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Factors Affecting the Transmission of *Salmonella* by Flies: Natural Resistance to Colonization and Bacterial Interference

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Groups of immobilized gnotobiotic houseflies, *Musca domestica* L., were monocontaminated with graded doses of *Salmonella typhimurium*, bicontaminated with *S. typhimurium* and *Proteus mirabilis*, and multicontaminated with *S. typhimurium* and a mouse fecal flora. Of 45 flies fed an average of 22 *Salmonella* cells alone, 26 showed bacterial multiplication, up to 1.4×10^7 in one case. There was a fairly consistent increase in percentage of *Salmonella* excreters with increasing input, so that all flies given 10^4 and 10^5 cells shed *Salmonella* some time during an observation period of 8 to 13 days. Multiplication, however, reached higher levels in flies given 10^2 or 10^3 organisms. A high input was not necessary to produce a high proportion of *Salmonella* excreters. Among 22 flies given an average of 90 *Salmonella* cells, multiplication occurred in 17 and 18 became excreters. Antagonism between *P. mirabilis* and *S. typhimurium*, which was previously described in the blowfly maggot tract, also occurred in the gut of the adult housefly. With an input of 10^3 cells each of *Salmonella* and *Proteus*, there was a reduction of *Salmonella* excreters to 27% on the first day and elimination by the second day. With a normal fecal flora which contained no demonstrable *P. mirabilis*, 37% of the flies were still excreting *Salmonella* on the fourth day, but not by day 6; among flies fed *Salmonella* alone at the same input, the organism was excreted by 87% on the first day and 62% on the second day, and outputs continued for at least 8 days. *Salmonella* was lodged mainly in the mid- and hindgut 3 days after the infective meal, minimizing transmission through vomit. Comparison studies with the common green bottle fly, *Phaenicia sericata* Mg., showed it to be more resistant than the housefly to establishment and multiplication of *S. typhimurium*.

The double life of a fly exposes both maggot and adult to pathogenic agents. We have previously found that the hostile environment of the larval gut, combined with antagonisms of the normal flora and effects of metamorphosis, minimize the maggot-adult transmission route (2, 3, 6). Thus, adults emerge from pupation with far fewer microbes in their digestive tracts than maggots possess. This is significant, as approximately 20% of newly emerged houseflies (1, 10) and 37% of green bottle flies (1) have been shown to have sterile digestive tracts. The remaining flies emerge with a variable burden of intestinal microorganisms.

We know of only two studies which have attempted to quantitate the fate of a pathogen in the adult fly (7, 9), and in neither was the microbial condition of the flies' guts known. The flies may have harbored a complex or simple flora in their digestive tracts. It is well known that the normal gut flora of conventional laboratory animals enhances resistance to *Vibrio comma*, *Shigella*, *Salmonella*, and other pathogens (8). However, almost nothing is known about this phenomenon in insect hosts, despite the obvious implications for disease transmission and insect pathology.

The purpose of this study was to determine the natural level of resistance of the housefly's gut to colonization by *Salmonella typhimurium*, to compare the minimal implantation dose in the housefly (*Musca domestica L.*) with that in the green bottle fly (*Phaenicia sericata* Meigen), and to learn whether the presence of other bacteria alters the natural level of resistance of the gut to the specific pathogen. Our approach was to monitor daily bacterial output from the feces of gnotobiotic flies which had ingested a known number of organisms.

