How Firebrats (Thysanura: Lepismatidae) Detect and Nutritionally Benefit From Their Microbial Symbionts Enterobacter cloacae and Mycotypha microspora

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ABSTRACT The phylogenetically ancient firebrats, Thermobia domestica (Packard) (Thysanura: Lepismatidae), lack any form of long-distance communication, yet are able to locate mates in sustained hot and humid microhabitats, typically within human habitations where they feed on dried goods, including cellulosic substrates. We have recently shown that firebrats aggregate in response to two symbiotic microorganisms in their feces, the bacterium Enterobacter cloacae and the fungus Mycotypha microspora. Our objectives were to determine how firebrats detect M. microspora and E. cloacae, and whether these microbial symbionts nutritionally benefit firebrats. Applied to a glass surface in bioassays, E. cloacae as well as the isolated exopolysaccharide of E. cloacae induced arrestment of firebrats, whereas M. microspora induced arrestment only in the presence of cellulosic substrate. When M. microspora and E. cloacae were grown aerobically on cellulose agar, only M. microspora yielded zones of clearing indicative of enzymatic cellulose degradation. Firebrats also arrested in response to D-glucose, which is a constituent of the exopolysaccharide and which is produced by the cellulase of M. microspora during cellulose degradation. First- to third-instar nymphs of firebrats that were fed E. cloacae, M. microspora, or a nutrient-rich diet developed equally well. By consuming E. cloacae and M. microspora, and by spreading them through feces, firebrats appear able to occupy nutrient-poor habitats that otherwise would not support development of their offspring.

KEY WORDS firebrat, Thermobia domestica, microbial symbiont, Enterobacter cloacae, Mycotypha microspora

Microorganisms possess an array of chemicals on their cell surface (Bartnicki-Garcia 1968, Hancock and Poxton 1988) and secrete chemicals that often react with the external environment (Burns and Dick 2002). Insects are known to be attracted to, or arrested by, many of these microbe-derived semiochemicals (message-bearing semiochemicals). For example, locusts aggregate in response to the semiochemicals guaiacol and phenol produced by bacteria in their feces (Dillon et al. 2000, 2002; Dillon and Charnley 2002), flour beetles aggregate in response to triglycerides in fungal mycelia (Starratt and Loschiavo 1971, 1972), and bark beetles aggregate in response to verbensols produced by intestinal bacteria from tree-derived α-pinene (Brand et al. 1975, Byers and Wood 1981).

Many insects ingest symbionts or their metabolites that provide nutrients or enzymes and thus improve the insects’ development and survival (Woodbury 2012). For example, larvae of microbe-exposed flour beetles (van Wyk et al. 1959, Sinha 1966), grape berry moths (Mondy and Corio-Costet 2000), and gall midges (Janson et al. 2009, Heath and Stireman 2010) develop more quickly and live longer than symbiont-free larvae because of nutrients they acquire from their symbionts. Alternatively, microbial symbionts of cockroaches (Cruden and Markovetz 1979, Gijzen et al. 1994), termites (Veivers et al. 1991, Hyodo et al. 2000), and longhorned beetles (Kukor and Martin 1986; Kukor et al. 1988) possess cellulase, which provides the insect host with cellulose-derived glucose (Buchholz et al. 1983). Firebrats, Thermobia domestica (Packard) (Thysanura: Lepismatidae), are phylogenetically ancient insects that are found almost exclusively within human habitations (Brett 1962, Sweetman 1965, Zungoli 1983). They feed on dried cellulosic goods such as paper and cotton-fabrics (Austini and Richardson 1941, Mallis et al. 1958, Zinkler and Götze 1987, Treves and Martin 1994) and lack any form of long-distance communication, yet are able to locate mates and suitable habitats by aggregating on substrates that other firebrats have contacted (Tremblay and Gries 2003, Woodbury and Gries 2008). Firebrats aggregate in response to the symbiotic bacterium Enterobacter cloacae (Enterobacteriaceae) and the symbiotic fungus Mycotypha microspora (Mycotyphaceae), which they carry in their digestive tract and deposit with their feces (Woodbury and Gries 2013a,b; Woodbury et al.)
Still unknown was whether firebrats aggregate in response to cell-surface chemicals, enzymes, or enzyme metabolites of *M. microspora* and *E. cloacae*.

