assay, greater than 99% similarity is needed for species identification, and 90–99% similarity is needed for genus identification.

2.17.2 Rearing of House Flies at Other Universities

The colony at the University of Minnesota was started with house flies hand-netted from the campus dairy in August 2005. Adult flies were kept at 30ºC, uncontrolled humidity and a 16:8 h light:dark regime, and were sustained on a diet of granulated sugar, skim-milk powder and water. Larval medium consisted of a 1:2:2 ratio (by volume) of Purina rat chow, hardwood sawdust and water.

The colony at the University of Florida had been in culture for more than 38 years, with flies that were probably captured in Florida. Adults were kept at 27ºC, 65–70% RH and a 12:12 h light:dark regime, and were sustained on a diet of granulated sugar, powdered skim milk and water. Larval medium consisted of a 2:3:1.5:8 ratio of calf protein supplement, wood chips, bran and tap water.

2.17.3 Supplementary Figures

Fig. S1. Biolog GN2 microplate data for oxidase-positive strains 1 and 2. The closest species match for each strain is given. <X> = positive; <X- = mismatched positive; X = negative; X+ = mismatched negative; {X} = borderline; and -X = less than A1 (control) well. Prob = the probability that the tested strain belongs to this species.

Fig. S2. Comparison of 16S rRNA sequence of *Pseudomonas fulva* versus oxidase-positive strain 1.

Fig. S3. Comparison of 16S rRNA sequence of *Chryseobacterium* sp. versus oxidase-positive strain 2.

Fig. S4. Comparison of 16S rRNA sequence of *Comamonas* sp. versus oxidase-positive strain 3.

Fig. S5. Comparison of 16S rRNA sequence of *Klebsiella oxytoca* versus oxidase-negative strain 1 (strain SFU-1).

Fig. S1

Oxidase-positive strain 1

Colour	1	2	3	4	5	6	7	8	9	10	11	12
A	0	4	25	{80}	19+	33	-0	-3	-3	{78}	0	0
B	2	54	11	13	10	<260>	0	-1	-2	-4	0	<105>
C	7	47	{76}	4	19	11	8	17	16	-1	{87}	46+
D	{87}	<285>	<242>	42+	1	2	<215>	4	3	7	45	22
E	33	<203>	4	<127>	10	<173>	5	{78}	{78}	-0	1	<177>
F	<156>	36	11	{74}	<116>	<200>	{72}	<324>	<287>	<299>	-1	10
G	<198>	9	26	12	14	<291>	<244>	23	<173>	42	{89}	<222>
H	5	47+	19	-1	-1	<262>	<188>	9	35	2	-1	0

	Species	PROB	SIM	DIST	TYPE
=>1)	*Pseudomonas fulva*	98	0.69	4.55	GN-NENT OXI+

Oxidase-positive strain 2

Colour	1	2	3	4	5	6	7	8	9	10	11	12
A	0	<440>	<396>	<391>	<190>	<223>	47	38	34	<82>	-113	-42
B	25	<422>	-120	-13	<431>	<349>	6	-15	-34	<401>	-61	<205>
C	41	<85>	<132>	36	-164	13	-25	<422>	<119>	-54	<146>	18+
D	<293>	<94>	<286>	-126+	-9	-7	19	<71-	-46	-137	-12	-86
E	-144	40	<290>	17	<158>	-36	21	<270>	10	-34	-224	-60
F	-261	8	-215	<84>	-32	<238>	<241>	<440>	<216>	<295>	<378>	<353>
G	-153	<118>	<234>	<210>	<220>	<283>	-57	-107	<304>	<207>	34	-200
H	-252	<166>	<241>	<170>	-305	-311	-266	21+	<213>	<384>	40+	-7+

	Species	PROB	SIM	DIST	TYPE
=>1)	*Chryseobacterium gleum/indologenes*	100	0.53	7.35	GN-NENT OXI+

Fig. S2

Query:8 aacgtcaaaattgcagagtattaatctacaacccttcctcccaacttaaagtgctttaca 67
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:465 aacgtcaaaattgcagagtattaatctacaacccttcctcccaacttaaagtgctttaca406

Query:68 atccgaagaccttcttcacacacgcggcatggctggatcaggctttcgcccattgtccaa127
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: atccgaagaccttcttcacacacgcggcatggctggatcaggctttcgcccattgtccaa346

Query:128 tattccccactgctgcctcccgtaggagtctggaccgtgtctcagttccagtgtgactga187
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:345 tattccccactgctgcctcccgtaggagtctggaccgtgtctcagttccagtgtgactga286

Query:188 tcatcctctcagaccagttacggatcgtcgccttggtgagccattacctcaccaacaagc247
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:285 tcatcctctcagaccagttacggatcgtcgccttggtgagccattacctcaccaacaagc226

Query:248 taatccgacctaggctcatctgatagcgcaaggcccgaaggtcccctgctttctcccgta307
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:225 taatccgacctaggctcatctgatagcgcaaggcccgaaggtcccctgctttctcccgta166

Query:308 ggacgtatgcggtattagcgttcctttcgaaacgttgtcccccactaccaggcagattcc367
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:165 ggacgtatgcggtattagcgttcctttcgaaacgttgtcccccactaccaggcagattcc106

Query:368 taggcattactcacccgtccgccgctgaatcmaggagcaagctcccgtcatccgctcgac427
|||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct:105 taggcattactcacccgtccgccgctgaatcaaggagcaagctcccgtcatccgctcgac 46

Query:428 ttgcatgtgttaggc442
|||||||||||||||
Sbjct:45 ttgcatgtgttaggc 31

Pseudomonas fulva gene for 16S rRNA, partial.
Identity = 434/435) 99.8%
Score = 860
Expect = 0

Fig. S3

```
Query:17  taggtttatccctatacaaaagaagtttacaacccatagggccgtcgtccttcacgcggg 76
          ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct:440 taggtttatccctatacaaaagaagtttacaacccatagggccgtcatccttcacgcggg381


Query:77  atggcttggatcaggctctcacccattgtccaatattcctcactgctgcctcccgtagga136
          |||||| |||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct:380 atggct-ggatcaggctcccacccattgtccaatattcctcactgctgcctcccgtagga322


Query:137 gtctggtccgtgtctcagtaccagtgtgggggatcaccctctcaggccccctaaagatcg196
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:321 gtctggtccgtgtctcagtaccagtgtgggggatcaccctctcaggccccctaaagatcg262


Query:197 tagacttggtgagccgttacctcaccaactatctaatcttgcgcgtgcccatctctatcc156
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:261 cagacttggtgagccgttacctcaccaactatctaatcttgcgcgtgcccatctctatcc202


Query:257 accggagttttcaatatcaagtgatgccactcgatatattatggggtattaatcttcctt316
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:201 accggagttttcaatatcaagtgatgccactcgatatattatggggtattaatcttcctt142


Query:317 tcgaaaggctatccccctgataaaggcaggttgcacacgtgttccgcacccgtacgccgc376
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:141 tcgaaaggctatccccctgataaaggcaggttgcacacgtgttccgcacccgtacgccgc 82


Query:377 tctcaagatcccgaaagatctctaccgctcggcttgcatgtgtta-gcctcccgctagcg435
          |||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct:81  tctcaagatcccgaaagatctctaccgctcggcttgcatgtgttaggcctcccgctagcg 22


Query:436 ttcatcctga445
          ||||||||||
Sbjct:21  ttcatcctga 12
```

Chryseobacterium sp. LDA39 G1966 4a 16s rRNA
Identity = (425/430) 98.8%
Score = 797
Expect = 0

Fig. S4

```
Query:1   gcacgtagt-agccggtgcttattcttacggtaccgtcatggaccctctttattagagag 59
          ||||||||| ||||||||||||||||||||||||||||||||| || |||||||||
Sbjct:487 gcacgtagttagccggtgcttattcttacggtaccgtcatggacccccctgtattagagag428

Query:60  agtcttttcgttccgtacaaaagcagtttacaacccgagggccttcatcctgcacgcggc119
          |||||||||||||||||||| |||||||||||||||||||||||| |||||||
Sbjct:427 agtcttttcgttccgtacaaaagtagtttacaacccgagggccttcatcctacacgcggc368

Query:120 attgctggatcaggctttcgccccattgtccaaaattccccactgctgcctcccgtagga179
          |||||||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct:367 attgctggatcaggctttcgccc-attgtccaaaattccccactgctgcctcccgtagga309

Query:180 gtctgggccgtgtctcagtcccagtgtggctggtcgtcctctcagaccagctacagatcg239
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:308 gtctgggccgtgtctcagtcccagtgtggctggtcgtcctctcagaccagctacagatcg249

Query:240 tcggcttggtaagcttttatcccaccaactacctaatctgccatcagccgctctagtagc299
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:248 tcggcttggtaagcttttatcccaccaactacctaatctgccatcagccgctctagtagc189

Query:300 gcaaggcccgaaggtcccctgctttcatccttagatctcatgcggtattagctactcttt359
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:188 gcaaggcccgaaggtcccctgctttcatccttagatctcatgcggtattagctactcttt129

Query:360 cgagtagttatcccccactactaggcacgttccgatgtgttactcacccgttcgccactc419
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:128 cgagtagttatcccccactactaggcacgttccgatgtgttactcacccgttcgccactc 69

Query:420 gtcagcgtccgaa-aactgttaccgttcgacttgcatgtgtaaagcatgcc469
          |||||| |||||| | ||||||||||||||||||||||||||||||||
Sbjct:68  gtcagcatccgaagacctgttaccgttcgacttgcatgtgtaaagcatgcc 18
```

Comamonas sp. TK41 partial 16S rRNA gene.
Identity = (462/471) 98.1%
Score = 839
Expect = 0

Fig. S5

```
Query:5   GGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATYGACRAGGTTA 64
          ||||||||||||||||||||||||||||||||||||||||||||||||| ||| ||||||
Sbjct:517 GGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGACAAGGTTA458

Query:65  TTAACCTYATCRCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATA124
          ||||||| ||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:457 TTAACCTTATCGCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATA398

Query:125 CACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCC184
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:397 CACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCC338

Query:185 CGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTA244
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:337 CGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTA278

Query:245 GGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCTAATCCCATCTGGGCACATC304
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:277 GGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCTAATCCCATCTGGGCACATC218

Query:305 TGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCT364
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:217 TGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCT158

Query:365 ACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCC424
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:157 ACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCC 98

Query:425 GCCACTCGTCACCCGAGAGCAAGCTCTCTGTGCTACCGTTCGACTTGCATGTGTTAGGCC484
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:97  GCCACTCGTCACCCGAGAGCAAGCTCTCTGTGCTACCGTTCGACTTGCATGTGTTAGGCC 38

Query:485 TGCCGCCAGCGTTCAATCTGAGCCAGGAACAAACTCT521
          ||||||||||||||||||||| || |||||||
Sbjct:37  TGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTCT  1
```

Klebsiella oxytoca isolate GR6 16S rRNA gene, partial sequence.
Identity = (511-517) 98.8%
Score = 998
Expect = 0

3: Bacteria on housefly eggs, *Musca domestica*, suppress fungal growth in chicken manure through nutrient depletion or antifungal metabolites[†]

3.1 Abstract

Female houseflies, *Musca domestica* (Diptera: Muscidae), lay their eggs in ephemeral resources such as animal manure. Hatching larvae compete for essential nutrients with fungi that also colonize such resources. Both the well-known antagonistic relationship between bacteria and fungi and the consistent presence of the bacterium *Klebsiella oxytoca* on housefly eggs led us to hypothesize (1) that *K. oxytoca*, and possibly other bacteria on housefly eggs, help curtail the growth of fungal resource competitors, and (2) that such fungi indeed adversely affect the development of housefly larvae. Bacteria washed from housefly eggs significantly reduced the growth of fungi in chicken manure. Nineteen bacterial strains and ten fungal strains were isolated from housefly eggs and chicken manure, respectively. Co-culturing each of all the possible bacterium–fungus pairs revealed that the bacteria as a group, but no single bacterium, significantly suppressed the growth of all fungal strains tested. The bacteria's adverse effect on fungi is due to resource nutrient depletion and/or the release of antifungal chemicals. Well-established fungi in resources significantly reduced the number of larval offspring that completed development to adult flies.

[†] This chapter was published in *Naturwissenschaften* **96**: 1127-1132 in 2009 with authors as follows: Lam, K., Thu, K., Tsang, M., Moore, M., and Gries, G.

Keywords: Houseflies . *Musca domestica* . *Klebsiella oxytoca* . Bacterial symbiont . Fungi . Communication ecology

3.2 Introduction

Bacteria and fungi interact with almost every living organism as well as with one another. As mutualistic symbionts, they fix nitrogen for plants (Rabie *et al.* 2008; Leigh *et al.* 2009), facilitate food digestion in vertebrate and invertebrate hosts (Vannini *et al.* 2004; Hongoh *et al.* 2008), counter pathogen invasion in insects (Oliver *et al.* 2003; Yoder *et al.* 2008), and contribute to insect signalling systems (Ryker and Yandell 1983; Lam *et al.* 2007).

As antagonists, bacteria and fungi cause animal and plant diseases (Karcher *et al.* 2008; Krejzar *et al.* 2008) or compete for resources with other organisms (Wertheim *et al.* 2002; Rohlfs 2006). When competing with insect larvae for ephemeral resources, such as manure or carrion, microorganisms can have adverse effects on larval development. For example, fungi have been shown to reduce the number of burying beetle larvae, *Nicrophorus quadripunctatus*, that complete development in carcasses, even though adult beetles apply chemicals that curtail fungal growth (Suzuki 2001). Similarly, when fungi establish on manure 3 days before houseflies, *Musca domestica*, deposit their eggs, all hatching larvae perish. When fungi are not established in advance, however, large numbers of fly larvae can outcompete fungi by releasing allelopathic chemicals that inhibit mycelial growth and disturb the sporogenic process of fungi (Zvereva 1986a, b).

Because the timing of resource colonization is such an important factor in the outcome of competition between housefly larvae and fungi, we have predicted that there might be mechanisms that suppress fungal growth even before larvae hatch. Considering (1) the numerous examples of bacteria strongly inhibiting fungal growth (e.g., Mille-Lindblom and Tranvik 2003; Brucker *et al.* 2008) and (2) the consistent presence of

proliferating bacteria on the surface of housefly eggs (Lam *et al.* 2007), we tested the hypotheses (1) that these egg-associated bacteria may not only serve as oviposition cues for adult flies but may also help curtail the growth of manure-colonizing fungi, and (2) that well-established fungal competitors adversely affect the development of housefly larvae.

3.3 Materials and methods

3.3.1 Do bacteria associated with housefly eggs suppress fungal growth in manure?

Houseflies used in all experiments were reared as previously described (Lam *et al.* 2007). In experiment 1 (n = 10), eggs (0.272 g) freshly deposited on sterile skim milk agar (SMA; 15 ml water, 0.12 g nutrient broth powder, 0.24 g skim milk powder, 0.3 g agar) were collected and shaken vigorously in distilled water (4.5 ml), and the resulting egg wash was poured through glass wool to remove eggs. Manure pats (0- to 24-h-old) from free-range organic chickens (Wind's Reach Farm, Langley, BC, Canada) were thoroughly mixed and 10-g aliquots packed tightly into sterile plastic Petri dishes (35×10 mm). Egg wash (400 µl) or distilled water was applied evenly over the manure surface of treatment or control dishes, respectively. The dishes were covered loosely with lids and incubated at 24°C and 85% relative humidity (RH). After 0, 12, 24, 36, and 48 h, 1.5-g samples were taken from each dish and fungal biomass was quantified by extraction and gas chromatographic–mass spectrometric analyses of ergosterol, a fungal-specific steroid and an indicator of fungal biomass (Saraf *et al.* 1997). For each sample, the area beneath the ergosterol peak was integrated and converted to a percent relative to the amounts of ergosterol in treatment and control samples at the onset of the experiment (time and

amounts set to 0 and 100%, respectively). Changes in ergosterol content over time in treatment and control dishes (Fig. 3.1) were analyzed by repeated measure analysis of covariance (ANCOVA; Proc Mixed procedure, SAS, Statistical Software, version 9.1).

3.3.2 Do specific bacterial isolates suppress growth of specific fungal strains?

In experiment 2 (n = 1–3), individual egg-associated bacterial strains and chicken manure-associated fungal strains were co-cultured to determine antifungal activity. To isolate bacterial strains, a wash of fresh housefly eggs was streaked and single-colony isolates re-streaked on SMA. Isolated strains were then maintained at 24°C and 85% RH on 90-mm Petri plates containing sterile nutrient agar (20 ml water, 0.16 g nutrient broth powder, and 0.4 g agar). Each strain was identified with 16S rRNA sequencing (using the 27f and 519r primer set; Lane 1991) and API 20E biochemical identification systems (Biomerieux, France).

To isolate fungal strains, ten manure pats (0- to 24-h-old) from free-range, organic chickens were collected, and a sterile inoculating loop was inserted into the center of each pat and streaked across potato dextrose agar (PDA; 20 ml water, 0.48 g potato dextrose broth powder, and 0.4 g agar). Mycelia from separate fungal colonies were isolated and incubated on PDA at 24°C and 85%RH. To identify these fungi, each strain was grown in a Sabouraud dextrose broth and a Qiagen DNA extraction performed using the QIAamp® DNA mini kit (Qiagen®, Canada, cat. no. 51304) according to manufacturer's instructions. Sequencing of 18S rRNA was performed using Fun 1 and Fun 2 primers (unpublished; designed by Dr. Bryne, Animal Health Centre, Abbotsford, BC, Canada) and the Genbank database.