MATERIALS AND METHODS

M. domestica and *P. sericata* were colonized from local populations and were reared gnotobiotically as previously described (5, 6). Teneral adults were cold-immobilized and fastened by their wings to paraffin on strips of aluminum foil; these strips were previously sterilized by exposure to ultraviolet light for 3 days. Aseptic procedures and sterile materials were used throughout the study. The mounted flies were placed in vials containing 2 ml of physiological saline, and were positioned vertically with their abdomens just above the saline. One day after mounting, dead flies were culled and remaining flies were fed 2 μliters of a bacterial solution of known concentration by means of a 10-μliter Hamilton syringe calibrated in tenths (error, $\pm 5\%$). Doses of about 100 organisms or less were plated directly from the syringe to avoid dilution errors. The infective dose was obtained by diluting a 24-hr broth culture of the organism in sterile 5% milk-5% sucrose solution to the approximate number desired. The organisms used were *Salmonella typhimurium*, resistant to 2,000 μg of streptomycin per ml, and *Proteus mirabilis*, reistant to 100 μg of oxytetracycline per ml; both had been used in previous studies (4, 6). A mouse fecal flora was obtained by triturating 260 mg of fresh feces from laboratory mice in 20 ml of 0.9% saline, and centrifuging the mixture at 500 rev/min for 2 min. Dilutions of the supernatant were prepared in milk solution to obtain the desired density of flora, and *Salmonella* cells were added to this before the flies were fed. A sample of the infective meal was plated on Brain Heart Infusion (BHI) agar in the monoflora studies; in the biflora study, MacConkey agar which contained streptomycin (1,000 μg/ml) or oxytetracycline (100 μg/ml) was used to permit exclusive and uninhibited outgrowth of *Salmonella* or *Proteus*, respectively. Mixed flora was plated on both BHI agar and MacConkey agar plus streptomycin, and the difference between the number of colonies on the two plates was assumed to be the amount of mixed flora present. The fecal output from flies was monitored from day 1, 24 hr after the infective meal.

Flies were kept in a hood at 25 C. Each morning, they were transferred to vials which contained 2 ml of 0.9% saline, and were fed to repletion from a cotton swab saturated with 5% milk-5% sucrose solution (one fly per swab); the feeding stimulated defecation. After 4 hr, the flies were returned to empty vials, and the vials with saline solution containing their defecations were sampled and plated on appropriate medium. These daily 4-hr samplings, corrected for dilution factors, represent a fly's fecal output of bacteria. Flies were again fed in the afternoon to improve their longevity. Tests showed that *Salmonella* did not multiply in the saline during the 4-hr period.

Bacterial colonies on the plates were counted after 48 hr at 37 C. *Salmonella* colonies were routinely checked in Triple Sugar Iron (TSI) agar, and occasionally in other media, as well as serologically, to confirm the identity of the organism and the absence of contaminants. *Proteus* colonies were checked in TSI agar and urea broth. A bacteriological examination of mouse feces was made to identify some of the types and numbers of bacteria which were present in the mixed flora fed to the flies. This included examination of aerobic platings on BHI, EMB, SS, Chapman Stone, and blood agars. The presence of anaerobes was confirmed with Brewer's anaerobic agar, with and without blood. Identification of organisms was based on a number of diagnostic biochemical tests.

RESULTS

The minimal criterion we adopted for multiplication of bacteria in the fly is a single or cumulative fecal output which is at least double the input.

S. typhimurium in monobiotic houseflies. Three groups of 15 flies each were fed average numbers of 17, 20, and 28 *S. typhimurium* cells. The dosage was averaged at 22 (SD, 7.3) and the flies were combined as group 1 because of significant overlap shown by an analysis of variance (Fig. 1, Table 1). In additional experiments, graded inputs of bacteria were used as follows: group 2, 50 *Salmonella* cells; group 3, 90 cells; group 4, 1,200 cells (Fig. 2); group 5, 13,000 cells; and group 6, 134,000 *Salmonella* cells. With the single exception of group 2, there was a consistent increase in percentage of *Salmonella* excreters as input was increased, until all flies became excreters with an input of 1.3×10^4. Table 1 summarizes the results.

In a related experiment, we studied the localization of *S. typhimurium* in the gut. Nine gnotobiotic flies were each fed 2.8×10^3 organisms, and their feces were sampled for 2 days. On the third day, their crop, midgut, and hindgut were aseptically dissected and separately homogenized, diluted, and plated. Table 2 shows *Salmonella* output for the first 2 days after the infective meal, and the distribution of the organism in the gut on day 3. The *Salmonella* cells were lodged mainly in the midgut; in some cases, they were in the hindgut, but they occurred minimally in the crop at this time.