Adult firebrats periodically leave aggregations to acquire water, whereas early-instar nymphs typically survive and develop normally without ever leaving aggregation sites (N. Woodbury, personal observation), suggesting that *M. microspora* or *E. cloacae* may provide water or nutrients that support development or prolong survival of nymphs. Our objectives were to determine 1) how firebrats detect *M. microspora* and *E. cloacae*, and 2) whether *M. microspora* and *E. cloacae* nutritionally benefit firebrats.

**Materials and Methods**

**Laboratory Colony of Firebrats.** Firebrats used for bioassays were obtained from a colony maintained at the insectary of Simon Fraser University (SFU) at 23–35°C, 70–85% relative humidity (RH), and a photoperiod of 8:16 (L:D) h (Woodbury and Gries 2007). The colony comprised insects of mixed age and gender that were obtained from autoclave rooms and boilers around SFU.

**Digestion of Cellulose, Chitin, and Uric Acid by Microbial Enzymes.** Cellulose-, carboxymethyl cellulose-, chitin-, chitosan-, and uric acid agar were each prepared according to Hendricks et al. (1995). Each agar type consisted of K2HPO4 (0.5 g), MgSO4 (0.25 g), agar powder (20 g), and autoclaved polysaccharide or uric acid powder (1.88 g) (all obtained from Sigma–Aldrich Inc., Mississauga, ON, Canada) added to distilled H2O (1,000 ml) and autoclaved. Chitin was first acid-swollen (Nahar et al. 2004) before being added to agar. Ten grams of chitin (purified powder from crab-shells, Sigma) was suspended in chilled o-phosphoric acid (88% wt:vol) and stirred at 0°C for 1 h, at which point the solid chitin became swollen and gelatinous. The acid-swollen chitin was then washed with chilled distilled H2O, filtered, washed with NaHCO3 (1% wt:vol), and filtered once more. H2O washing and filtering were repeated until the acid-swollen chitin was neutralized to pH 7.

Each plate of polysaccharide agar or uric acid agar was inoculated with *M. microspora* and *E. cloacae*, *Aspergillus niger* (a fungus known to produce cellulase, chitinase, and uricase; serving as a positive control), or a sterile wire loop (negative control), and maintained at 35°C and 80% RH within a growth chamber (Conviron, Winnipeg, Manitoba, Canada). After 72 h, each microorganism was visually assessed for substrate utilization (Yeoh et al. 1985). Each agar plate was flooded with a 5-ml aqueous solution of L1 (1% wt:vol) and KI (2% wt:vol) mixed with 1 ml HCl (0.1 M). After 3–5 min, the iodine-stained agar was gently rinsed with running tap water for 30 s to remove excess stain. After rinsing, iodine-stained agar appeared reddish brown in color, and clear yellow zones became apparent wherever microbes had digested polysaccharide or uric acid substrates.

**Response of Firebrats to Enzymes and Enzyme–Substrate Digests.** The following enzymes or enzyme–substrate mixtures (all obtained from Sigma–Aldrich) were each dissolved in distilled water (2 ml), shaken at 30°C, applied to a microscope slide (25 mm²), and air-dried: 1) cellulose (0.03 g) from *A. niger* (EC 3.2.1.4), 2) cellulase (0.03 g) from *A. niger* (EC 3.2.1.4) shaken for 48 h with powdered cellulose (0.5 g), 3) chitinase (0.02 g) from *Streptomycetes griseus* (EC 3.2.1.14), 4) chitinase (0.02 g) from *S. griseus* (EC 3.2.1.14) shaken for 48 h with acid-swollen chitin (0.5 g), 5) uricase (0.01 g) from *Candida* sp. (EC 1.7.3.3), and 6) uricase (0.01 g) from *Candida* sp. (EC 1.7.3.3) shaken for 60 min with uric acid (0.5 g) (Cochran 1976). The response of firebrats to microscope slides inoculated with enzyme or enzyme–substrate was tested in dual-choice olfactometers consisting of two lateral Pyrex glass petri dishes connected to a central dish (all dishes 3 by 9 cm inner diameter) via a Pyrex glass tubing (2.5 by 2 cm inner diameter). This olfactometer design mimics the natural still-air shelters of firebrats. Before the start of bioassays, a folded cone of sterile filter paper (Whatman no. 1, 125 mm in diameter) was placed (tip facing central chamber) into each lateral dish to serve as an artificial shelter. The treatment microscope slide and control microscope slide (carrying plain water) were randomly assigned to each paper cone.