To determine the effect of each bacterial isolate on the growth of each fungal isolate, 90-mm Petri plates of potato nutrient agar (20 ml water, 0.48 g potato dextrose broth powder, 0.16 g nutrient broth powder, and 0.4 g agar) were inoculated with a fungal strain at their center and a bacterial strain (as a single point and a semicircular streak) 20 mm away from the fungal inoculum (Fig. 3.2a). Antifungal activity of each bacterial isolate against each fungal isolate was recorded by observations and photographs taken every 8–12 h (e.g., Fig. 3.2b, c). Bacteria were classified as: N, having no effect on fungal growth across the agar plate; B1–B3, forming a barrier to fungal growth; and I1–I3, forming a zone of inhibition against fungal growth (see Fig. 3.2 and Supplementary Material).

3.3.3 Do bacteria suppress fungal growth by nutrient depletion or antifungal chemicals?

Experiments 3 and 4 (n = 3–30) were designed to determine whether housefly egg-associated bacteria decrease fungal growth in manure through competition for nutrients (experiment 3) and/or the release of fungistatic chemicals (experiment 4). In experiment 3, liquid cultures (100 ml water, 2.4 g potato dextrose broth powder, 0.8 g of nutrient broth powder) of each bacterial strain were prepared and incubated in 250-ml Erlenmeyer flasks (24°C, uncontrolled RH, 150 rpm) for 66 h, thus allowing time for bacterial growth and metabolism. Following incubation, each broth culture was centrifuged at 9,000 rpm for 15 min at 24°C (Beckman Coulter Allegra 64R centrifuge with C0650 rotor) and the supernatant filtered (0.22-µm Millex GP filter unit, Millipore. S.A.S., 67120 Molsheim, France) to remove all bacteria. Aliquots (5 ml) of each filtrate were transferred to glass test tubes (14.3 mm ID×125 mm) and each aliquot was

inoculated with a loopful of hyphae of a different fungal strain. Control tubes contained fresh liquid culture media without bacterial exposure.

All tubes were incubated at 24°C and 40 rpm for 2 weeks. Fungal growth was monitored daily through visual estimates of the volume occupied by fungi. The fungal volume observed in treatment tubes after 2 weeks was converted to a percentage [%=(treatment fungal volume/control fungal volume)×100], and the mean percentage of each treatment compared via a two-tailed one-sample t test with the mean (set to 100%) to be expected when a bacterial strain has no effect on fungal growth.

The protocol of experiment 4 was identical to that of experiment 3 except that nutrients depleted by bacterial growth were replenished by adding an aliquot of concentrated potato nutrient broth (0.46 ml water, 0.043 g nutrient broth, 0.13 g potato dextrose broth) prior to adding the fungal strain to each test tube. Because the complement of fungi that colonize manure may differ daily, fungal strains were re-isolated from fresh chicken manure for experiments 3 and 4 and 5–7.

3.3.4 Do fungi decrease survival of housefly larvae?

In experiments 5–7 (n = 8–10 each), housefly eggs were reared on sterile artificial larval diet with (treatment) or without (control) prior colonization with a particular fungal isolate (*Fusarium* spp., *Phoma* spp., or *Rhizopus* spp.) from fresh chicken manure. Sterile larval diet was prepared from autoclaved wheat bran (100 g; two liquid cycles for 45 min at 121°C) and an autoclaved mixture of brewers yeast (6.5 g), molasses (1.3 ml), and water (200 ml). This diet was mixed thoroughly with a fungal isolate that had been grown for 1 week on PDA. The control diet was mixed without addition of a fungal isolate. Jars

(100×170 mm) containing treatment or control diets were incubated at room temperature for 3 days, after which 800 housefly eggs were buried 5 cm deep in each medium and provisioned with a supplemental protein paste, prepared from pasteurized skim milk powder and water. In each experiment, jars were kept at room temperature until all adult houseflies had emerged. Numbers of adult houseflies were counted and data analyzed by a two-tailed two-sample t test using JMP IN (version 5.1; SAS Institute) with α=0.05.

3.4 Results and discussion

In experiment 1, the amount of ergosterol (indicative of fungal biomass) increased at a slower rate in manure treated with housefly egg wash than in water-treated (control) manure (Fig. 3.1; F=6.79, P=0.0111, n=10), suggesting that egg wash bacteria reduced fungal growth. In contrast, filter-sterilized (bacteria-free) egg wash did not reduce fungal growth in earlier studies with in vitro potato dextrose agar cultures (data not shown). Our results suggest that bacteria from housefly eggs help curtail the growth of fungi growing on natural chicken manure, which contains many other bacteria.

In experiment 2, 19 bacterial strains were isolated from housefly egg wash and ten fungal stains from fresh chicken manure. Growth of each bacterium impeded the subsequent growth of at least one fungal strain, although some bacteria (e.g., *Klebsiella oxytoca* and *Bacillus cereus*) reduced the growth of most fungal strains tested (Supplementary Material, Table 1). These bacterial effects ranged from slowing fungal growth while crossing bacterial growth lines to exhibiting >3-mm zones of complete fungal inhibition (e.g., Fig. 3.2b–d).

In experiment 3, culturing of bacterial strains followed by fungal strains reduced the growth of many fungi (Fig. 3.3). This could have been due to nutrient depletion by bacteria and/or their release of fungistatic chemicals. That some of these adverse effects were retained in experiment 4 (Fig. 3.3), despite replenishment of bacteria-depleted nutrients prior to fungal inoculation, implicates bacteria-derived chemicals interfering with fungal growth. For example, the growth of *Eremedothis angulata* and that of both *Ascobolus crenulatus* strains were inhibited by several bacterial strains in experiments 3 and 4. In contrast, the growth of *Mucor* sp. was inhibited by *Acinetobacter baumanii*, *Chryseobacterium gleum/indologenes*, *K. oxytoca*, and *Pseudomonas fulva* in experiment 3, but not in experiment 4, suggesting that these bacteria deplete some nutrients essential for the growth of this fungus.

In experiments 5–7, significantly fewer houseflies completed development in diets inoculated with *Fusarium* spp., *Phoma* spp., or *Rhizopus* spp. than in diets not pretreated with these fungi (Fig. 3.4). This implies that establishment of manure-associated fungi is harmful to housefly larvae and that suppression of fungal growth can significantly increase larval survival.

Our data suggest that the diverse bacteria present on housefly eggs may have an important biological role in limiting competition from fungi on ephemeral resources. They may deprive fungi of essential nutrients and/or release chemicals that interfere with fungal growth. These chemicals could be bacterial metabolic by-products that inhibit fungal growth directly or they may act through a change in pH or osmotic measure. Alternatively, they may be secondary metabolites that are released in direct response to fungal presence.

In experiment 4, *Acinetobacter* sp. and *B. cereus* significantly increased the growth of *Mucor* sp. relative to the control. This stimulation of growth may be due to the bacterial synthesis of vitamins and/or other cofactors that benefit the growth of this particular fungus. That there are bacterial strains which inhibit some fungal strains while benefiting others highlights the need for a variety of egg-associated bacteria to control all fungi. Indeed, all bacteria together, but not one single bacterium alone, curtailed the growth of most fungi. The diversity of bacterial strains on housefly eggs may be needed to cope with the diversity of fungal strains that can colonize animal manure. *Klebsiella oxytoca* exhibited the broadest antifungal activity (Fig. 3; Supplementary Material, Table 1), suggesting that it may serve the dual role as an oviposition cue for gravid female houseflies (Lam *et al.* 2007) and an antifungal agent (this study). Spruce bark beetles, *Dendroctonus rufipennis*, also deploy bacteria against competitive fungi by secreting a bacteria-containing oral exudate which curtails fungal growth in their gallery system (Cardoza *et al.* 2006). Other interactions between insects and fungi may entail Allee effects between insect larvae (Rohlfs *et al.* 2005) and avoidance of microbe-infested resources (Atkinson 1981; Rozen *et al.* 2008).

Some bacteria compete with macroorganisms for ephemeral resources (Burkepile *et al.* 2006), but their use of nutrients does not interfere with the development of housefly larvae. On the contrary, bacteria are essential dietary constituents for fly larvae (Schmidtmann and Martin 1992; Rochon *et al.* 2004), and egg-derived bacteria in particular have been shown to significantly increase larval survival to adults (Lam *et al.*, unpublished data). Thus, bacteria associated with housefly eggs not only ameliorate the resource for the development of larvae by suppressing fungal growth but also become a

part of the larvae's diet. A thorough understanding of the many mutual or reciprocal actions between insect- and manure-derived bacteria, fungi, and fly larvae in the manure community awaits further studies.

3.5 References

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3.6 Figures

Fig. 3.1 Percent ergosterol (indicator of fungal biomass; amounts at time 0 set to 100%) present in chicken manure at various time intervals after treatment with either a wash of housefly eggs or a water control. The rate of fungal growth differed significantly between treatment and control applications (ANCOVA, P=0.011, n=10 for each diagram pair).

Fig. 3.2 Representative photographs of agar plates with **a** an early stage of growth of one fungal strain (*Fusarium* sp.) inoculated at the center and one or two bacterial strains (marked by arrows) inoculated in point and streak form. **b**-**d** Bacterial strains with: **b** no effect on fungi ("N"); **c** a strong barrier to fungal growth ("B3"); and **d** a wide zone of inhibition of fungal growth ("I3").

Fig. 3.3 Effect of prior bacterial growth in broth culture on subsequent fungal growth when bacteria-depleted nutrients were replenished (experiment 4) or not (experiment 3). In each experiment, fungal growth is reported as a percent relative to growth in broth with no prior bacterial growth (control). An asterisk indicates a significant difference (*=0.01<P<0.05; **=P<0.01); numbers in parentheses indicate the number of replicates. The number of replicates varied due to duplicate fungal and bacterial strains identified after experiment completion.

Fig. 3.4 Mean numbers of adult houseflies completing development to adults when 800 eggs were reared in sterile larval diet incubated for 3 days with (treatment) or without (control) experimental infestation with a fungal isolate.

Fig. 3.1

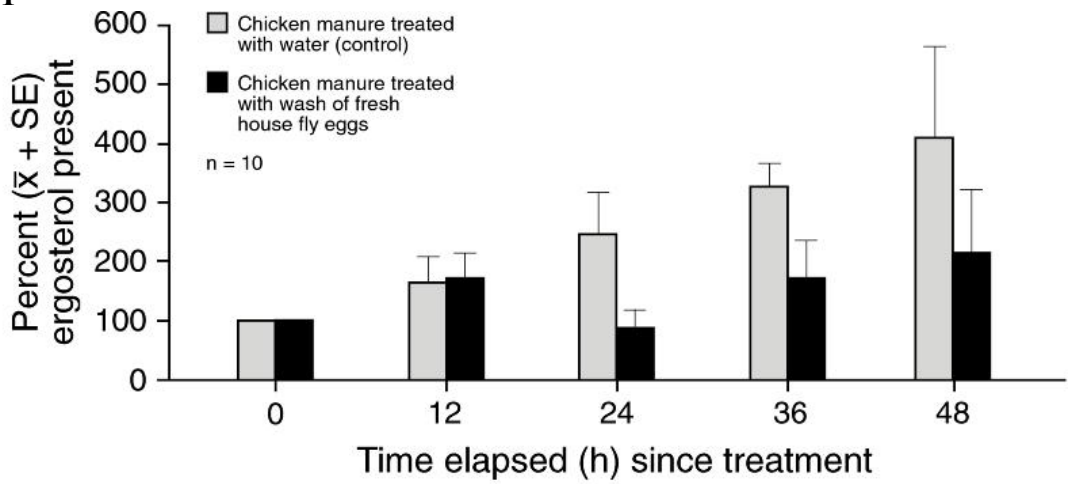

Chicken manure treated with water (control)
Chicken manure treated with wash of fresh house fly eggs
n = 10
Percent ($\bar{x}$ + SE) ergosterol present
600
500
400
300
200
100
0
0
12
24
36
48
Time elapsed (h) since treatment