S. typhimurium in monobiotic P. sericata. Gnotobiotic green bottle flies of group 7 were fed an average dose of 100 *Salmonella* cells. Multiplication occurred in 7 of 20 flies (35%), and 9 flies (45%) excreted *Salmonella* some time during a 6-day period (Fig. 3, Table 3). In group 8, which received 730 *Salmonella* cells, bacterial multiplication occurred in four of eight flies, and seven of these passed the organism in their feces. The maximal single output was 6.0×10^4 on the tenth and terminal day of a fly which had a consistently high output. In group 9, which received 7.9×10^3 *Salmonella* cells, 6 of 10 flies (60%) showed multiplication, with the highest output exceeding 10^7. All but one fly were bac-

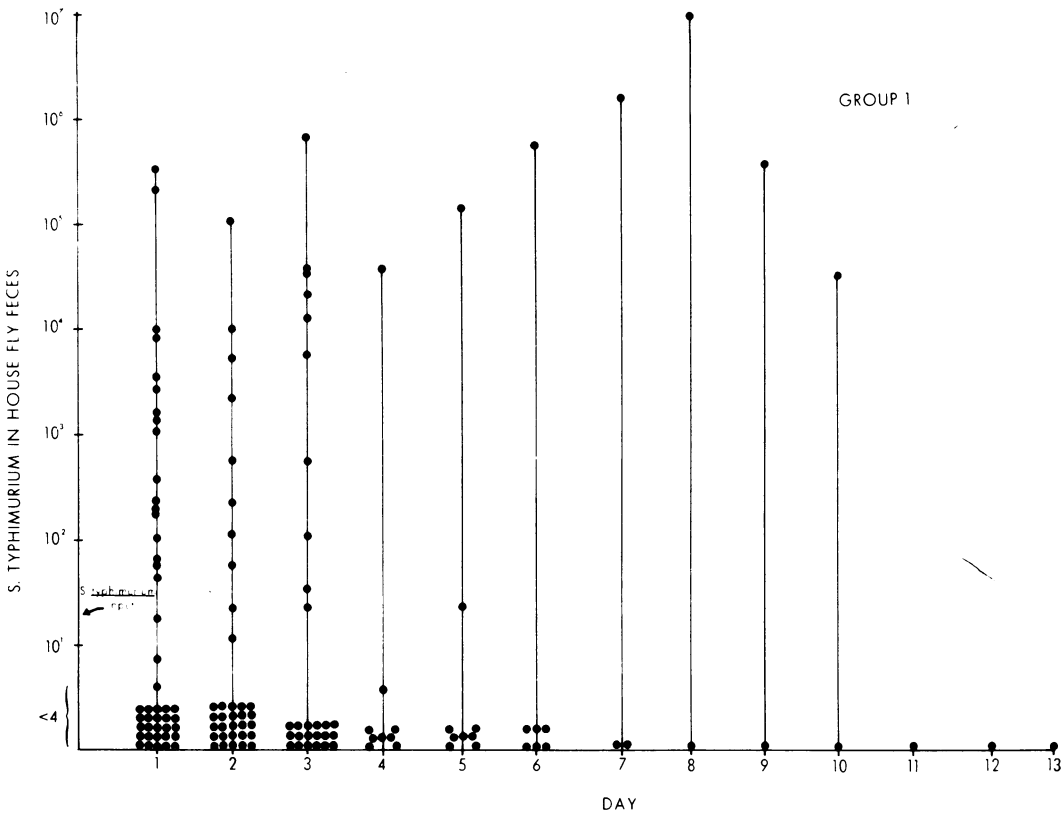

FIG. 1. *Daily fecal output of S. typhimurium in monobiotic houseflies fed an average of 22 organisms. A dot represents a single fly's output per 4-hr sampling period.*

TABLE 1. *S. typhimurium in monobiotic M. domestica*

Input of *Salmonella* cells	No. of flies excreting *Salmonella*/ no. tested	No of flies showing *Salmonella* multiplication/no. tested	Maximal *Salmonella* output
22 (group 1)	27/45 (60%)	26/45 (58%)	1.4×10^7
50 (group 2)	9/16 (56%)	7/16 (44%)	8.0×10^4
90 (group 3)	18/22 (81%)	17/22 (77%)	1.8×10^7
1,200 (group 4)	13/15 (87%)	11/15 (73%)	1.3×10^7
13,000 (group 5)	11/11 (100%)	2/11 (18%)	4.2×10^4
134,000 (group 6)	10/10 (100%)	3/10 (30%)	2.2×10^6

terial excreters; the maximal period of excretion of *Salmonella* was 9 days (Table 3).