All experimental replicates were run at 21 ± 2°C and 30–60% RH, which are conditions conducive to the response of firebrats (Tremblay and Gries 2003; Woodbury and Gries 2007, 2008). For each replicate (n = 30–32 in each experiment), a single adult female firebrat was released into the central chamber of the olfactometer and allowed to explore all chambers. Each female was tested only once. Females were selected as bioassay insects because they exhibit the same response as males and nymphs (Tremblay and Gries 2003; Woodbury and Gries 2008, 2013) and were most abundant in the laboratory colony.

As firebrats are nocturnal foragers that arrest in shelters during daylight, each insect was released into an olfactometer during the dark period, allowing it to explore the chambers during 10 h of darkness, and to arrest in one of the shelters during 6 h of light. After this time (16 h total), the insect’s position within the olfactometer was recorded. Any insects found not to be in contact with the treatment or control cone were recorded as nonresponders. Choices of insects in each experiment were analyzed by χ² test, by using JMP software tables (SAS, Cary, NC). All experimental replicates were run at 21 ± 2°C and 30–60% RH. Olfactometers were washed with LiquiNox detergent (Alconox Inc., White Plains, NY) and were oven-dried (Pacific Combustion Engineering Co., Torrance, CA) between each bioassay. Voucher specimens were deposited in the museum collection of SFU.

**Response of Firebrats to Monosaccharides, Polysaccharides, Vitamins, Sterols, Amino Acids, or Salts.** The following chemicals (all Sigma–Aldrich) in 0.2-ml aliquots (dissolved or suspended in 0.25 ml water) were each applied to a one-eighth piece of filter paper, and
Response of Firebrats to Microorganisms on Glass Rather Than Paper Surfaces. A 0.5-ml (wet-weight) aliquot of Enterobacter cloacae (1 by 10^8 cfu) or Mycomycota microspora (1 by 10^8 cfu) was swept off the surface of a GlcNAc agar culture by using a micropatula, transferred to a microscope slide (25 mm²), and air-dried. The moist surface of sterile GlcNAc agar (free of microorganisms) was also swept and transferred to control slides. Responses of firebrats to treatments and control slides were tested in dual-choice olfactometers and analyzed as previously described.

Response of Firebrats to the Cell Membrane and to Water-Soluble Compounds of E. cloacae and M. microspora. Microbial cell-surface compounds were isolated according to the methods by Hancock and Poxton (1988). Ten micrograms (wet-weight) of E. cloacae (2 by 10^7 cfu) or M. microspora (2 by 10^7 cfu) were transferred from GlcNAc agar culture to a watch-glass, submerged in 2 ml of distilled water, and ultrasonicated for 5 min by using a Tide Buzz 50-kHz ultrasonicating probe (Black & Decker). Ultrasonicated microbial microorganisms were vortexed in 5 ml of phosphate buffer (pH 7) for 30 min and centrifuged (Allegra 64R, Beckman Coulter Inc., Mississauga, ON, Canada) at 10,000 g for 10 min at 4°C. The resulting pellet (containing disrupted cell walls, membranes, and large insoluble organelles) was collected, resuspended in 4 ml of sterile water, applied in 0.5-ml aliquots to one-eighth pieces of filter paper, and air-dried. The centrifuge supernatant was added to 20 ml of 0.5 cm² piece of filter paper inoculated with one of the following mono- and polysaccharides tested, only d-glucose GlcNAc agar to test for sterility. Each sterile egg was transferred to a sterile, transparent vial containing a 0.5-cm² piece of filter paper inoculated with one of the following autolysed diets: 1) 1 mg Heinz oatmeal cereal and moist cotton, 2) 1 mg living E. cloacae isolated from GlcNAc agar, 3) 1 mg living M. microspora isolated from GlcNAc agar, 4) moist cotton alone, and 5) 1 mg Heinz oatmeal cereal without moist cotton. All vials were maintained at 30°C and 70% RH in sterile conditions. Starting at the time of hatching, the body length of each nymph (excluding antennae and caudal filaments) was measured daily, by using the micrometer eyepiece of a dissecting microscope. Ten nymphs were exposed to each of the five experimental diets. Their lifespan and total body length after molting to third instar were analyzed by analysis of variance followed by the Tukey-Kramer multiple comparison test (P < 0.05), using JMP software (SAS).