Fig. 3.2

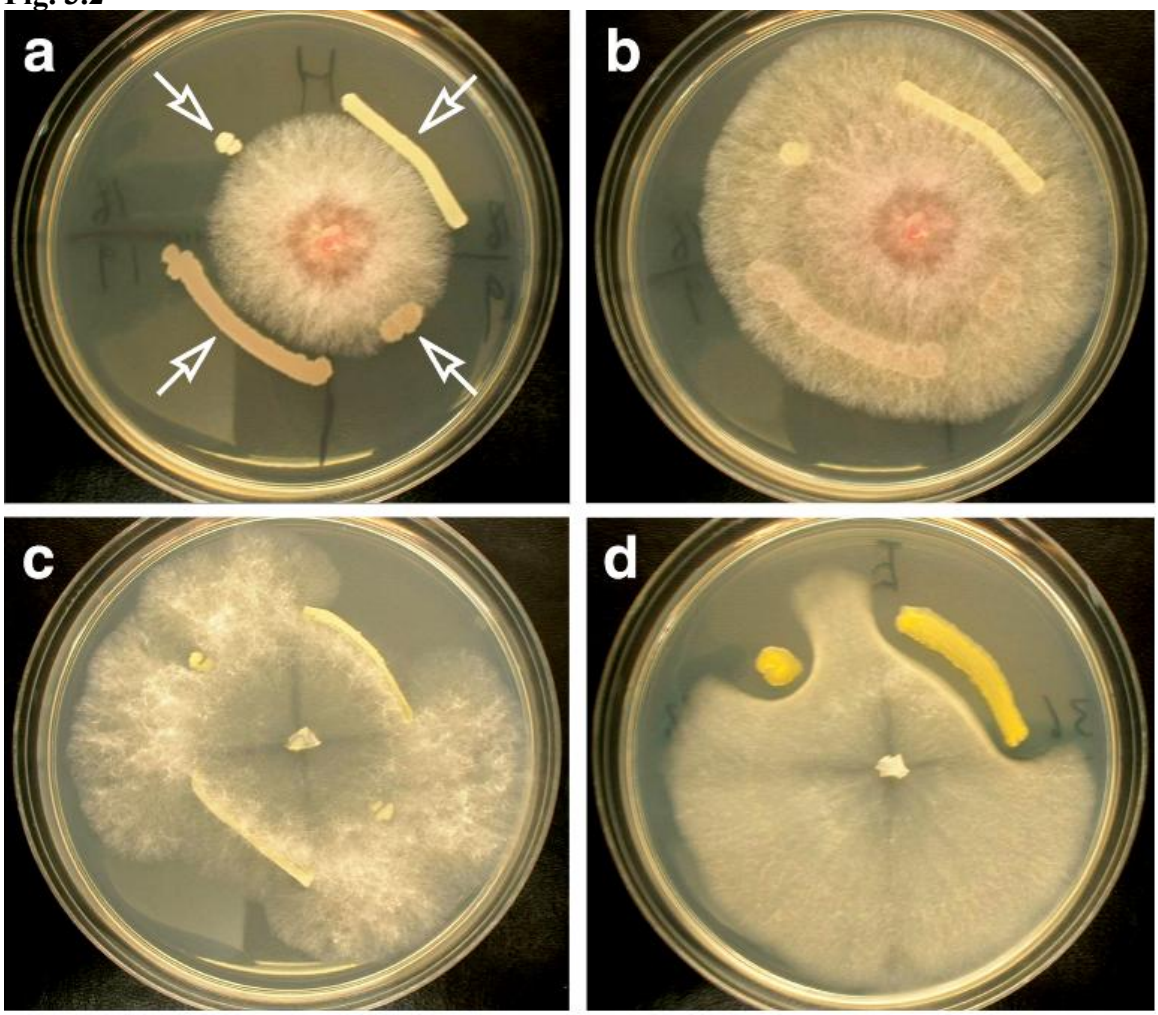


Fig. 3.3

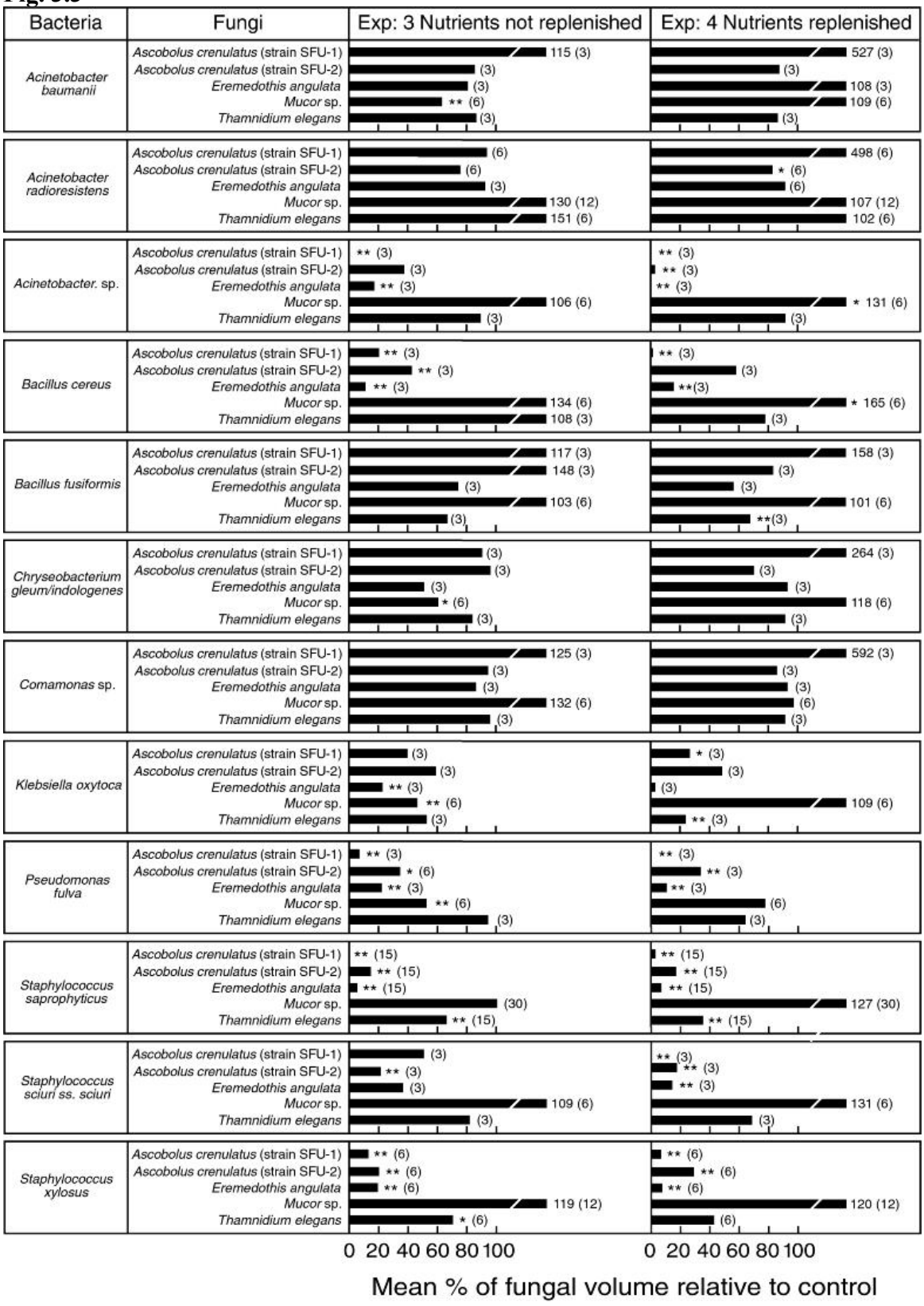
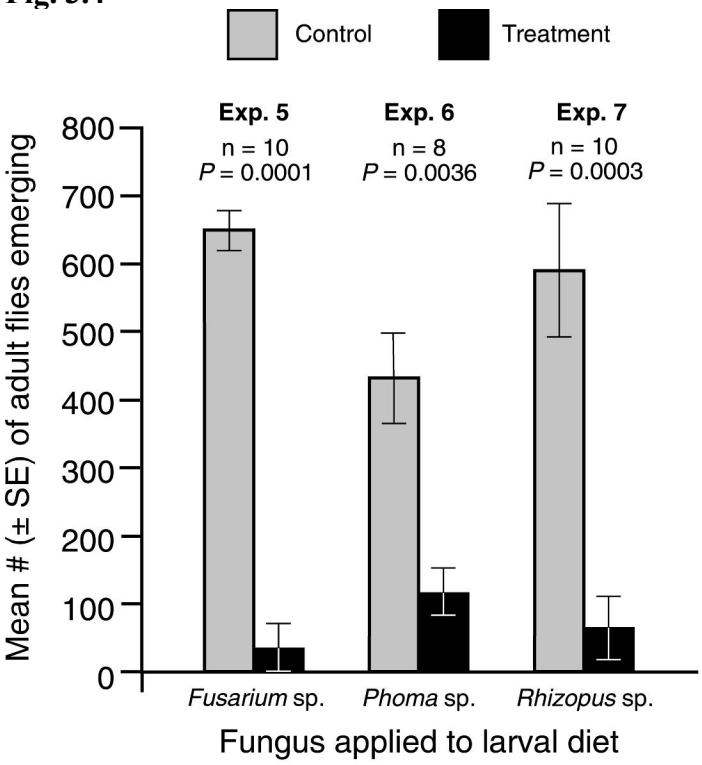

Bacteria
Fungi
Exp: 3 Nutrients not replenished
Exp: 4 Nutrients replenished
Acinetobacter baumanii
Acinetobacter radioresistens
Acinetobacter. sp.
Bacillus cereus
Bacillus fusiformis
Chryseobacterium gleum/indologenes
Comamonas sp.
Klebsiella oxytoca
Pseudomonas fulva
Staphylococcus saprophyticus
Staphylococcus sciuri ss. sciuri
Staphylococcus xylosus
Ascobolus crenulatus (strain SFU-1)
Ascobolus crenulatus (strain SFU-2)
Eremedothis angulata
Mucor sp.
Thamnidium elegans
0 20 40 60 80 100
0 20 40 60 80 100
Mean % of fungal volume relative to control

Fig. 3.4


Control
Treatment
Exp. 5
n = 10
P = 0.0001
Exp. 6
n = 8
P = 0.0036
Exp. 7
n = 10
P = 0.0003
800
700
600
500
400
300
200
100
0
Mean # (± SE) of adult flies emerging
Fusarium sp.
Phoma sp.
Rhizopus sp.
Fungus applied to larval diet

3.7 Supplementary material

Supplementary Table 1. Effect of each bacterial strain, isolated from the surface of house fly eggs, on the growth of each fungal strain, isolated from chicken manure, in experiment 2. Nomenclature as follows: N = no effect; B1, B2: fungal growth slowed slightly (B1) or significantly (B2) while crossing bacterial growth line; B3: fungal growth cannot cross bacterial line and must grow around; I1 – I3: zones of fungal inhibition, ranging from small (< 1 mm; I1), medium (1-2 mm; I2) to wide (> 2 mm; I3), are present around bacterial growths. Numbers in parentheses indicate the number of replicates.

Supplementary Table 1

Bacterial isolate	Fungal isolate									
	Fusarium sp.	*Galactomyces geotrichum*	*Mucor ramosissimum*	*Mucor* sp. (SFU - 1)	*Mucor* sp. (SFU - 2)	*Penicillium* sp.	*Penicillium expansum*	*Rhizopus stolonifer* (SFU - 1)	*Rhizopus stolonifer* (SFU - 2)	*Rhizopus stolonifer* (SFU - 3)
Acinetobacter baumannii	N (3)	I2 (1)	N (1)	N (1)	N (3)	N (1)	B2 (1)	B1 (1)	B1 (1)	
Acinetobacter radioresistens (SFU-1)	N (3)	I2 (1)	B3 (1)	N (1)	N (3)	N (1)	B2 (1)	B1 (1)	B2 (1)	
Acinetobacter radioresistens (SFU-2)	N (3)	N (1)	N (1)	N (2)	N (3)	N (1)	N (1)	B1 (1)	B2 (1)	N (1)
Acinetobacter sp.	N (3)	N (1)	B1 (1)	N (1)	N (3)	N (1)	N (1)	N (1)	B1 (1)	B1 (1)
Bacillus cereus	B1 (3)	I3 (1)	I3 (1)	B1 (2)	B1 (3)	B2 (1)		I3 (1)	I2 (1)	I3 (1)
Bacillus fusiformis	B1 (3)	B2 (1)	B2 (1)	N (1)	N (3)	N (1)	N (1)	B2 (1)	B2 (1)	
Chryseobacterium sp.	N (3)	B3 (1)	B2 (1)	B1 (1)	B1 (3)	N (1)	B3 (1)	B2 (1)	B1 (1)	B3 (1)
Comamonas sp.	I2 (3)		B2 (1)	I2 (3)	I2 (3)	N (1)	N (1)	N (1)	B1 (1)	I3 (1)
Klebsiella oxytoca	B1 (3)	B3 (1)	B3 (1)	B1 (1)	B1 (3)	B2 (1)	B3 (1)	B3 (1)	B2 (1)	B2 (1)
Leucobacter sp.	N (3)	N (1)	N (1)	N (2)	N (3)	N (1)	N (1)	N (1)	N (1)	N (1)
Pseudomonas fulva	I3 (3)		B2	I3 (1)	I3 (3)	N (1)		B1 (1)	N (1)	
Staphylococcus saprophyticus (SFU-1)	B2 (2)	I2 (1)	B2 (1)	N (2)	N (3)	B2 (1)	B1 (1)	B3 (1)	B1 (1)	
Staphylococcus saprophyticus (SFU-2)	N (3)	N (1)	B1 (1)	N (1)	N (3)	N (1)	N (1)	N (1)	N (1)	N (1)
Staphylococcus saprophyticus (SFU-3)	B1 (3)	N (1)	N (1)	N (3)	B1 (3)	N (1)	N (1)	N (1)	N (1)	N (1)
Staphylococcus saprophyticus (SFU-4)	B2 (3)	I2 (1)	I2 (1)	N (2)	B1 (3)	N (1)	B2 (1)	B2 (1)	B1 (1)	I1 (1)
Staphylococcus saprophyticus (SFU-5)	B1 (3)	N (1)	N (1)	N (1)	B1 (3)	N (1)	N (1)	N (1)	N (1)	B2 (1)
Staphylococcus sciuri ss sciuri	N (3)	N (1)	N (1)	N (3)	B1 (3)	N (1)	N (1)	N (1)	N (1)	N (1)
Staphylococcus xylosus (SFU-1)	N (3)	B1 (1)	N (1)	B1 (1)	N (3)	N (1)	N (1)	N (1)	N (1)	N (1)
Staphylococcus xylosus (SFU-2)	N (3)	N (1)	N (1)	B1 (2)	B1 (3)	N (1)	N (1)	N (1)	N (1)	N (1)

4: Semiochemical-mediated oviposition avoidance by female house flies, *Musca domestica*, on animal feces colonized with harmful fungi[‡]

4.1 Abstract

House flies, *Musca domestica*, utilize ephemeral resources such as animal feces for oviposition and development of larval offspring, but face competition with fungi colonizing the same resource. We have predicted that house flies avoid oviposition on feces well-colonized with fungi, thereby reducing fungal competition for larval offspring. Working with fungal isolates from chicken feces, we have previously shown that prior establishment of *Phoma* spp., *Fusarium* spp., or *Rhizopus* spp. on feces significantly reduced oviposition by house flies. Here we report that, in the headspace volatiles of these three fungal genera, five compounds (dimethyl trisulfide, an unknown, 2-phenylethanol, citronellal, norphytone) elicit responses from house fly antennae. In behavioural bioassays, dimethyl trisulfide and 2-phenylethanol significantly reduced oviposition by house flies. We conclude that fungus-derived volatiles serve as semiochemical cues that help house flies avoid resources colonized with fungal competitors for the development of larval offspring.

Keywords: House flies *Musca domestica* *Phoma* spp. *Rhizopus* spp. Fungi Animal Feces Resource competition Oviposition Semiochemicals Dimethyl trisulfide 2-Phenylethanol

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4.2 Introduction

Microorganisms competing with insect larvae for ephemeral resources can adversely affect larval development, particularly when these microorganisms are first to colonize the resource. For example, microorganisms have been shown to reduce the number of burying beetle offspring, *Nicrophorus quadripunctatus*, completing development (Wilson *et al.* 1984). Competition with fungal (Fuentes-Contreras *et al.* 1998; Askary and Brodeur 1999), viral (Hochberg 1991) or bacterial (Chilcutt 1997) pathogens of host insects adversely affects even parasitoid larvae, which exploit living organisms. The outcome of this competition is dependent upon the relative timing of infection. Within pea aphids, *Acyrthosiphon pisum*, the presence of bacteria is so detrimental to larvae of the parasitoid *Aphidius ervi* that these bacteria persist as beneficial endosymbionts of the aphid (Oliver *et al.* 2003).

Competition with fungi is particularly costly for larvae of the house fly, *Musca domestica.* Females lay their eggs on fleeting organic resources such as manure (Keiding 1974; Fatchurochim *et al.* 1989). Eggs hatch within 24 h and larvae develop through three instars in 5-7 days. After a 5-d pupal period, adult males and females eclose. When fungi were established on a fleeting resource 3 days prior to house fly oviposition, all of the resulting larvae perished (Zvereva 1987).

When the relative timing of resource colonization strongly affects the outcome of competition, harmful competitors are often recognized and avoided. Many parasites and parasitoids recognise host-marking pheromones and avoid oviposition on the same host (Li 2006; Stelinki 2007). The mistletoe-feeding pierid butterflies *Delias argenthona* and *D. nigrina* have overlapping host ranges but oviposit more selectively on specific host

species when heterospecific competitors are present (Orr 2008). Mosquitoes avoid larval habitats with intra- or interspecific competitors (Munga *et al.* 2006). Adult ladybirds avoid oviposition on plants with other aphid predators already present (Sarmento *et al.* 2007). Leaf beetles (Roeder *et al.* 2007) and Australian weevils (Rayamajhi *et al.* 2006) avoid plants already colonized with a rust fungus, and larvae and adults disperse rapidly when placed on fungus-infected host plants. Finally, the hymenopteran parasitoid *Aphidius ervi* avoids oviposition on plants where aphid hosts were killed by the entomopathogenic fungus *Pandora neoaphidis* (Baverstock 2005).

All of the above examples are interactions between specific insects and specific microorganisms. In contrast, house flies face diverse fungi as resource competitors, each with a different establishment rate and competitive effect on house fly larvae. To determine whether houseflies have adapted to avoid fungus-infested resources, we isolated fungal strains from different types of animal feces, and tested the hypotheses that house flies avoid oviposition on feces that was colonized with competitive fungi, and that the avoidance is due to fungus-derived semiochemicals.

4.3 Methods and Materials

4.3.1 Experimental Insects

Adult house flies were kept in cages at 50-80% relative humidity (RH), 22-30°C and a 16L:8D photoperiod, and were provisioned with water, sugar cubes and skim milk powder *ad libitum*. House fly eggs were collected on glass Petri dishes (50 × 10 mm) containing sterile skim milk agar (SMA; 15 ml water, 0.12 g nutrient broth powder, 0.24 g skim milk powder, 0.3 g agar) and reared to adult insects in artificial diet prepared from

wheat bran (400 g), brewers yeast (15 g), molasses (15 ml) and water (700 ml), with a supplemental protein paste prepared from skim-milk powder and water (Lam *et al.* 2007).

4.3.2 Isolation and Identification of Fungal Strains

To isolate feces-colonizing fungi, feces pats from free-range organic chickens, sheep, horses and wild barn swallows were collected at the Wind's Reach Farm, Langley, B.C., Canada. A sterile inoculating loop was inserted into the centre of each pat and streaked across Petri plates (90 × 15 mm) containing feces agar (20 ml water, 2.5 g chicken feces that had been sterilized by stirring and autoclaving >3 times at 121°C for 45 min, plus 0.4 g agar). This procedure was to select for fungi that can grow on chicken feces nutrients alone. Mycelia from separate fungal colonies were isolated and incubated on Petri plates (90 × 15 mm) containing potato dextrose agar (PDA; 20 ml water, 0.48 g potato dextrose broth powder, 0.4 g agar) at 24°C and 85% RH. To identify the fungi that expressed behavioural activity in experiments 1-6, each strain was grown in a Sabouraud dextrose broth, and DNA extraction was performed using the QIAamp® DNA Mini Kit (Qiagen®, Canada, Cat. No. 51304) according to the manufacturer's instructions. Sequencing of 18S rRNA was performed using Fun 1 and Fun 2 primers (unpublished; designed by Dr. Bryne, Animal Health Centre, Abbotsford, B.C., Canada). Fungi were identified based on colony morphology and by comparing 18S rRNA sequences to the Genbank database.

4.3.3 Hypothesis 1: House flies avoid Oviposition on Feces colonized with Fungi

In two-choice experiments 1-6 (n = 10 each), we compared oviposition by house flies on sterilized feces (control) and sterilized feces mixed with one of six fungal isolates

(treatment) as follows: *Phoma* sp., *Rhizopus* sp., *Fusarium* sp. (SFU-1), *Fusarium* sp. (SFU-2), Unknown 1, and unknown 2.

Chicken feces pats (0- to 24-h-old) were blended and sterilized. An 8-g aliquot of treatment feces was then thoroughly mixed with the mycelia and spores (if present) of a one-week-old PDA culture of one fungal isolate. An 8-g aliquot of control feces was mixed without addition of fungi. Aliquots (8 g) of each feces mixture were packed tightly into separate sterile plastic Petri dishes (35 × 10 mm). To generate an attractive crevice for oviposition, a 5-mm diameter well was poked into the centre of each dish using a sterile metal spatula.

Control and treatment dishes were then incubated separately for 3 days in a sterile glass chamber (15 cm ID × 28 cm) through which filtered and humidified air was drawn at 0.5 L/min. Control and treatment dishes were then placed in randomly-assigned opposite corners of a mesh cage (30 × 30 × 45 cm) containing 20 male and 20 female house flies. When oviposition ceased after 3-6 h, the eggs on each dish were removed and weighted or counted (~15,000 eggs/g).

The weights/counts of eggs deposited on treatment and control oviposition sites within each replicate pair were converted to two separate percentages adding up to 100% of total eggs deposited. This approach was chosen because of variation in the total amount of eggs deposited by the flies in each replicate. Two-tailed one-sample bootstrap tests on the means were conducted to determine whether the mean percentages of eggs deposited on treated feces was significantly different from 50%. For each bootstrap test, 1000 bootstrap samples were randomly generated with replacement, and each sample contained the same number of replicates as the original data set. The mean percentage of

the original data set was then compared with the mean percentages of its bootstrap samples (SAS, ver. 9.1; SAS Institute, Cary, North Carolina, U.S.A.) with $\alpha = 0.05$.