Dibiotic houseflies. Among 15 flies of group 10, which were fed 1,000 cells each of *S. typhimurium* and *P. mirabilis*, 4 passed *Salmonella* on the first day, and 2 of these showed possible multiplication of the organism. From the second day on, no *Salmonella* cells were recovered from any fly. *Proteus*, on the contrary, multiplied in 10 flies (67%), and was excreted by 11 flies for a maximum of 9 days, at which time the last fly died (Fig. 4).

S. typhimurium and mouse fecal flora in gnotobiotic houseflies. The mouse feces yielded the following bacteria per milliliter of suspension: *Escherichia coli*, 10×10^4; *Micrococcus* sp., 103×10^4; *P. vulgaris*, 1; and, on anaerobic agar, 46×10^4 cells of a diplococcus type. It is significant that *P. mirabilis* was not isolated. The infective meal given the flies of group 11 contained 1.5×10^8 *Salmonella* cells and 1.2×10^4 organisms from the fecal suspension (Fig. 5). In the presence of the mixed flora, the pathogen multiplied in 9 of 14 cases, and 13 flies passed

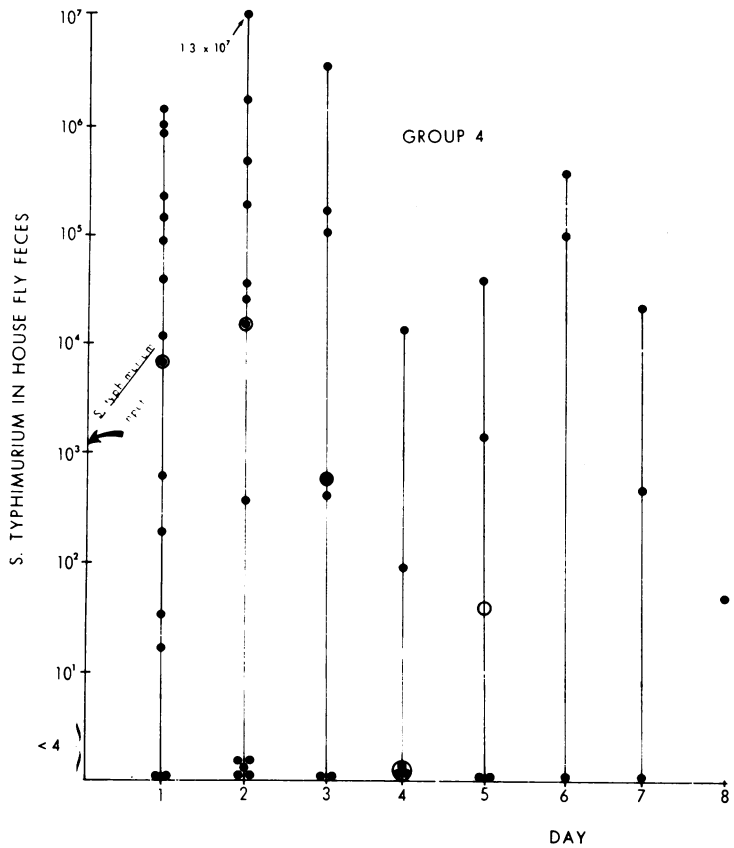

FIG. 2. *Same as Fig. 1, with input of 1.2 × 10³ S. typhimurium. Open circles are medians.*

TABLE 2. *S. typhimurium in the feces and gut of monofloral houseflies fed 2.8 × 10³ organisms*