Results and Discussion

Digestion of Cellulose, Chitin, and Uric Acid by Microbial Enzymes. When cultured aerobically, M. microspora produced zones of clearing in each of the cellulose-, chitin-, and uric acid agar after 72 h, indicating that the fungus produced cellulase, chitinase, and uricase enzymes in response to their respective substrates (Fig. 1). Aerobically cultured E. cloacae did not produce zones of clearing in any of the agar types tested, indicating that the bacteria did not produce cellulase, chitinase, or uricase (Fig. 1).

Response of Firebrats to Enzymes and Enzyme–Substrate Digests. Pure cellulase, chitinase, or uricase and substrate digest of each enzyme failed to induce arrestment of firebrats in bioassays (Fig. 1A), indicating that firebrats do not respond to microbe-derived enzymes.

Response of Firebrats to Monosaccharides, Polysaccharides, Vitamins, Sterols, Amino Acids, or Salts. Of all mono- and polysaccharides tested, only d-glucose...
induced arrestment of firebrats (Fig. 2B). A mixture of KH₂PO₄ and Na₂HPO₄ salts also induced arrestment, whereas ergosterol, cholesterol, or mixed essential amino acids did not (Fig. 2B). These results are in accordance with previous findings that firebrats consume glucose (Berger 1945, Lau–Greig 1992), and that their antennae detect glucose and salts (Hansen–Delkeskamp 2001).

Response of Firebrats to Microorganisms on Glass Rather Than Paper Surfaces. Glass surfaces previously exposed to firebrats or covered with *E. cloacae* induced arrestment of firebrats (Fig. 3). In contrast, *M. microspora* on glass surfaces did not induce arrestment of firebrats but did arrest them when placed on paper (Fig. 3). These results suggest that firebrats respond to the presence of *E. cloacae*, and to metabolites of *M. microspora* produced on cellulose substrate.

Response of Firebrats to the Cell Membrane and to Water-Soluble Compounds of *E. cloacae* and *M. microspora*. Ultrasonicated *E. cloacae* and *M. microspora* each induced arrestment of firebrats (Fig. 4A). Ultrasonication and centrifugation of *E. cloacae* and *M. microspora* each produced an insoluble pellet (containing cell membranes and cell walls) and a supernatant (containing water-soluble components) (Hancock and Poxton 1988, Ruiz–Herrera 1992). The supernatant, but not the pellet, of *E. cloacae* induced arrestment of firebrats (Fig. 4A). Neither the pellet nor the supernatant of *M. microspora* arrested firebrats (Fig. 4B).

The bacterium *E. cloacae* is known to produce enzymes under anaerobic conditions (Sami et al. 2008, Rezaei et al. 2009), but it did not produce cellulase, chitinase, or uricase aerobically on agar (Fig. 1). That *E. cloacae* induced arrestment of firebrats in the absence of any enzymatic substrate (Fig. 3) implicates the surface chemistry of *E. cloacae* to mediate the arrestment response. The surface of *E. cloacae* is known to be coated with a thick layer of colanic acid, a polysaccharide that consists of D-glucose, D-galactose, D-glucuronic acid, and L-fucose (Fig. 5) (Sutherland 1969, Rättö et al. 2006). When bacterial cells are ultrasonicated and centrifuged, colanic acid is disrupted and dissolved in the water-soluble supernatant (Ruiz–Herrera 1992), which elicits firebrat arrestment (Fig. 4A). Moreover, of the monosaccharide constituents of colanic acid, only D-glucose induces firebrat