4.3.4 Hypothesis 2: Fungi produce Semiochemicals that inhibit Oviposition by Houseflies

4.3.4.1 Acquisition of Headspace Volatiles

Ten Petri plates (90 × 15 mm) containing potato dextrose + feces agar (20 ml water, 0.48 g potato dextrose broth powder, 2.5 g sterilized chicken feces, 0.4 g agar) were inoculated with mycelia of a fungal isolate that inhibited oviposition in experiments 1-6. Plates in staggered form were placed into a sterile glass chamber (15 cm ID × 28 cm) through which filtered and humidified air was drawn at 1.0 L/min for 1 wk. Volatiles were adsorbed on 0.2 g of Porapak Q (50-80 mesh, Waters Associates Inc. Milford, Massachusetts, USA) inside a Pyrex glass tube (3.8 × 40 mm), and desorbed with 2 mL of pentane. Headspace volatiles of sterile potato dextrose + feces agar served as a control and were also adsorbed on Porapak Q.

To ensure that clean air entered the autoclaved aeration chamber and to prevent introduction of bacteria onto the fungal plates, air was filtered through 3.5 g of autoclaved (45 min at 121°C) and oven-dried (overnight at 110°C) Porapak Q that was tightly-packed into a glass tube (140 × 8 mm ID). Each day, agar plates were visually monitored for bacterial and fungal contamination, but none was observed.

4.3.4.2 Analyses of Headspace Volatiles

To identify volatiles that elicited responses from house fly antennae, Porapak Q extracts were concentrated and 1-µl aliquots analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn *et al.* 1975; Gries *et al.* 2002).

Analyses were carried out using a Hewlett Packard (HP) 5890A gas chromatograph equipped with a GC column (30 m × 0.25 mm ID) coated with DB-5 (J&W Scientific, Folsom, California, USA) and helium as a carrier gas (35 cm^{-1}) with the following temperature program: 50°C (1 min), 20°C/min to 280°C. For GC-EAD recordings, an antenna was pulled from a live female house fly and suspended between two glass capillary electrodes (1.0 × 0.58 × 100 mm) (A-M Systems, Inc., Carlsborg, Washington, USA) filled with saline solution (Staddon and Everton, 1980). Mass spectra of antenna-stimulating components were obtained with a Saturn 2000 Ion Trap (Varian) fitted with a DB-5 column. Compounds were identified by comparing their retention indices (Van den Dool and Kratz 1963) and mass spectra with those reported in the literature [dimethyl trisulfide (Swearingen *et al.* 2006), phenylacetaldehyde, norphytone (Pherobase), citronellal, 2-phenylethanol (Adams 1989)] and with those of authentic samples.

4.3.4.3 Two-choice Experiments with Candidate Semiochemicals.

Experiments 7-11 (n = 10-24 each) tested each of the candidate semiochemicals (dimethyl trisulfide, phenylacetaldehyde, 2-phenylethanol, cironellal, norphytone) to determine its effect on oviposition by house flies. Two glass Petri dishes (50 × 10 mm) containing sterile skim milk agar (15 ml water, 0.12 g nutrient broth powder, 0.3 g agar) served as oviposition sites. A thin strip of agar (width < 5 mm) was excised across the middle of the plate to provide a crevice conducive for oviposition. The neat candidate semiochemical was released from a 5-µL micro-capillary tube which was sealed with plasticine at one end and placed within the crevice such that the open end was near the centre of the plate. An empty tube was prepared analogously and placed into the crevice of the control plate. In each replicate, the two plates were randomly assigned to opposite

corners of a mesh cage (30 × 30 × 45 cm) that contained 20 male and 20 female house flies. Resulting egg counts/weights were analysed as described above. In all replicates, release rates (µg/h) of candidate semiochemicals were determined volumetrically by measuring with a digital calliper the capillary tubes' length of released chemical, and by taking into account (*i*) the total length of the tube (40 mm), (*ii*) its total holding capacity (5 µL), (*iii*) the compound's density, and (*iv*) the duration (16 h) of the bioassay.

4.4 Results

4.4.1 Hypothesis 1: House flies avoid Oviposition on Feces colonized with Fungi

In experiments 1 and 2, sterilized feces inoculated with *Phoma* sp. or *Rhizopus* sp. received significantly fewer eggs from female house flies than sterilized feces with no added fungi (Fig. 4.1, $P < 0.001$). In contrast, in experiments 3-5 the presence of *Fusarium* sp. (SFU-1 or SFU-2) or Unknown Fungal Isolate 1 had no effect on oviposition decisions (Fig. 4.1, $P > 0.05$). In experiment 6, feces inoculated with Unknown Fungal Isolate 2 received significantly more eggs than feces devoid of fungi (Fig. 4.1, $P < 0.05$).

4.4.2 Hypothesis 2: Fungi produce Semiochemicals that inhibit Oviposition by House flies

GC-EAD analyses of headspace volatile extracts of *Phoma* and *Rhizopus* fungi (which strongly inhibited oviposition by female house flies) and of *Fusarium* sp. (SFU-1) (which appeared to have some effect on oviposition), revealed six compounds that consistently elicited responses from house fly antennae (Fig. 4.2). By comparing their mass spectra and retention indices with those of authentic standards, antennal-stimulatory components 1, 2, 4, 5 and 6 were identified as dimethyl trisulfide, phenylacetaldehyde, 2-

phenylethanol, citronellal and norphytone, respectively. Component 3 remains unknown. Of these six components, only phenylacetaldehyde was also found in headspace volatiles of sterile feces agar (data not shown). Thus, phenylacetaldehyde was excluded form experiments 7-10, which tested the effect of each of the fungal volatiles, dimethyl trisulfide, 2-phenylethanol, citronellal or norphytone, on oviposition decisions by house flies. In experiments 7-10, dimethyl trisulfide and 2-phenylethanol strongly reduced oviposition by female house flies (Fig. 4.3A, experiments 7-9), whereas neither citronellal nor norphytone had a behaviour modifying effect (Fig. 4.3A, experiment 9,10). These contrasting results could not be attributed to contrasting release rates of semiochemicals because citronellal and norphytone had greater release rates than the oviposition-inhibiting semiochemicals (Fig. 4.3B).

4.5 Discussion

Our data show that female house flies avoid oviposition on ephemeral resources that have been colonized by specific fungal resource competitors, likely due to detection of volatiles produced by fungi.

The presence of particular genera of fungi such as *Phoma* and *Rhizopus*, rather than just the presence of any fungus, affected oviposition by house flies on feces. *Phoma* is a genus of common soil fungi that contains many plant pathogenic species causing rots and blight (Gray *et al.* 2008; Davidson *et al.* 2009), but it also contains species affecting insects. *Phoma aspidioticola*, for example, is highly pathogenic to the scale insect *Aspidiotus destructor* but not to the plant host *Syzygium cumini* (Narendra and Rao 1974). *Rhizopus* is a genus of molds that includes fungi found in soil, decaying fruit and vegetables, old bread and animal feces (El-Mougy *et al.* 2008; Rodriguez *et al.* 2008;

Serrano-Garcia *et al.* 2008, Takahashi *et al.* 2008). However, some species are plant pathogens (Prom and Perumal 2008; Zhao *et al.* 2008), cause infections in humans and animals (Landlinger *et al.* 2008), or affect arthropods. For example, *Rhizopus thailandensis* has demonstrated experimental pathogenicity to *R. sanguineus* ticks (Casasolas-Oliver *et al.* 1991).

By avoiding oviposition sites colonized with potentially pathogenic fungi, female house flies make decisions that enhance their fitness. Larval diet inoculated with mycelia and/or spores of the oviposition-inhibiting fungi *Fusarium* spp., *Phoma* spp. and *Rhizopus* spp. had detrimental effects on the number of house fly larvae that completed development (Lam *et al.* 2009). This decrease in larval survival may not occur with other fungi [e.g. *Fusarium* spp. (SFU-1)] that had little or no impact on oviposition by female house flies. Alternatively, these fungi may simply lack the semiochemical cues that indicate the presence of (harmful) fungi.

Release rates of synthetic semiochemicals from glass capillary tubes in oviposition experiments 7-10 exceeded those from agar cultures of *Rhizopus* spp. by 100-500 times. This, however, seemed justified because levels of semiochemical emission from *Rhizopus* spp. are likely to have varied greatly during the 7-d aeration period, with peak emissions assumed to have coincided with a short (~1 d) period of rapid fungal growth (when nutrients were plentiful), and with much lower levels of emissions before and after that period. Each fungal isolate with adverse effects on the development of house fly larvae (Lam *et al.* 2009) emitted dimethyl trisulfide and 2-phenylethanol, each of which strongly reduced oviposition by house flies on feces. Neither of these two semiochemicals has previously been reported to affect oviposition decisions by flies, but

2-phenylethanol is a bacteria-derived putative oviposition semiochemical for *Anopheles gambiae* mosquitoes (Lindh *et al.* 2008).

Dimethyl trisulfide and 2-phenylethanol are well known in diverse ecological contexts. For examples, 2-phenylethanol is released from the hair pencils of courting males in noctuid and pyralid moths (Kuwahara 1980; and references cited therein). As a plant semiochemical, 2-phenylethanol adds to the attractiveness of plants to their respective herbivores (e.g., Zilkowski *et al.* 1999), pollinators (e.g., Knutsen and Tollsten 1993) or predators in tritrophic systems (e.g., Zhu and Park 2005). Dimethyl trisulfide is a major constituent of the flower of the voodoo lily, *Sauramutum guttatum,* and of the stinking mushroom, *Phallus impudicus* (Borg-Karlson *et al.* 1994). It is also present in floral volatiles of bat-pollinated plants, and in the essential oils of garlic, *Allium sativum* (Harborne *et al.* 1999; Kim *et al.* 2004). It is a major aroma component of cooked brassicaceous vegetables (Marujama, 1970), an indicator of decaying meat (Brown 1982), and a semiochemical of the dead-horse arum, *Helicodiceros muscivorus,* a plant which fools flies into pollinating it by emitting a smell like a rotting carcass (Stensmyr *et al.* 2002). Dimethyl trisulfide is also a volatile odour constituent of various types of animal manure (Cai *et al.* 2007), and, as a single component, it is known to attract some calliphorid and one muscid fly (Nilssen *et al.* 1996).

Manure-emitted dimethyl trisulfide may, at least in part, be produced by fungi colonizing it. That some flies are attracted to dimethyl trisulfide (Nilssen *et al.* 1996) implies that it may serve as a foraging cue indicative of food or oviposition sites. Feces are rich in protein and could provide nutrients to adult flies even though they may decide not to use it as a resource for larval offspring. These decisions may be based on absolute

or relative amounts of compounds that are unambiguous indicators of both the presence and infestation level of harmful fungi. All such fungal isolates in our study released both dimethyl trisulfide and 2-phenylethanol, and either compound alone reduced oviposition by female flies, possibly due to release rates indicative of significant fungal infestations.

It would now be intriguing to determine whether volatile profiles of fungal isolates that are harmful or harmless to larval offspring of house flies differ, and how the differences would facilitate oviposition decisions by female flies. It would also be worth investigating whether the semiochemicals, or the fungi producing them, could be exploited as a tactic within programs for managing house flies in livestock production facilities.

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4.7 Figures

Fig. 4.1 Percent of house fly eggs oviposited on sterile chicken manure incubated for 3 days with (treatment) and without (control) experimental infestation with a fungal strain. Replicates in which no oviposition occurred were omitted.

Fig. 4.2 Results of gas chromatographic-electroantennographic detection analysis of headspace volatiles captured from *Rhizopus* spp. growing on manure agar. No additional responses from house fly antennae were observed with the volatiles of the other two oviposition-inhibiting fungal strains (data not shown). Compounds that consistently (n = 3) elicited antennal responses are marked with arrows and are labelled: (1) dimethyl trisulfide; (2) phenylacetaldehyde; (3) unknown; (4) 2-phenylethanol; (5) citronellal; and (6) norphytone. Phenlyacetaldehyde was found in headspace volatiles of *sterile* manure agar, and thus was omitted from experiments 7-10.

Fig. 4.3 (A) Percent of house fly eggs oviposited on artificial agar oviposition sites with (treatment) or without (control) a candidate semiochemical released from a micro-capillary tube at the centre of the oviposition site; (B) Rates of evaporation of each candidate semiochemical from micro-capillary tubes (5 μm ID × 40 mm).

Fig. 4.1

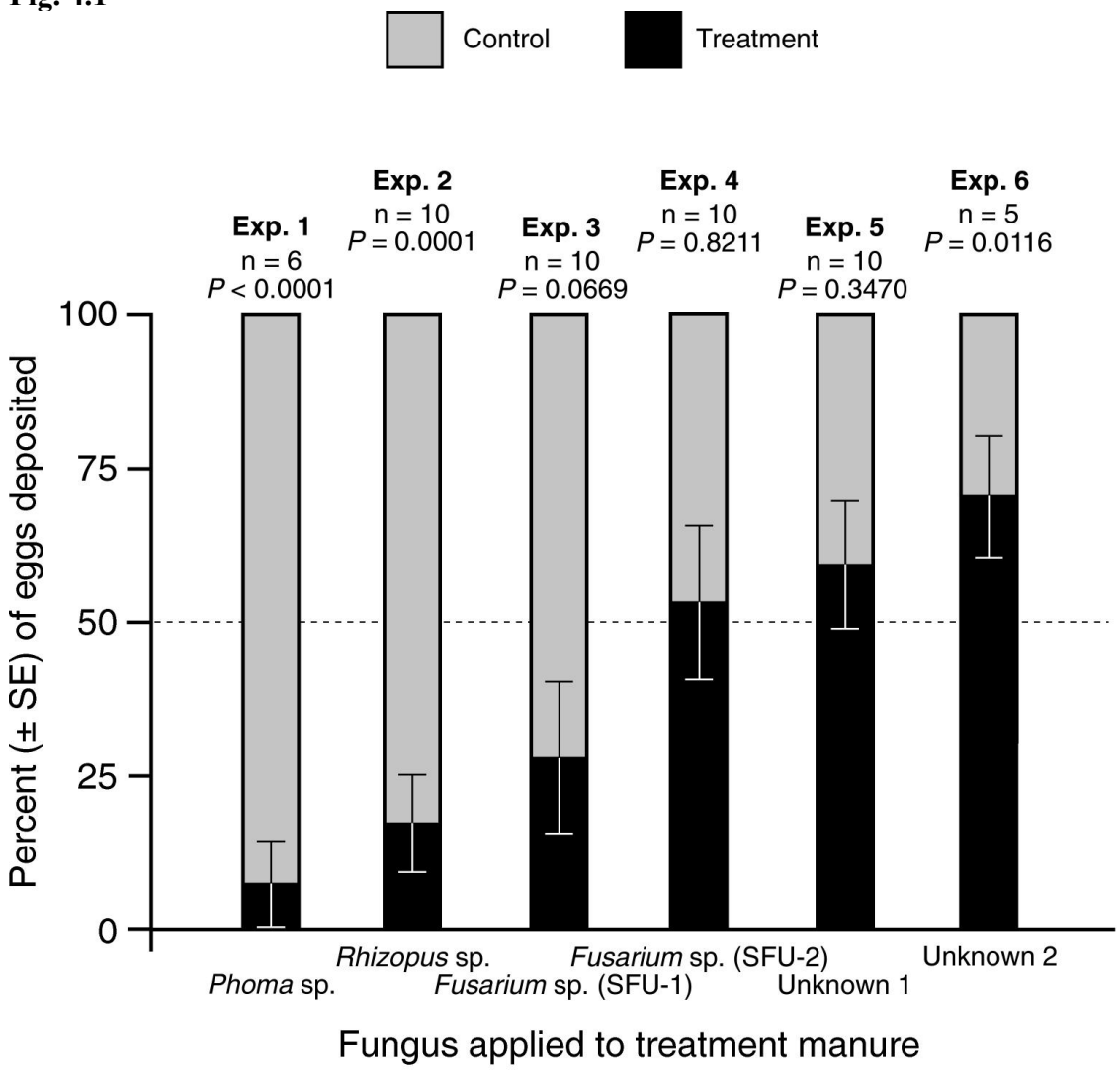

Control
Treatment
Exp. 1
n = 6
P < 0.0001
Exp. 2
n = 10
P = 0.0001
Exp. 3
n = 10
P = 0.0669
Exp. 4
n = 10
P = 0.8211
Exp. 5
n = 10
P = 0.3470
Exp. 6
n = 5
P = 0.0116
100
75
50
25
0
Percent (± SE) of eggs deposited
Phoma sp.
Rhizopus sp.
Fusarium sp. (SFU-1)
Fusarium sp. (SFU-2)
Unknown 1
Unknown 2
Fungus applied to treatment manure