Specimen	Fecal output[a]		No. of *S. typhimurium* in gut on day 3[b]		
	Day 1	Day 2	Crop	Midgut	Hindgut
1	4.4×10^1	2.0×10^5	<4	6.0×10^4	5.6×10^1
2	1.0×10^3	1.2×10^3	<4	1.2×10^3	<4
3	2.0×10^3	8	<4	<4	<4
4	3.0×10^3	1.5×10^4	<4	1.6×10^3	<4
5	2.6×10^3	8.0×10^2	<4	1.6×10^3	2.0×10^1
6	—	2.4×10^3	<4	8.0×10^2	1.6×10^1
7	—	—	3.9×10^2	1.1×10^3	6.3×10^2
8	4.8×10^4	<4	<4	4.0×10^2	8.0×10^2
9	<4	2.0×10^2	<4	4.8×10^2	1.2×10^1

[a] Per 4-hr sampling period.
[b] Each segment of gut homogenized in 2 ml of saline.

Salmonella. *Salmonella* outputs above 10^4 occurred in a number of these flies, and maximal output was 1.3×10^6, compared with an initial input of 1.5×10^3. No *Salmonella* cells were recovered after day 5. Tests of the fecal flora were only begun on day 7 and were continued through day 11. The output of the fecal flora continued at high levels during this period, with peak output reaching 1.6×10^8, compared with an initial input of 1.2×10 .

S. typhimurium and mouse fecal flora in gnotobiotic green bottle flies. The fecal flora was es-

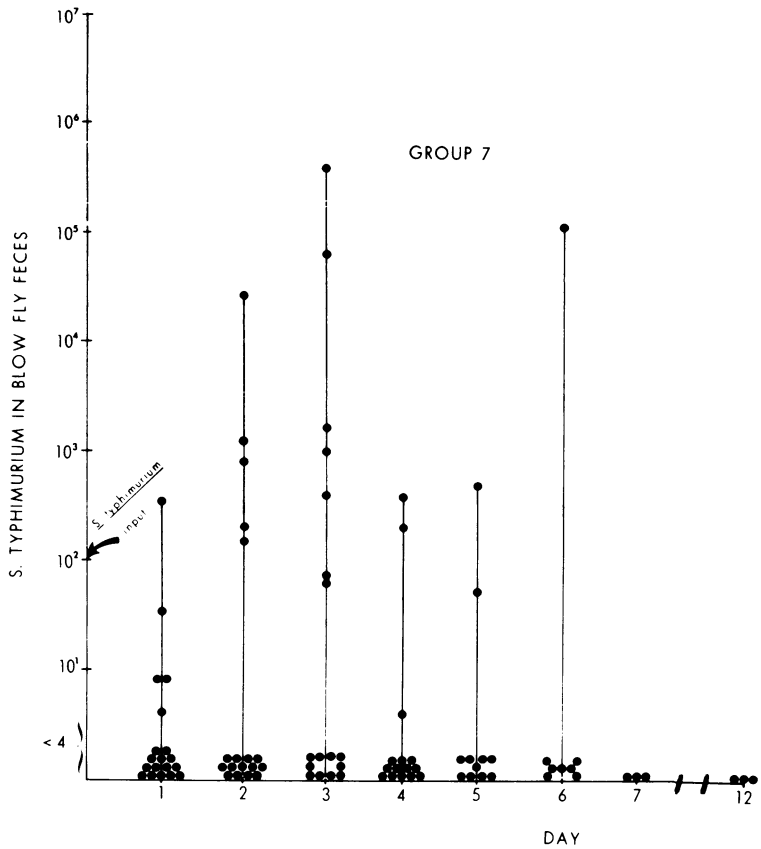

FIG. 3. *Daily 4-hr fecal output of S. typhimurium in monobiotic green bottle flies, P. sericata, fed an average of 100 organisms.*

TABLE 3. *S. typhimurium in monobiotic P. sericata*

Input of *Salmonella* cells	No. of flies excreting *Salmonella*/ no. tested	No. of flies showing *Salmonella* multiplication/no. tested	Maximal *Salmonella* output
100 (group 7)	9/20 (45%)	7/20 (35%)	4.1×10^5
730 (group 8)	7/8 (88%)	4/8 (50%)	6.0×10^4
7,900 (group 9)	9/10 (90%)	6/10 (60%)	1.0×10^7

sentially that given to group 11, but the numbers of bacteria fed to the 15 flies of group 12 were 2.7×10^4 fecal flora and 5.4×10^3 *Salmonella*, a ratio of 4:1 (flora to pathogen), instead of a 10-fold difference as in group 11. *Salmonella* multiplied in 5 flies to a maximum of 1.3×10^6 and was excreted by 12 flies. Presence of the organism was terminated by death of surviving flies between days 7 and 8. The fecal flora attained a 7-log superiority over *Salmonella* based on comparison of median outputs, including a single 4-hr output of 4.6×10^8 which was the highest recorded for any fly in this study (Fig. 6).