![Fig. 2.](image-url)
arrestment (Fig. 2B), indicating that it is the D-glucose in colanic acid that mediates the arrestment response. Analogously, the cell wall of zygomycete fungi is known to possess GlcNAc, D-glucosamine, D-mannose, and D-glucuronic acid in the form of chitin, chitosan (Bartnicki-Garcia 1968, Ruiz-Herrera 1992), mucor, and mucoric acid (Bartnicki-Garcia and Reyes 1968, Miyazaki and Irino 1971, Bartnicki-Garcia and Lindberg 1972) (Fig. 5B). However, none of these monosaccharides induced arrestment responses in threrats (Fig. 2B), implying that the arrestment-mediating compound is not present on the surface of M. microspora. Instead, it seems to be associated with the enzymatic activity of M. microspora on cellulosic substrate. M. microspora produces cellulase aerobically (Fig. 1), which, in turn, releases D-glucose and/or cellulobiose (Buchholz et al. 1983). As firebrats respond to M. microspora only in the presence of a cellulosic substrate (Fig. 3), it is the cellulase or its metabolites (D-glucose or cellulobiose) that mediate firebrat arrestment. Moreover, as firebrats respond to D-glucose (Fig. 2B), but not to cellulase (Fig. 2A) or cellulobiose (Fig. 2B), it is apparently D-glucose produced by the cellulase of M. microspora that causes firebrats to arrest.

Analysis of firebrat gut contents (Zinkler and Götzke 1987) and antibiotic elimination of cellulose-producing organisms from firebrat guts (Treves and Martin 1994) demonstrated that firebrats produce their own endogenous cellulases that enable them to acquire glucose from cellulose. Therefore, it is more beneficial for firebrats to acquire glucose than to acquire cellulase. This would explain why firebrats arrested in response to glucose (Fig. 2B), the glucose-rich portion of E. cloacae (Fig. 4A), and glucose produced by M. microspora on paper (Fig. 3), but not in response to cellulase (Fig. 2A).

Micorales fungi closely related to M. microspora are known to contain large quantities of phosphate within their hyphae (Bartnicki-Garcia and Nickerson 1962, Datema et al. 1977). Phosphate salts caused arrestment of firebrats (Fig. 2B), suggesting that phosphates contained within M. microspora contribute to firebrat arrestment. Why cellulase-exposed cellulose did not arrest firebrats (Fig. 2A) remains unknown. Cellulase disassociated from M. microspora may have experienced suboptimal conditions for enzymatic activity, or the concentration of cellulase was too low to produce glucose at a quantity sufficiently high for firebrats to respond.

Growth and Survival of Axenic Firebrats Provisioned With a Nutrient-Rich Diet, or With E. cloacae or M. microspora, With and Without Access to Water. Firebrat nymphs deprived of water or nutrients had significantly shorter bodies at third instar than nymphs fed E. cloacae or M. microspora (Fig. 6A). Nymphs provided with E. cloacae or M. microspora, or with

<table>
<thead>
<tr>
<th>Test stimulus</th>
<th>Number of females responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firebrats exposed to glass slide (3d)</td>
<td>23 **</td>
</tr>
<tr>
<td>Control</td>
<td>4 (0)</td>
</tr>
<tr>
<td>E. cloacae on glass slide</td>
<td>23 *</td>
</tr>
<tr>
<td>Control</td>
<td>9 (2)</td>
</tr>
<tr>
<td>M. microspora on glass slide</td>
<td>12</td>
</tr>
<tr>
<td>Control</td>
<td>10 (2)</td>
</tr>
<tr>
<td>M. microspora on paper</td>
<td>27 *</td>
</tr>
<tr>
<td>Control</td>
<td>12 (11)</td>
</tr>
</tbody>
</table>

Fig. 3. Number of female firebrats arresting on a 1.5-cm² piece of glass that was 1) exposed to firebrats for 3 d, 2) inoculated with 1 by 10⁸ colony forming units (cfu) of E. cloacae, or 3) inoculated with 1 by 10⁶ cfu of M. microspora. Numbers within bars indicate the number of insects responding to the treatment stimulus (upper bar) or control stimulus (lower bar). An asterisk (*) indicates a significant preference for a particular test stimulus (χ² test; **P ≤ 0.01). Numbers in brackets indicate numbers of nonresponding insects.