Fig. 4.2

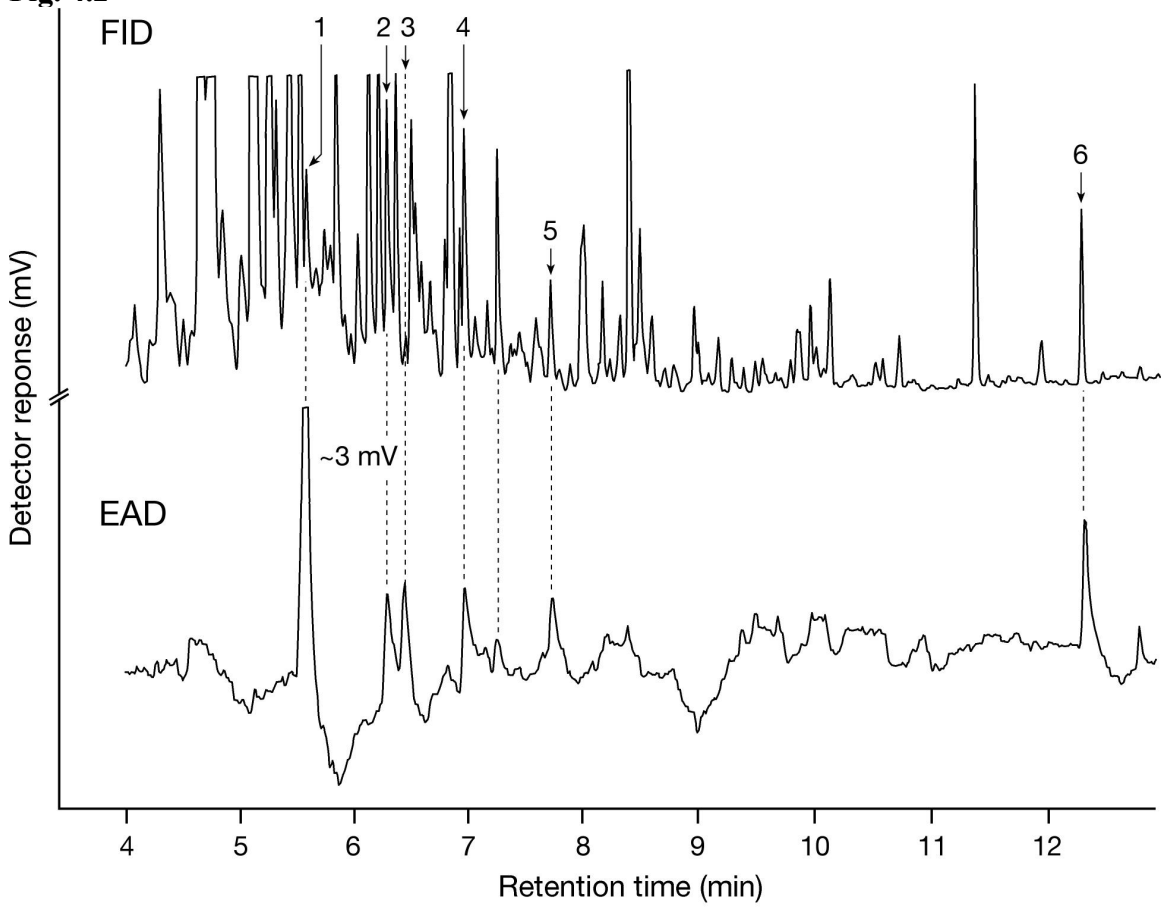
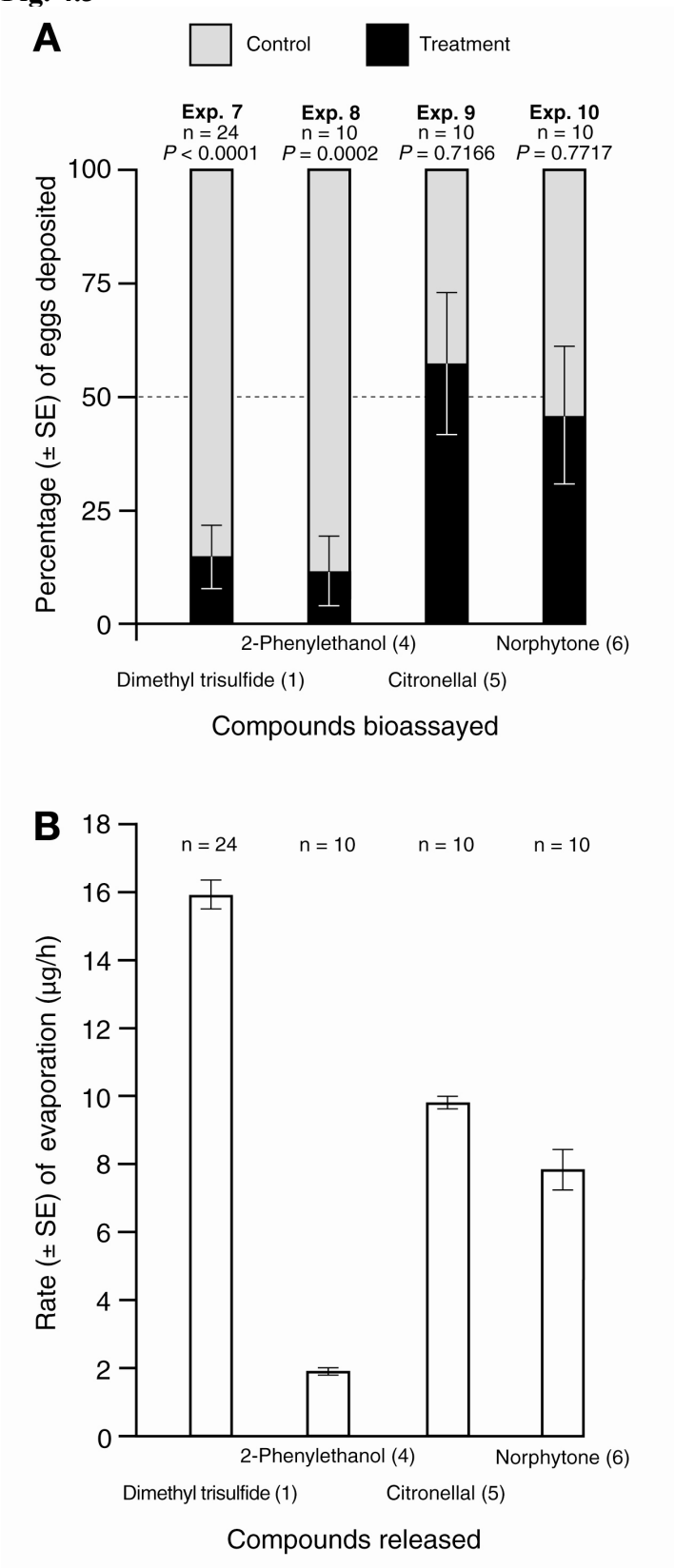

FID
1
2 3
4
5
6
~3 mV
EAD
Detector reponse (mV)
4
5
6
7
8
9
10
11
12
Retention time (min)

Fig. 4.3


A
Control
Treatment
Exp. 7
n = 24
P < 0.0001
Exp. 8
n = 10
P = 0.0002
Exp. 9
n = 10
P = 0.7166
Exp. 10
n = 10
P = 0.7717
Percentage (± SE) of eggs deposited
100
75
50
25
0
2-Phenylethanol (4)
Norphytone (6)
Dimethyl trisulfide (1)
Citronellal (5)
Compounds bioassayed
B
n = 24
n = 10
n = 10
n = 10
Rate (± SE) of evaporation (µg/h)
18
16
14
12
10
8
6
4
2
0
2-Phenylethanol (4)
Norphytone (6)
Dimethyl trisulfide (1)
Citronellal (5)
Compounds released

5: Ovipositing female house flies provision offspring larvae with bacterial food[§]

5.1 Abstract

Symbiotic bacteria on house fly eggs, *Musca domestica* L. (Diptera: Muscidae), provide ovipositional cues for conspecific female flies and curtail the growth of fungi that compete with fly larval offspring for resources. Because bacteria are also essential dietary constituents for developing larvae, we tested the hypothesis that egg-derived bacteria support development of larvae to adults. From house fly eggs, we isolated and identified 12 strains of bacteria, 8 and 4 of which were previously shown to induce and inhibit oviposition, respectively. When larvae were provisioned with a total dose of 10^6–10^7 colony-forming units of bacteria from either the oviposition-inducing or -inhibiting group, or from both groups together, significantly more larvae completed development. Thus, egg-associated bacteria could be a fail-safe mechanism that ensures a bacterial food supply for larval offspring, particularly if the resource selected by parent females is poor in bacterial food.

Key words: *Musca domestica*, oviposition, bacteria, nutritional resource, larval survival, symbiosis, Diptera, Muscidae, induction, inhibition

[§] This chapter was published in *Entomologia Experimentalis et Applicata* 133: 292-295 in 2009, with authors as follows: Lam, K., Geisreiter, C., and Gries, G.

5.2 Introduction

Micoorganisms are essential sources of nutrition for many insects. For example, cyanobacterial mats in ponds induce oviposition by mosquitoes and serve their developing larvae as food (Benzon & Apperson, 1988; Rejmankova *et al.*, 1996, 2000). Microbes associated with decaying plant tissues constitute an essential nutritional component for larval development in drosophilid flies (Begon, 1982), and adult flies transfer yeasts to larval feeding sites (Gilbert, 1980; Fogleman & Foster, 1989; Morais *et al.*, 1994; Rohlfs &Hoffmeister, 2005).

Many bacterial strains have been isolated from the surfaces of eggs oviposited by female house flies, *Musca domestica* L. (Diptera: Muscidae), on ephemeral resources such as manure (Lam *et al.*, 2007). Some of these egg-associated bacterial strains induce further oviposition by conspecific flies, whereas others inhibit it (Lam *et al.*, 2007). Specific strains increase in abundance over time, resulting in inhibition of oviposition. Among these strains, *Klebsiella oxytoca* (Flügge) Lautrop plays a key role in reversing the ovipositional response of gravid female house flies from induction to inhibition (Lam *et al.*, 2007).

Bacteria are essential dietary constituents of house fly larvae (Watson *et al.*, 1993). Bacteria may be found naturally in the resource selected as an oviposition site, or they could be deposited by the female at the time of oviposition. Bacterial deposition by female flies would ensure that hatching larvae are well provisioned with food, irrespective of bacterial abundance in the oviposition site. Here, we tested the hypothesis that bacteria associated with house fly eggs are a nutritional resource that help house fly larvae complete development to adults.

5.3 Materials and methods

5.3.1 Isolation of bacterial strains from house fly eggs

House flies were reared as previously described (Lam *et al.*, 2007). Eggs from hundreds of females that oviposited for >6 h were collected on glass Petri dishes (50 × 10 mm) containing sterile skim milk agar (SMA; 15 ml water, 0.12 g nutrient broth powder, 0.24 g skim milk powder, and 0.3 g agar). The eggs were shaken vigorously in distilled water, and the resulting wash poured through glass wool to remove eggs. This wash was then streaked, and single-colony bacterial isolates re-streaked on SMA. Isolated strains were maintained at 24 °C and 85% relative humidity (r.h.) on 90 × 15 mm plastic Petri plates containing sterile nutrient agar (20 ml water, 0.16 g nutrient broth powder, and 0.4 g agar). Each strain was identified with 16S rRNA sequencing (using 27f and 519r primer set; Lane, 1991) and the API 20E biochemical identification system (bioMérieux, Marcy-l'Etoile, France). Although not all bacterial strains that were washed from house fly eggs grow on SMA, we obtained a broad sample of the various bacterial strains present on house fly eggs.

5.3.2 Effect of bacteria on number of larvae completing development to adults

Larvae that had hatched from surface-sterilized eggs were reared on sterile larval diet, in the presence (treatment) or absence (control) of bacterial isolates (Table 6.1) at total doses of 10^2 to 10^7 colony-forming units (CFUs) per plate.

The sterile larval diet was adapted from Watson *et al.* (1993) and consisted of 500 ml of water, containing 4 g of nutrient broth powder, 1.3 g of alfalfa meal, and 3 g of agar powder. The mixture was autoclaved at 121 °C for 30 min and cooled to 45 °C before adding two chicken egg yolks from surface-sterilized eggs and half of a surface-sterilized

multivitamin tablet (Multi Plus; London Drugs, Richmond, BC, Canada). The intact chicken eggs were sterilized in 95% ethanol for 30 min prior to yolk isolation (Watson *et al.*, 1993), and multivitamin tables were irradiated for 5–10 min on all sides with a UV lamp (G30T8 30W Germicidal; Sylvania, Mississauga, Ontario, Canada). After mixing, 20-ml aliquots were poured into sterile 60 × 20 mm plastic Petri dishes.

House fly eggs were collected on sterile SMA, surface-sterilized by rinsing them in sequence for 12 min each with a bleach solution (0.026 g l^{-1} NaOCl) and then sterile water before being transferred to dishes of sterile larval diet at room temperature until larvae hatched. Potential growth of any bacteria possibly still associated with surface-sterilized eggs on the larval diet was carefully monitored, with the intention to discard intermediate dishes with bacterial growth. Within 12 h of hatching, 30 larvae were transferred with a sterile camel-hair brush to an experimental dish containing sterile larval diet that had just been inoculated with either bacterial isolates or sterile distilled water as a bacteria-free control (Table 5.1). Allowing larvae to hatch in intermediate dishes ensured that larvae from those eggs that were not completely sterilized, or that were damaged during sterilization, were not transferred onto experimental dishes.

Experimental treatments (Table 5.1) included: (A) sterile distilled water (as a bacteria-free control), (B–D) three mixtures of bacterial strains isolated from house fly eggs, and (E) the mixture of bacteria washed off fresh house fly eggs. Treatments B–D were prepared by growing each bacterial strain on nutrient agar for 24 h, and by suspending and combining equal CFUs of target strains in sterile distilled water. A dilution series (10×) of each treatment mixture (B–E) was plated on nutrient agar to estimate the total number of CFUs of bacteria applied per plate. Each treatment was

replicated five times per dose, and all treatments and doses were tested concurrently. The highest dose tested for treatment E (Table 5.1) was 10^5 CFUs.

Experimental plates with larval diet were maintained at 24 °C and 85% r.h. until larvae pupated. Pupae were transferred with flame-sterilized forceps to sterile plastic Petri dishes (60 × 20 mm) where they dried and left until adults emerged. The number of adults that eclosed from each dish was recorded. In preliminary experiments, the type of treatment to which larvae were exposed did not affect the length and dry weight of eclosing flies (data not shown).

For each dose of bacteria tested, the mean number of flies that eclosed from each of the five treatments was compared with an analysis of variance (ANOVA), followed by a Tukey's HSD multiple comparison where indicated (JMP 7; SAS Institute, Cary, NC, USA).

5.4 Results and discussion

When grown in the presence of egg-wash bacterial isolates at doses of 10^6 and 10^7 CFU per plate, significantly more larvae completed development to adults than larvae without bacterial supplements ($P<0.0001$; Figure 5.1). Either separately or together, the oviposition-inducing or -inhibiting bacteria had the same positive effect on the survival of larvae. These results support our former hypothesis (Lam *et al.*, 2007) that oviposition-inhibiting bacteria are not harmful to larval offspring; rather, their presence indicate to female flies where and when to deposit their complement of eggs.

At doses of $\leq 10^5$ CFUs, the effect on larval development varied with the bacterial group(s) applied, and the effect became inconsistent (Figure 5.1). The finding that at

doses of 10^2–10^4 CFU per plate the egg-wash bacteria (treatment E) had a more positive effect on larval development than any other group(s) of bacteria suggests that some bacterial strains that were not present in treatments B, C, or D better supported larval development. Alternatively, nutrients washed off the egg surface may have been responsible for the enhanced effect of treatment E. The overall effects of bacterial supplementation on larval development diminished with decreasing numbers of CFUs (Figure 5.1), suggesting that the abundance of bacteria in natural resources must remain above a critical threshold to sustain development of house fly larvae. Conceivably, bacteria derived from house fly eggs could supplement the community already present in any oviposition site. For example, in just 24 h – the time between oviposition of eggs and hatching of larvae – the abundance of *K. oxytoca* on eggs increased by more than 10^8 CFUs (Lam *et al.*, 2007).

Other insects have been shown to deposit microorganisms that supplement larval nutrition, particularly in ephemeral resources. Females of the vinegar fly *Drosophila subobscura* Collin transfer a critical amount of yeast to oviposition substrates, thereby improving survival of larval offspring in a variety of substrates (Rohlfs & Hoffmeister, 2005). The generalist cactophilic fly *Drosophila serido* Vilela & Sene vectors various types of yeasts, with adults and larvae apparently exhibiting a different preference for types of substrate and yeast (Morais *et al.*, 1994). Finally, the gall midge *Schizomyia galiorum* Kieffer uses a needle-like ovipositor to inoculate host plants with fungal conidia that are essential for gall formation and larval nutrition (Rohfritsch, 2008).

Our data support the hypothesis that bacteria associated with house fly eggs may supplement the diet of hatching and developing larval offspring. Having already

demonstrated that these bacteria affect oviposition decisions by female house flies (Lam *et al.*, 2007), and suppress the growth of fungal competitors on larval resources (Lam *et al.*, 2009), egg-associated bacteria obviously serve multifunctional roles.

5.5 References

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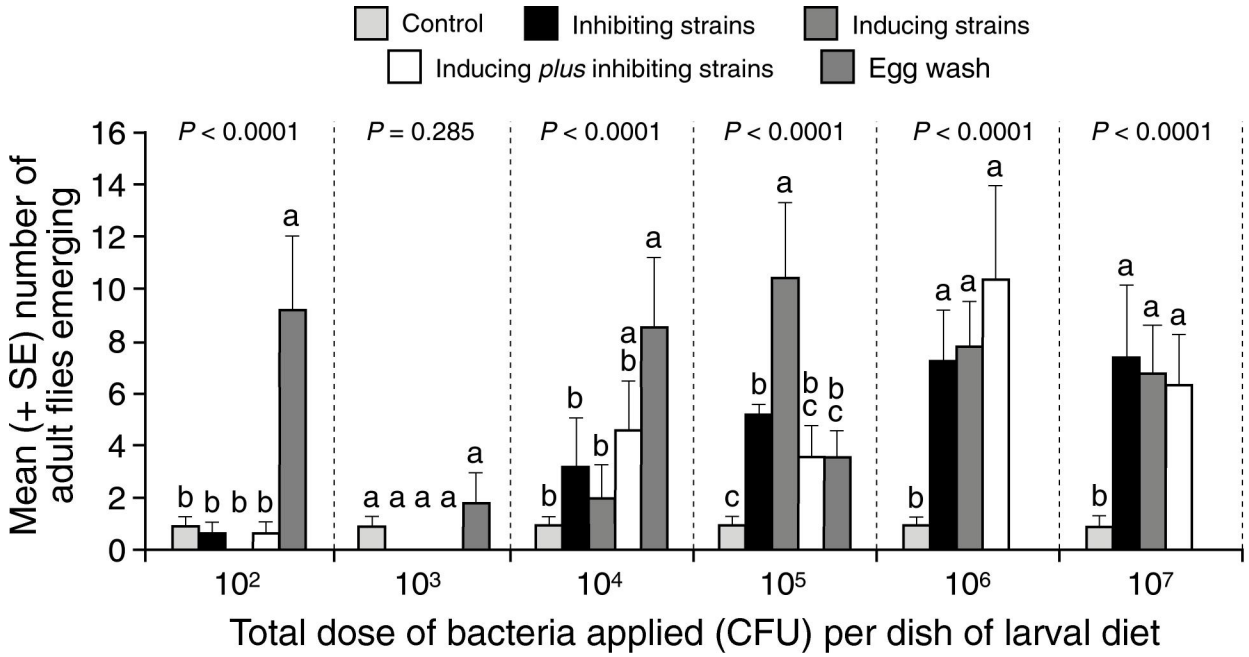
5.6 Figures

Fig. 5.1 Mean (+SE) number of house fly, *Musca domestica*, larvae completing development to adults in response to the presence or absence of bacteria isolated from *M. domestica* eggs. The bacterial species belong to treatment groups described in Table 5.1. For each exposure dose (n = 5 per treatment), data were subjected to analysis of variance followed by a Tukey's HSD multiple comparison test. Within each dose, *P*-values refer to ANOVA statistics and different letters above bars indicate a statistically significant difference (Tukey's HSD multiple comparison, $\alpha = 0.05$). The maximum dose tested for treatment E (Table 5.1) was 10^5 colony-forming units.

Fig. 5.1


Control
Inhibiting strains
Inducing strains
Inducing *plus* inhibiting strains
Egg wash
Mean (+ SE) number of adult flies emerging
P < 0.0001
P = 0.285
P < 0.0001
P < 0.0001
P < 0.0001
P < 0.0001
Total dose of bacteria applied (CFU) per dish of larval diet

5.7 Tables

Table 5.1 Bacteria isolated from eggs of house flies, *Musca domestica*, and applied to sterile larval diet, just prior to the placement of sterile *M. domestica* larvae, for assessment of nutritional bacterial effects on larval development (Figure 5.1)

Table 5.1

Treatments tested[1,2,3]

A.	Sterile distilled water (bacteria-free control)
B.	Bacteria inhibiting oviposition by house flies:[4] *Chryseobacterium gleum* (Holmes et al.)/ *indologenes* (Yabuuchi et al.) Vandamme et al. *Comamonas* sp. *Klebsiella oxytoca* (Flugge) Lautrop *Pseudomonas fulva* Iizuka and Komagata
C.	Bacteria inducing oviposition by house flies:[4] *Acinetobacter baumannii* Bouvet and Grimont *Acinetobacter radioresistens* Nishimura et al. *Acinetobacter* sp. *Bacillus cereus* Frankland and Frankland *Bacillus fusiformis* Meyer and Neide *Staphylococcus saprophyticus* (Fairbrother) Shaw et al. *Staphylococcus sciuri* sensu stricto *sciuri* Kloos et al. *Staphylococcus xylosus* Schleifer and Kloos
D.	All bacteria of B and C
E.	Bacteria in egg wash (positive control), containing most of the bacterial isolates used in treatment D plus some additional bacteria that could not be cultured.

[1]The total dose of bacterial isolates tested ranged from 10^2 to 10^7 colony-forming units (CFUs) per plate of larval diet.
[2]For each dose, the total number of CFUs was identical for each treatment (except the control not containing bacteria).
[3]Treatments B–D were prepared using an equal number of CFUs of each bacterial strain.
[4]Data from Lam *et al.* (2007).

6: Vertical transmission of *Klebsiella oxytoca*, a multi-functional mutualistic symbiont of house flies, *Musca domestica*[**]

6.1 Abstract

In some mutualistic symbioses, vertical transmission of microbial symbionts must evolve for hosts to specialize on the most effective symbiont strains, and for subsequent mutualistic coevolution to occur. *Klebsiella oxytoca* plays several important and specific roles as a bacterial symbiont of house flies, *Musca domestica.* It serves as an ovipositional cue for conspecific flies, an antifungal agent, and as a nutritional supplement for house fly larvae. The multiple fitness benefits imparted by *K. oxytoca*, and its close association with house flies, strongly suggest that *K. oxytoca* is vertically transmitted by house flies. Here, we applied green fluorescent protein (pEGFP)-expressing *K. oxytoca* to the surface of house fly eggs. When we reared these eggs to adults in a sterile environment, we demonstrated that hatching house fly larvae obtain GFP-*K. oxytoca* from the egg surface and retain it internally from the larval to the adult stage. *Klebsiella oxytoca* was present on eggs deposited in crevices accessible only by the flies' extended ovipositor, suggesting that eggs are coated with *K. oxytoca* before or during oviposition. While horizontal transmission of *K. oxytoca* by house flies is likely prevelant, our laboratory data suggest that vertical transmission alone is sufficient for

[**] This chapter is presented in manuscript form to be submitted for publication with authors as follows: Lam, K., Pinto, L., Labrie, A., Tsang, M., Moore, M., and Gries, G.

perpetuating this symbiotic association. Further research is needed to conclusively show how *K. oxytoca* is transmitted between all stages of house fly development.

Keywords: House flies, *Musca domestica, Klebsiella oxytoca*, vertical transmission, oviposition, symbiotic bacteria.

6.2 Introduction

Mutualistic microbial symbionts affect the ecology and fitness of many living organisms. For example, symbiotic gut microflora of herbivorous animals digest plant cellulose (Anand and Sripathi, 2004; Warnecke *et al.*, 2007; Toyoda *et al.*, 2009). Symbiotic viruses and yeast-like microorganisms of parasitoids suppress the immune systems of the parasitoids' hosts (Dupas *et al.*, 1996; Renault *et al.*, 2002; Kim *et al.*, 2004; Amaya, 2005, Lawrence & Matos, 2005). Larval offspring of fruit flies, and of flies that oviposit in other plant and organic materials, obtain food supplements from symbiotic yeasts (Morais *et al.,* 1994; Rohlfs & Hoffmeister, 2005). Cultivated fungal 'crops', the primary food for leafcutter ants (Mikheyev *et al.*, 2007), are kept mould-free by symbiotic bacteria (and their antimicrobial products) associated with the ants (Caldera *et al.*, 2009; and references therein). Furthermore, microbial symbionts produce semiochemical cues that result in aggregated oviposition in onion maggot flies, *Delia antiqua* (Judd and Borden, 1992), and density-dependent repellence in mountain pine beetles, *Dendroctonus ponderosae* (Ryker & Yandell, 1983).

Mutualistic symbionts can be acquired by individual hosts through vertical and/or horizontal transmission. For example, *Burkholderia* spp. are mutualistic bacterial symbionts in the midgut of both *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae) (Kikuchi *et al.*, 2005). They can either be transmitted vertically from one generation of hosts to the next or horizontally between different populations, and even across species, when they live freely in soil environments (Kikuchi *et al.*, 2005).

While horizontal transmission of symbionts is often possible, particularly for hosts inhabiting nutrient- or microbe-rich environments, vertical transmission is

necessary for hosts to specialize on the most effective symbiont strains, and for mutualistic coevolution to occur between hosts and microbial symbionts (Douglas, 1998). This theoretical concept is best conveyed by quoting Douglas (1998), as follows: "In symbioses with horizontal transmission, hosts can generally form associations with a broad range of symbionts, including taxa from which they derive little or no benefit. Hosts that specialized on the most effective symbionts would be selected against if these symbionts were rare in the environment. There is, however, one route by which this selection pressure against specialization may be circumvented: vertical transmission. A host lineage in which symbionts are transmitted faithfully from parent to offspring has 'captured' an effective symbiont taxon. Further, the vertically transmitted symbionts are generally under selection pressure to maintain and enhance their effectiveness because they are dependent on the sustained performance, especially fecundity, of their host."

We have shown that *Klebsiella oxytoca* is a key bacterial symbiont on the surface of house fly, *Musca domestica* L. (Diptera: Muscidae), eggs (Lam *et al.*, 2007, 2009a, b). Females lay their eggs on fleeting organic resources such as animal feces (Keiding, 1974) and eggs hatch within 24 h and develop through three instars in 5-8 d. After a 5- to 8-d pupal period, adult males and females eclose. The fitness of house flies is enhanced by *K. oxytoca* in several ways. As *K. oxytoca* proliferates on egg surfaces, its presence and abundance signals to other gravid female house flies the progressive aging of eggs, and thus an increasing probability of larval and egg cannibalism (Lam *et al.*, 2007). *Klebsiella oxytoca* also inhibits the growth of many fungi on feces, thereby protecting larvae from both scramble-type competition for resources (Lam *et al.*, 2009a) as well as the larvicidal compounds produced by some fungi (Zvereva, 1986). Moreover, *K.*

oxytoca supplements the diet of house fly larvae in oviposition sites that lack appropriate bacteria for consumption (Lam *et al.*, 2009b).

Klebsiella oxytoca is found on eggs of house flies from several geographical locations in North America, and is associated with house fly colonies that have been laboratory-reared for several decades (Lam *et al.*, 2007). This close association, along with the multiple benefits that *K. oxytoca* already provides, led us to predict that vertical transmission of *K. oxytoca* is an essential life history trait of house flies. Furthermore, because house flies oviposit on, and larvae develop in, many different types of ephemeral resources with rapidly changing physical, chemical and microbiological conditions (Larsen *et al.*, 1966; Bradley & Sheppard, 1984; Amano, 1985; Lysyk & Axtell, 1987), we further predict that house flies internally retain *K. oxytoca* from eggs to adults without reliance upon re-acquiring it from their environment or through horizontal transmission.

Even though female house flies often deposit eggs in a narrow crevice that is accessible only by their extended ovipositor, we predict that they are still capable of transferring *K. oxytoca* to eggs.

In this study, we tested the hypotheses (1) that *K. oxytoca* is obtained from the surface of eggs by hatching larvae and retained internally from the larval to the adult stage; and (2) that it is transferred from female adults to eggs, even within deep crevices. To test these hypotheses, we constructed a strain of *K. oxytoca* that constitutively expresses green fluorescent protein so that we could track its occurrence through the developmental stages of flies.

6.3 Methods and results

6.3.1 Experimental Insects

Adult house flies from laboratory stock were kept in cages at 50-80% relative humidity (RH), 22-30°C and a 16:8 h light:dark regime, and were provided with water, sugar cubes and skim milk powder *ad libitum*. House fly eggs were collected on 50-mm glass Petri dishes containing sterile skim milk agar (SMA; 15 ml water, 0.12 g nutrient broth powder, 0.24 g skim milk powder, and 0.3 g agar) and reared to adult flies on an artificial diet prepared from wheat bran (400 g), brewers yeast (15 g), molasses (15 ml) and water (700 ml), with a supplemental protein paste prepared from skim milk powder and water (Lam *et al.*, 2007).

6.3.2 Hypothesis 1: *K. oxytoca* is obtained from the surface of eggs by hatching larvae and retained internally from the larval to the adult stage

6.3.2.1 Methods

General experimental design

In experiment 1 (n = 3), we tested whether *K. oxytoca* on the surface of house fly eggs is obtained by hatching larvae and retained internally through larval, pupal and adult stages. The experiment was designed to determine whether vertical transmission alone is sufficient to perpetuate the symbiotic association between house flies and *K. oxytoca*.

To ensure that the *K. oxytoca* in all developmental stages originated from the egg surface, we transformed *K. oxytoca* to GFP-Ko with a plasmid containing the enhanced green fluorescent protein gene (pEGFP) (see below). We then replaced egg-surface bacteria with GFP-Ko, and reared the eggs in a sterile environment. We also reared GFP-Ko-free control eggs to ensure that GFP-Ko was not introduced to either cohort of flies

after the initial inoculation. Replicates in which the sterility of the flies was compromised, as indicated by GFP-Ko in control flies, were discarded (see Results and Discussion).

Production and isolation of pEGFP+kan plasmids

The pEGFP plasmid in *Escherichia coli* ATCC 33456 was generously provided by Dr. Eric Gilbert (Department of Biology, Georgia State University, Atlanta, GA, USA). It is a pUC19-based vector that encodes ampicillin resistance and a red-shifted *egfp* gene. *E. coli*–pEGFP was grown in 50 mL of Luria-Bertani broth (LB; 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1L dH_20, pH 7.5, autoclaved at 121°C for 25 min) with 100 µg/ml ampicillin at 37°C and 250 RPM for 18 h. The pEGFP plasmid was isolated from 5 mL of this broth culture, using the GeneJET Plasmid Miniprep kit (Fermentas Life Sciences, Burlington, ON, Canada).

Our house fly-derived *K. oxytoca* (strain SFU-1; Lam *et al.*, 2007) was ampicillin-resistant but sensitive to kanamycin when grown on LB Agar with 50, 100, 250, or 500 µg/ml of antibiotic. Therefore, for positive selection, a kanamycin-resistance cassette was ligated into the EcoR1 site of pEGFP. The kanamycin resistance gene and promoter were amplified from the plasmid pCR2.1 (Invitrogen, Burlington, ON, Canada) by PCR using forward primer CCGAATTCAGCTTGCAGTGGGCTTACAT, reverse primer CCGAATTCAAAGGGAATAAGGGCGACAC (Eco RI restriction sites underlined) and TAQ Polymerase (Fermentas Life Sciences). The PCR product, cut with Eco RI, and the pEGFP plasmid, cut with EcoR1 and treated for 30 min with shrimp alkaline phosphatase (Fermentas Life Sciences), were ligated with T4 ligase (Fermentas Life Sciences) following the manufacturer's protocol. A control ligation was performed without insert

DNA. A 10-µL aliquot of the undiluted ligation product was used immediately to transform 150 µL of chemically competent *E. coli* DH5α. Controls included transformation of *E.coli* using 10 µl water with no insert ligation. The transformation mixtures were diluted with 1 ml LB and the cells were allowed to recover with gentle shaking at 37°C before plating on LB and LB + kanamycin (30 µg/ml).

Kanamycin-resistant colonies were screened for fluorescence on a Zeiss Axioskop 2 plus microscope equipped with a Colibri LED light source for epifluorescence, using 470 nm incident light. The brightest clone was selected as the source for plasmid DNA pEGFP-kan. The pEGFP-kan plasmid was isolated from *E. coli* as noted above, and used to transform chemically competent *K. oxytoca* Strain SFU-1.

Transformation of *K. oxytoca* to GFP-Ko with pEGFP

As there is no protocol in the literature describing the production of chemically competent *Klebsiella*, we followed the method usually used for *E. coli* (Sambrook *et al.*, 1989). To produce competent *K. oxytoca* cells, a 5-mL overnight preculture (kept at 37°C and 250 RPM) was used to inoculate 100 mL of LB broth, which was incubated at 37°C and 250 RPM until log phase (OD_{600} = 0.45-0.55). The cultured cells were chilled on ice, pelletted (3500 g for 10 min in a chilled centrifuge), and re-suspended in 50 mL of ice-cold 100-mM $CaCl_2$. The tubes were kept on ice for 30 min with occasional swirling, then centrifuged at 3000 G, and decanted carefully. The cells were re-suspended in 1 mL of ice-cold 100-mM $CaCl_2$ containing 15% glycerol, and kept on ice for 1 h before snap-freezing in 100-µl aliquots in liquid nitrogen, for subsequent storage at -80°C. The aliquots of competent *K. oxytoca* cells were then transformed with either 4 or 40 ng of pEGFP+kan plasmid, using a 105-s heat shock (at 38, 38.9, 39.6, 40.5, 41.2 or 42°C).

Almost every plasmid-concentration + shock-temperature combination resulted in successful transformations, but the most brightly-fluorescent, kanamycin-resistant strain of transformed *K. oxytoca* (GFP-Ko) (Figure 6.1) was made with 4 ng pEGFP-kan plasmid and a 42°C heat shock.