<table>
<thead>
<tr>
<th>Test stimulus</th>
<th>Number of females responding</th>
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<tr>
<td>Ultrasonicated E. cloacae</td>
<td>32 **</td>
</tr>
<tr>
<td>Control</td>
<td>11 (6)</td>
</tr>
<tr>
<td>E. cloacae pellet</td>
<td>18</td>
</tr>
<tr>
<td>Control</td>
<td>14 (8)</td>
</tr>
<tr>
<td>E. cloacae supernatant (ppt)</td>
<td>38 **</td>
</tr>
<tr>
<td>Control</td>
<td>15 (4)</td>
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<tr>
<th>Test stimulus</th>
<th>Number of females responding</th>
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<tr>
<td>Ultrasonicated M. microspora</td>
<td>36 **</td>
</tr>
<tr>
<td>Control</td>
<td>11 (10)</td>
</tr>
<tr>
<td>M. microspora pellet</td>
<td>19</td>
</tr>
<tr>
<td>Control</td>
<td>16 (5)</td>
</tr>
<tr>
<td>M. microspora supernatant (ppt)</td>
<td>19</td>
</tr>
<tr>
<td>Control</td>
<td>15 (6)</td>
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</tbody>
</table>

Fig. 4. Number of female firebrats arresting on a piece of filter paper inoculated with (A) E. cloacae or (B) M. microspora, either ultrasonicated, or, ultrasonicated, centrifuged (10,000 × g) and obtained as a pellet or supernatant precipitate (ppt). Numbers within bars indicate the number of insects responding to the treatment stimulus (upper bar) or control stimulus (lower bar). An asterisk (*) indicates a significant preference for a particular test stimulus (χ² test; **P ≤ 0.01). Numbers in brackets indicate numbers of nonresponding insects.
water alone, survived equally long, and longer than nymphs provided with diet only; however, for nymphs to survive beyond the third instar (20–30 d), they required a perpetual supply of both nutrients and water (Fig. 6B).

Firebrats live longest (>50 d; Adams 1933a,b; Sweetman 1938) when they have access to both water and a nutrient-rich diet. Depriving firebrat nymphs of nutrients slowed their growth (Fig. 6A), and depriving them of water limited their survival to first instar (<10 d; Fig. 6B). Even in the absence of any food or water, *E. cloacae* or *M. microspora* could sustain firebrat nymphs for 22 or 26 d, respectively, but then these microbes apparently did not yield enough water to sustain further nymph survival (Fig. 6A and B). Perpetual supply of fresh *E. cloacae* and *M. microspora* within a shelter where adult firebrats deposit these microbes with their feces (Woodbury and Gries 2013, Woodbury et al. 2013) may give nymphs the adequate moisture to survive beyond third instar. As firebrats feed mainly on cellulose and glucose (Jackson 1886, Spencer 1930, Austin and Richardson 1941, Wall and Swift 1954, Lau–Greig 1992), which are deficient in amino acids, sterols, and other important nutrients, the amino acids and sterols associated with *E. cloacae* and *M. microspora* (Fitt and O’Brien 1985, Ruiz–Herrara 1992, Niyazi et al. 2004) are likely a major source of nutrients for firebrats. Dietary amino acids and sterols are crucial to survival and development of many insects (Clayton 1964, Douglas 2009).

In conclusion, firebrats detect the presence of *E. cloacae* based on its exopolysaccharide glycocalyx, and most likely respond to D-glucose as a constituent thereof. They respond to *M. microspora* only in the presence of cellulose, suggesting that enzymatic cellulolic metabolites of *M. microspora* (such as D-glucose) serve as the arrestment cue. Both microbes benefit their firebrat host by mediating aggregation behavior in a suitable shelter (Woodbury and Gries 2008, 2013a; Woodbury et al. 2013), and when they are consumed, they afford nymphs with water and nutrients that facilitate growth and survival. Ultimately, *E. cloacae* and *M. microspora* allow firebrats to locate and occupy nutrient-poor habitats that otherwise would not support nymphal development.

Acknowledgments

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