Application of GFP-Ko to surface-sterilized egg masses

A house fly egg mass was collected overnight (~14 h) from hundreds of house flies on sterile SMA (see above) and was surface-sterilized by rinsing, in sequence for 12 min each, with a dilute bleach solution (0.026 g/l NaOCl) and sterile water (Lam *et al.*, 2009b). Two sterile glass jars (100 ×170 mm) containing sterile larval diet [prepared from autoclaved wheat bran (100 g; two liquid cycles for 45 min at 121°C); an autoclaved mixture of brewers yeast (6.5 g), molasses (4 mL), and water (350 ml); and 10.5 mg of kanamycin to facilitate retention of the pEGFP+kan plasmid by GFP-Ko] were prepared, and ~5,000 surface-sterilized house fly eggs were buried 5 cm deep in the diet of each jar. This large number of eggs was deemed necessary to compensate for the anticipated high insect mortality (~90%) resulting from the sterilization processes and the addition of kanamycin to the water supplies of larvae and adults.

Before the sterilized (treatment) egg mass was placed into a jar, it received 2.5×10^6 CFU (colony forming units) of GFP-Ko suspended in 0.1 mL of LBK broth. For this application, GFP-Ko was grown overnight in 80 ml LBK broth at 37°C and 250 RPM, then measured via OD_{600}, and diluted with fresh LBK. The amount of *K. oxytoca* found naturally on the surfaces of house fly eggs increases rapidly over time, from 3.78×10^6 CFU per 0.2 g of 0-h-old eggs to 1.4×10^8 CFU per 0.2 g of 24-h-old eggs (Lam *et al.*, 2007). The application dose of GFP-Ko equates with that of *K. oxytoca* expected on a

similar number of living house fly eggs at the same age (Lam *et al.*, unpubl. data). The sterilized control egg mass received 0.1 mL of sterile LBK broth. Jars with treatment or control egg masses received two tablespoons of a thick paste made from pasteurized skim milk powder and sterile dH_2O, and were then covered with two layers of autoclaved paper towel tightly fastened with several rubber bands.

Jars with larval diet were placed in a running/closed Class II Type A2 biological safety cabinet (NuAire Inc., Plymouth, MN), maintaining a sterile environment for one week. From each jar, pupae were removed, surface-sterilized (submerged in 70% ethanol for 15 min and rinsed with sterile distilled water), and transferred to separate autoclaved glass jars (100 × 170 mm) with an autoclaved wire-mesh top. Isolation and surface-sterilization of pupae ensured that any *K. oxytoca* on or in adult female flies originated from within pupae, and were not acquired by adults as they emerged from the larval diet. Adult flies were provisioned with sterile water (containing 30 μg/ml kanamycin), autoclaved sugar, and pasteurized skim milk powder (sterility confirmed via plating on nutrient broth agar) *ad libitum*. Jars housing adult flies were held in the biological safety cabinet at room temperature for two additional weeks, after which egg collection was attempted on sterile SMA.

Monitoring presence and abundance of GFP-Ko in developmental stages of house flies

After an egg mass had been treated with GFP-Ko or sterile LBK, three insects (larvae, pupae or adults) were removed from each jar each week. To quantify the number of CFUs of GFP-Ko on the insects' integument, they were shaken in nutrient broth (NB: 1 ml water, 0.008 g nutrient broth powder), and washes were plated on LBKA as a 20× dilution series. To isolate bacteria from the interior of insects, they were surface-

sterilized (submerged in 70% ethanol for 15 min and rinsed with sterile distilled water), homogenized with a sterile glass rod in 1 mL of sterile NB, and a 20× dilution series of the homogenate was plated on LBKA. Bacterial colonies resembling GFP-Ko in colony morphology and colour were counted visually, and wet mounts of these bacteria were evaluated for bright-green fluorescence, using a fluorescent microscope with 470 nm incident light. The number of CFUs of GFP-Ko recorded in the three washes or homogenates were averaged, and each average was used as a single data point. Three independent data points (n=3) were run for each treatment and control pair at each developmental stage.

When treatment and control adult flies were 28 d old, their eggs were collected separately overnight on SMA, washed, and then homogenized at a concentration of ~ 300 eggs/ml of NB. The wash and homogenate were plated separately on LBKA as a 20× dilution series.

6.3.2.2 Results

In three replicates, GFP-Ko was absent from all control jars and present in homogenates of larvae, pupae, and gravid female flies in all treatment jars. Adults of only one of these three replicates survived sufficiently long to lay eggs (see discussion). This was likely due to either the toxicity of Kanamycin in both the larval media and water supply of adults, or in-jar crowding of adults. Treatment females of this one replicate laid eggs with abundant GFP-Ko on their surface, whereas control females did not (Figure 6.2).

In four additional replicates, control jars were accidentally contaminated with GFP-Ko during the handling or incubation of house fly eggs in larval media.

Consequently, GFP-Ko was detected in homogenates of larvae, pupae, and gravid female flies in both treatment and control jars. In two of these four replicates, female flies from both treatment and control jars laid eggs that were covered with GFP-Ko.

6.3.3 Hypothesis 2: *K. oxytoca* is transferred from adult female flies to eggs

6.3.3.1 Methods

In experiment 2 (n = 6), we tested whether *K. oxytoca* is present on house fly eggs deposited in sterile crevices so narrow and deep that they are accessible only by the flies' extended ovipositor (Figure 6.3) and not by their feet or any other body part. Under sterile conditions, SMA (20 ml) was poured into glass Petri dishes (90 mm ID ×15 mm). A 16-mm diam. well was cut into the centre of the solidified SMA using a cork borer. Glass capillary tubes (5 μm ID × 20 mm) were slid underneath the agar around the well to create narrow horizontal crevices for oviposition (Figure 6.3). Autoclaved wire mesh separated ten gravid female house flies in the upper portion of the Petri dish from the agar, such that only their extended ovipositors could reach into the horizontal crevices. The flies were given 9 h to oviposit, after which the agar was carefully peeled back to retrieve only those eggs >2 mm within the crevice. These eggs were washed by shaking them vigorously in 0.3 ml of NB, and a 20× dilution series of this wash was plated on SMA. Colonies resembling *K. oxytoca* in colony morphology and colour were identified with 16S rRNA sequencing (using the 27f and 519r primer set; Lane 1991) and API 20E biochemical identification systems (Biomerieux, France). Due to variation in the total number of eggs deposited in each replicate, the amounts of *K. oxytoca* were adjusted to 0.2 g of eggs.

6.3.3.2 Results

In all six replicates of experiment 2 (Figure 6.4), *K. oxytoca* was recovered from house fly eggs that were deposited deep within horizontal crevices that could be reached only by extended ovipositor (Figure 6.3), suggesting that *K. oxytoca* is applied to eggs prior to or during oviposition.

6.4 Discussion

The consistent presence of GFP-Ko in homogenates of surface-sterilized larvae, pupae and adult flies that were raised from surface-sterilized eggs treated with GFP-Ko (Figure 6.2), support our hypothesis that *K. oxytoca* can be transmitted vertically by its house fly host. Based on our laboratory data, hatching larvae can obtain *K. oxytoca* from the egg surface, and can retain it internally through larval, pupal and adult stages (Figure 6.2) without having to re-acquire it from their environments. We also found that *K. oxytoca* can be transferred from gravid female flies to eggs, even when only the extended ovipositor can come in contact with the deposited eggs (Figures 6.1-6.3). These results suggest that vertical transmission alone is sufficient for perpetuating the symbiosis between *K. oxytoca* and house flies, even when external/horizontal sources of *K. oxytoca* are not available.

GFP-Ko was consistently found in every developmental stage of treatment insects in every replicate of experiment 1 (Figure 6.2). The large error bars reflect the small number of successful replicates[††] and the variability in the amount of bacteria present, but not the absence of GFP-Ko from treatment jars of any replicate. The variability in

[††] additional replicates will be completed prior to preparing this chapter as a manuscript

bacterial concentration may have been due to slight differences in physical factors between replicates such as temperature and humidity, which were difficult to control in the biological safety cabinet. It may also have been caused by biological factors such as the unexpectedly large proportion of house fly eggs and pupae that survived the surface-sterilization processes, which in turn caused overcrowding of larvae and adult flies.

Contamination of control eggs/larvae with GFP-Ko occurred in four additional replicates of experiment 1, which greatly reduced the number of useful replicates. However, detection of GFP-Ko in fly larvae, pupae, adults and next-generation eggs from both treatment and control jars of all four replicates offers further evidence that GFP-Ko can be transmitted vertically, even when it is introduced accidentally at a very low dose. Nevertheless, more contamination-free replicates will be needed to substantiate the data.

The high incidence of mortality of adult flies in experiment 1 was likely caused by kanamycin in the flies' water supply. In three additional replicates (data not shown), we found that a 50% reduction in kanamycin concentration increased the rate of house fly survival to the extent that we could collect eggs from females in all three replicates. This change in kanamycin concentration should be implemented for all future replicates.

Klebsiella oxytoca was present on house fly eggs even when they were oviposited in sterile crevices that could be accessed only by the flies' extended ovipositor. Hence, we propose that female house flies transfer *K. oxytoca* either prior to or during oviposition, via some unknown mechanism. GFP-Ko was detected in the flies' feces (data not shown). Therefore, ovipositing flies may defecate onto their eggs if they are not obstructed by physical constraints of the oviposition site. Defecating on eggs or covering deposited eggs with frass (a combination of feces and plant material) is quite common in

the Insecta. For example, frass covers on eggs reduce the level of wasp parasitism in andromeda lace bugs, *Stephanitis takeyai* (Tsukada, 2000), decrease oviposition by conspecifics in Japanese pine sawyer beetles, *Monochamus alternatus* (Anbutsu and Togashi, 2002), and facilitate embryonic development of eggs of the leaf-feeding beetle *Weiseana barkeri* (Nahrung and Marohasy, 1997).

The mechanism for transferring symbiotic microorganisms between host stages and generations varies with each symbiotic relationship. For example, queen leafcutter ants carry pieces of maternal fungal gardens to start new gardens of their own (Mikheyev *et al.*, 2007). Some beetles and *Sirex* wood wasps have specialized organs (mycangia) that retain specific fungi essential for killing and exploiting host trees (Farrell *et al.*, 2001; Francke-Grosmann, 1939; Whitney & Farris, 1970). Inoculated into host trees by parent insects, these microorganisms are re-acquired by the mycangia of next-generation adults that carry them to new hosts (Raffa, 1988). Females of various insect species, including cockroaches, aphids and leafhoppers, may transmit symbiotic microorganisms transovarially (Kellner, 2002).

To understand exactly how *K. oxytoca* is vertically transmitted by house flies, further research is required to determine the structure(s) that retains *K. oxytoca* in gravid female house flies and the mechanism(s) for depositing *K. oxytoca* on eggs before or during oviposition. Immunohistochemical staining of sections of fixed house fly adults may be a useful next step.

We have previously shown that house flies benefit from *K. oxytoca* because it indicates the age of deposited eggs (Lam *et al.*, 2007), suppresses growth of competitive fungi (Lam *et al.*, 2009a), and supplements the larval diet (Lam *et al.*, 2009b). In turn, *K.*

oxytoca may benefit from its association with house flies in several ways. The surface and nutritional composition of house fly eggs support bacterial proliferation (Lam *et al.*, 2007). Furthermore, hatching larvae spread *K. oxytoca* throughout the resource and bring it into contact with new nutrients (Lam *et al.*, unpubl. data). Finally, dispersal by adult flies facilitates the spread of *K. oxytoca* to a wide range of geographic locations.

While we have found evidence that *K. oxytoca* may be transmitted vertically, it is likely also transmitted horizontally. Female house flies often aggregate during oviposition (Jiang *et al.*, 2002), which would allow bacterial transfer between adjacent eggs. Consequently, hatching larvae could acquire *K. oxytoca* not only from their own female parent but also from other parents. While tunnelling through the resource, larvae could distribute and ingest *K. oxytoca* of diverse origins. Cannibalism of younger larvae by older larvae would be yet another mode of horizontal transmission. Even if all of the above modes of horizontal transmission of *K. oxytoca* were absent, house flies can still maintain their association with *K. oxytoca* through vertical transmission alone (Figure 6.2). The sterile laboratory conditions of experiment 1 differed from the flies' natural environment, but were necessary to prove that vertical transmission can occur without external sources of *K. oxytoca*.

In summary, we present evidence that house flies can transmit *K. oxytoca* vertically without reliance on horizontal transfer. However, further experimentation is needed to understand the underlying mechanisms of this vertical transfer. Vertical transmission is expected in a mutualistic symbiosis, particularly when ovipositional resources of the insect host vary and make horizontal transfer potentially unreliable, and when the symbiont, such as *K. oxytoca,* offers multi-functional benefits to the insect host.

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6.6 Figures

Fig. 6.1 Wet mounts of Wild-type *Klebsiella oxytoca* (**a-c**) and GFP-Ko (*Klebsiella oxytoca* transformed with a pEGFP+kan plasmid; **d-f**) viewed under a Wave FX Spinning Disc confocal microscope (Zeiss Axio Observer Z1 inverted microscope with Hammamatsu EMCCD camera) at 1000 × magnification. DIC = differential interference contrast microscopy. Fluorescent = epifluorescent microscopy with 491 nm incident light from a HAL 100 light source. Merged = an overlay of DIC and Fluorescent images.

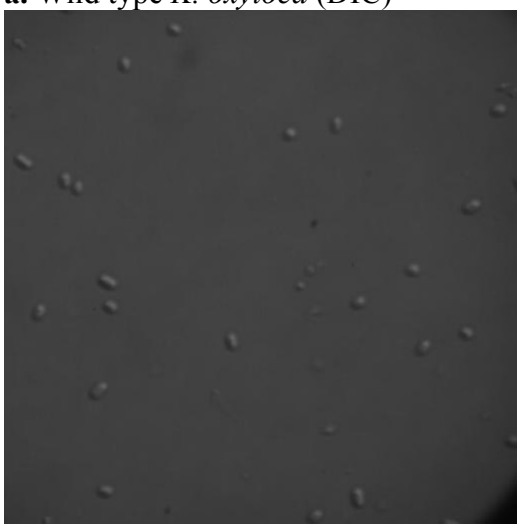
Fig. 6.2 The abundance of green fluorescent protein-*Klebsiella oxytoca* (GFP-Ko) [log (CFU)] recorded on the integument and interior of house flies in experiment 1. At each developmental stage, each data point represents the values obtained and averaged from 2-3 insects. GFP-Ko was not found on or in control insects at any stage of development, whereas GFP-Ko was found on and/or in all treatment insects. New eggs were collected from treatment and control adults of only one replicate, due to early mortality of adult flies. GFP-Ko was not found inside any eggs. CFU = colony forming units.

Fig. 6.3 Assembly for experiment 2, consisting of a plastic Petri dish (90 mm ID × 15 mm), sterile skim milk agar with a 16-mm diam. well in its centre, autoclaved wire mesh separating 10 gravid female house flies from the agar, and glass capillary tubes (5 μm ID × 20 mm) creating narrow horizontal crevices accessible only by the flies' extended ovipositors. *Klebsiella oxytoca* was consistently found on eggs collected from >2 mm within these crevices.

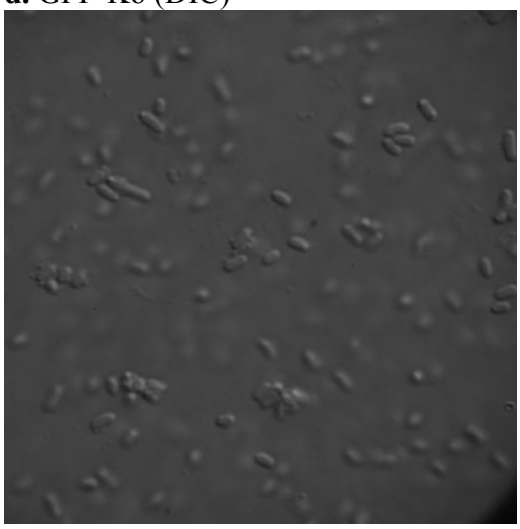
Fig. 6.4 The number of *Klebsiella oxytoca* [mean log (CFU) ± SE] recorded on the integument of house fly eggs (adjusted to 0.2 g of eggs) deposited >2 mm deep within a sterile crevice in experiment 2. CFU = colony forming units.

Fig. 6.1

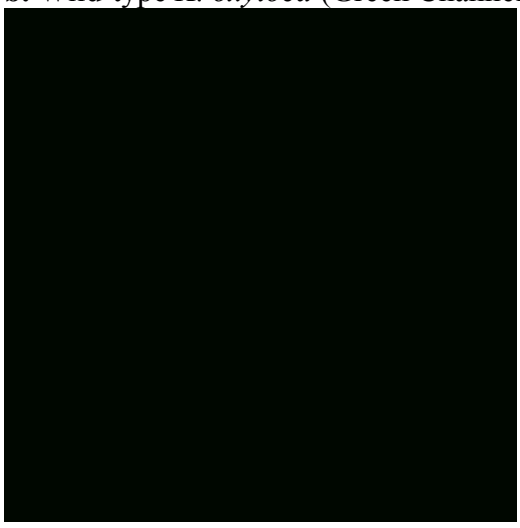
a. Wild type *K. oxytoca* (DIC)



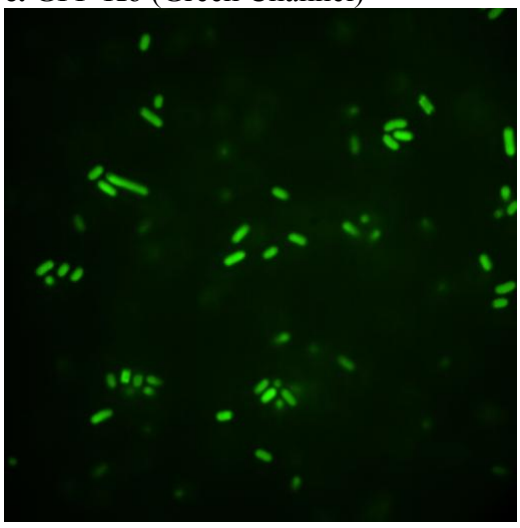
d. GFP-Ko (DIC)



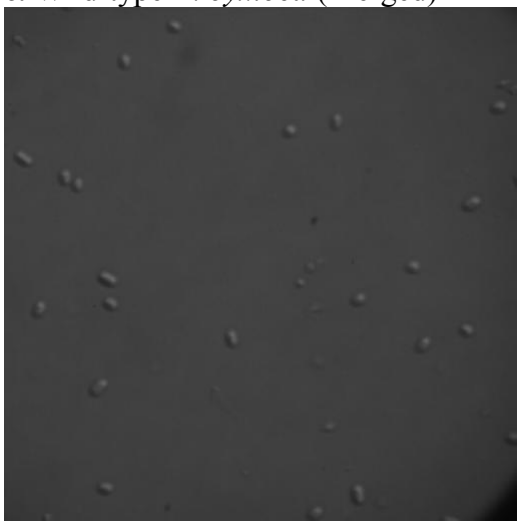
b. Wild-type *K. oxytoca* (Green Channel)



e. GFP-Ko (Green Channel)



c. Wild-type *K. oyxtoca* (Merged)



f. GFP-Ko (Merged)

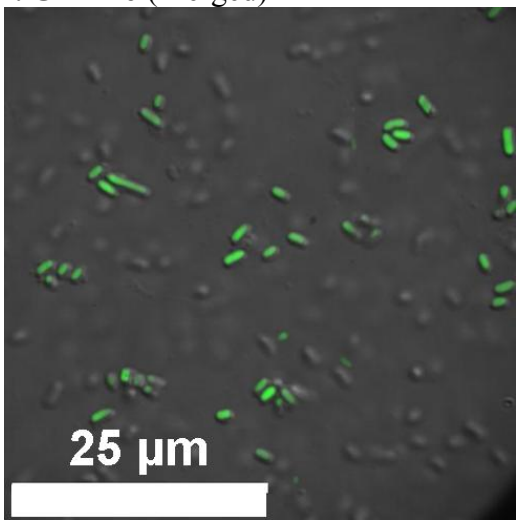


Fig. 6.2

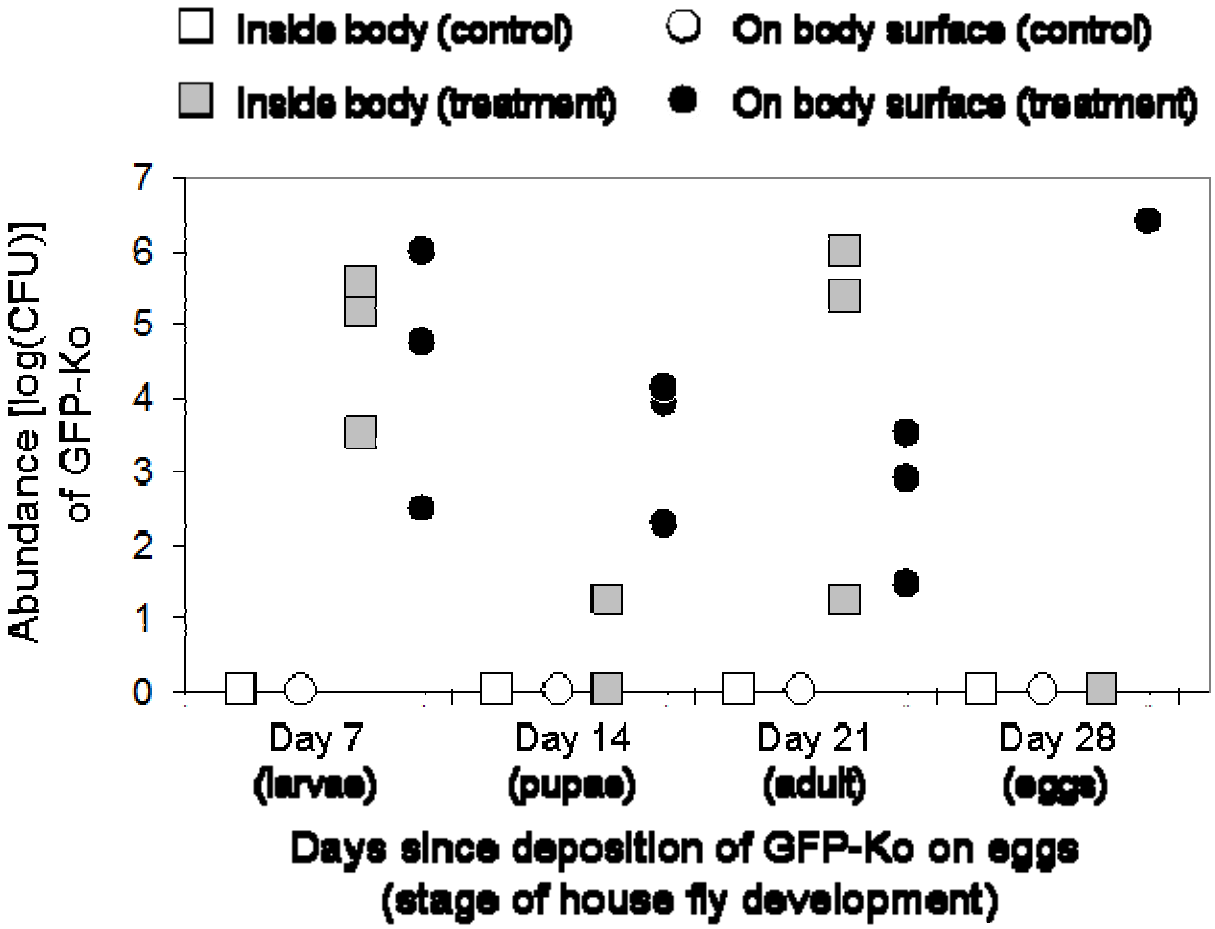

Inside body (control)
On body surface (control)
Inside body (treatment)
On body surface (treatment)
Abundance [log(CFU)] of GFP-Ko
0
1
2
3
4
5
6
7
Day 7
(larvae)
Day 14
(pupae)
Day 21
(adult)
Day 28
(eggs)
Days since deposition of GFP-Ko on eggs
(stage of house fly development)

Fig. 6.3

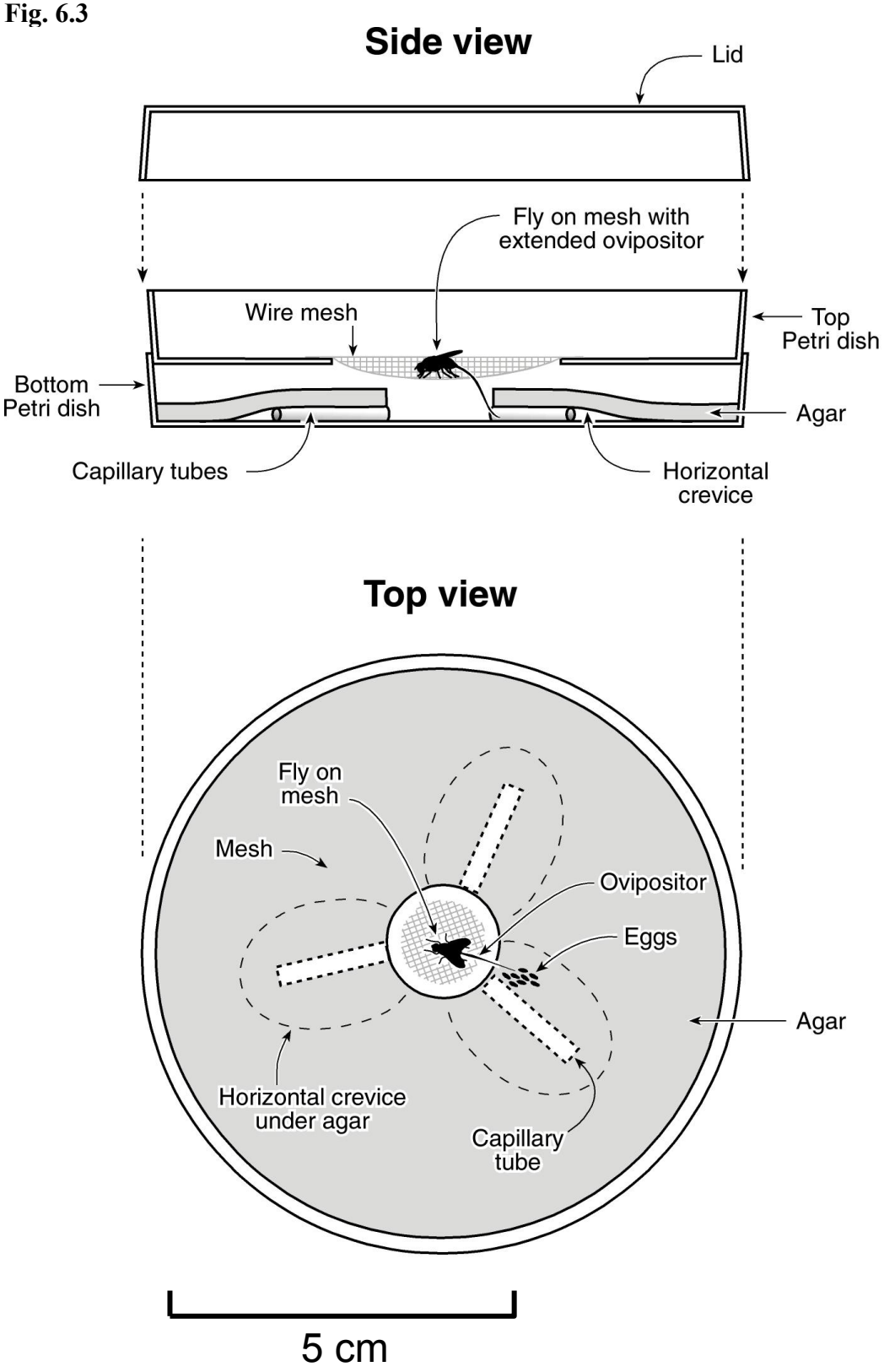
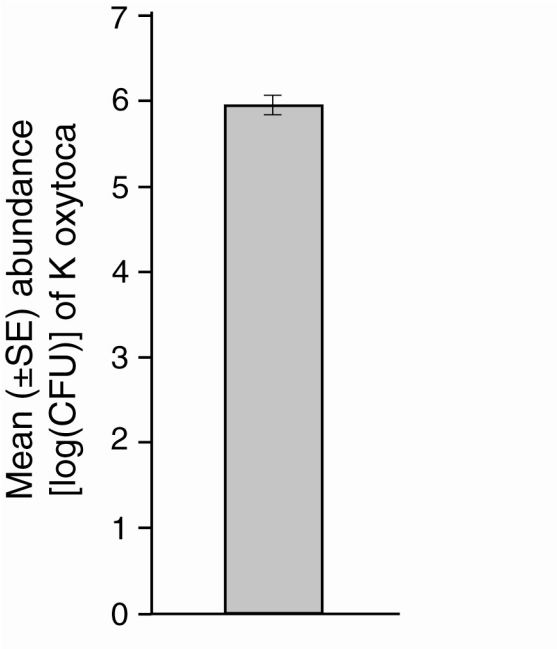

Side view
Lid
Fly on mesh with extended ovipositor
Wire mesh
Top Petri dish
Bottom Petri dish
Agar
Capillary tubes
Horizontal crevice
Top view
Fly on mesh
Mesh
Ovipositor
Eggs
Agar
Horizontal crevice under agar
Capillary tube
5 cm

Fig. 6.4



7: General Conclusions

7.1 Summary of main findings

My study of the oviposition ecology of house flies revealed that microorganisms play important roles in oviposition decisions of gravid female flies. Aggregated oviposition by house flies is adaptive because large numbers of even-aged larvae are more likely to outcompete fungal resource competitors. However, cannibalism of young house fly larvae by older larvae (Lam, unpubl. data) has detrimental fitness consequences to late-arriving adult females. Thus, I predicted that house flies have a type of cue that evolves from oviposition induction to inhibition some time after eggs are deposited on a resource. I found that the evolving cues depend upon a key bacterial species, *Klebsiella oxytoca*. *Klebsiella oxytoca* proliferates over time on the surface of deposited eggs, and at a threshold density it inhibits further oviposition. By deploying such evolving cues, female house flies can visit an oviposition site just once and deposit cues that will stimulate immediate oviposition induction of conspecifics, followed by delayed inhibition, thereby ensuring conditions conducive for offspring development.

Fungi competing with insect larvae for ephemeral resources, such as manure or carrion, can have adverse effects on larval development. When fungi establish on manure 3 days before houseflies deposit their eggs, all hatching larvae perish. However, when fungi are not established in advance, large numbers of fly larvae can outcompete fungi. Because the timing of resource colonization is such an important factor in the outcome of competition between house fly larvae and fungi, I predicted that there might be

mechanisms that suppress fungal growth even before larvae hatch. I have found that *K. oxytoca*, along with several other egg-associated bacteria, can inhibit the growth of a variety of fungal strains in chicken feces. This inhibition may be due to resource nutrient depletion and/or the release of antifungal chemicals. While some bacterial strains, including *K. oxytoca*, are able to inhibit the growth of several fungal strains, it appears that a group of different bacteria associated with house fly eggs is needed to inhibit all of the different fungi that I found in chicken feces.

When the relative timing of resource colonization strongly affects the outcome of competition, harmful competitors are often recognized and avoided. I have predicted, and experimentally shown, that well-established fungi in resources significantly reduce the number of larval offspring that complete development to adult flies. I have further predicted that house flies avoid oviposition on animal feces colonized with competitive fungi, and that the avoidance is due to fungus-derived semiochemicals. The fungal isolates (*Phoma* spp., *Fusarium* spp., *Rhizopus* spp.) from chicken feces release semiochemicals (dimethyl trisulfide and 2-phenylethanol) that significantly reduce oviposition by house flies, helping them avoid resources colonized with fungal competitors for the development of larval offspring.

Bacteria are essential dietary constituents of house fly larvae. Bacteria could either be present in the resource that parent house flies select for oviposition, or they could be deposited by female flies during oviposition and then proliferate, utilizing the resource. Bacterial deposition by female flies would ensure that hatching larvae are well provisioned with bacterial food, irrespective of bacterial abundance in the oviposition site. I have predicted that bacteria associated with house fly eggs are a nutritional

resource for house fly larvae. I have shown that significantly more larvae completed development to adults when they were provisioned with a total dose of 10^6–10^7 colony-forming units of bacteria isolated from house fly eggs. Thus, egg-associated bacteria could be a fail-safe mechanism that ensures a bacterial food supply for larval offspring, particularly if the resource selected by parent females is poor in bacterial food.

Egg-associated bacteria, in particular *K. oxytoca*, provide many benefits for house flies. I predicted that house flies can maintain and vertically-transmit these beneficial symbionts. I have shown that pEGFP-transformed *K. oxytoca,* when deposited to the surface of house fly eggs, are retained throughout larval, pupal, and adult stages, and are transferred to the surface of eggs during oviposition.

Insect-microbe systems with interactions as diverse and complex as those between house flies and their bacterial symbionts have rarely been studied. In the house fly-bacteria system, I investigated both intra- and inter-specific interactions and their mutualistic and antagonistic effects. I studied microbe-based oviposition signals and the fitness consequences of oviposition decisions. I explored the role of bacterial symbionts in ameliorating resources for larval development and curtailing the impact of fungal resources competitors. Finally, I have shed some light on the mechanisms that help maintain the long-term symbiotic relationship between house flies and their bacterial symbionts. Based on all my results and an exhaustive literature review, I conclude that there only few mutualistic symbionts known that impart so many different beneficial functions to their hosts as *K. oxytoca* does for house flies.

7.2 Future work

My research has revealed a number of aspects that warrant further investigation. For example, the mechanism by which house flies assess the abundance of *K. oxytoca* on house fly eggs is yet to be determined. Despite an exhaustive effort, I could not isolate volatile or contact chemical cues associated with *K. oxytoca*. The mechanisms by which various egg-associated bacteria inhibit the growth of manure-associated fungal strains warrant further investigation and may yield new fungistatic compounds. A broader survey of headspace volatiles released by additional feces-infesting fungi may reveal further semiochemicals that house flies exploit to avoid fungi-infested oviposition sites. Not all feces-infesting fungi inhibited oviposition by female house flies, suggesting that some fungi are more harmful than others to developing house fly larvae. It would be intriguing to explore whether harmful and harmless fungi release different volatiles blends, and adult female house flies can discern between them. It should also be explored whether egg-associated bacteria proliferate more readily in the oviposition site, and thus have a greater nutritional value for house fly larvae, than bacteria already present in that site. Finally, studying the vertical transmission of *K. oxytoca* and other beneficial bacterial strains with immunohistochemical staining of sections of fixed house fly adults may help us better understand where adult house flies carry *K. oxytoca* within their body and how they deposit it on their eggs so reliably.