DISCUSSION

The adult fly is better suited than the maggot for studies of monitored input and output of bacteria. We could feed the flies accurately determined numbers of organisms and quantitate their fecal output. Immobilization, however, sharply reduced average longevities to 6.8 and 7.0 days for houseflies and green bottle flies, respectively.

Longevity was an important factor in the experiments. Data obtained during the first few days were more meaningful, because there was a considerable degree of mortality toward the

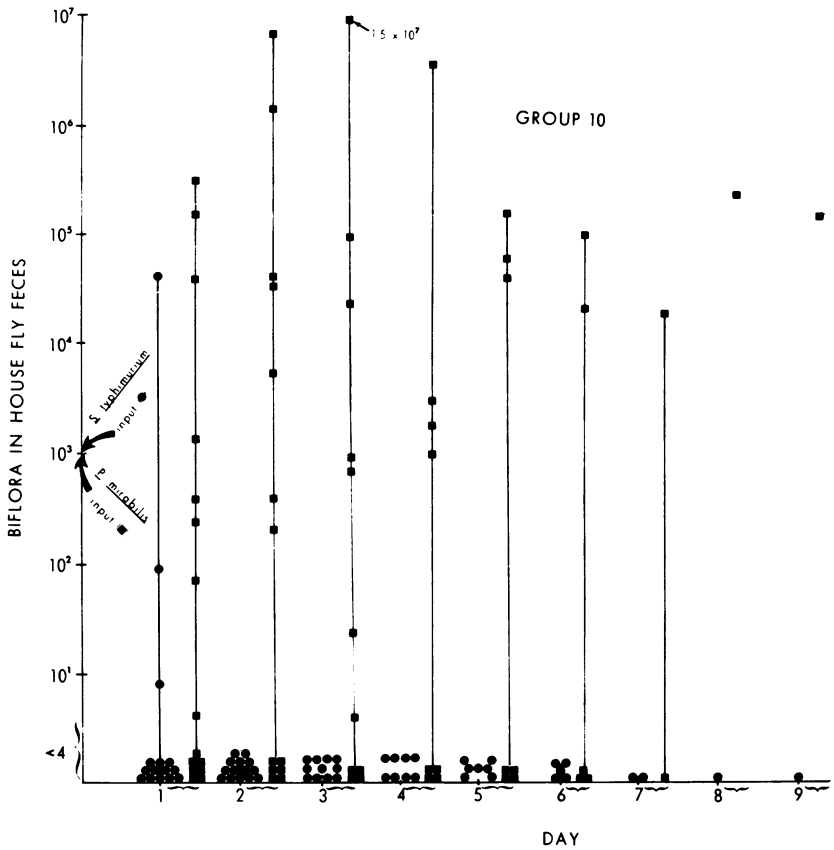

FIG. 4. *Daily 4-hr fecal output of S. typhimurium and P. mirabilis from dibiotic houseflies.*

end of an experiment. However, it is important to note in the comparisons made that in no experiment did the added flora have a significant effect on the longevity of either species of fly; nor was there a significant difference in the longevity of 30 germ-free houseflies when they were maintained under the same conditions and on the same diet without microorganisms. Also, the average longevities of the two species, and therefore the observation periods, were very close.

The natural resistance of the housefly to implantation with *S. typhimurium* is low indeed. About 20 cells fed to 1-day-old germ-free flies were enough to produce colonization in 26 of 45 flies. These flies excreted *Salmonella* at some time during 10 days following the infective meal; a peak output of 1.4×10^7 *Salmonella* cells per 4-hr sampling period was obtained.

As input increased, there was a generally consistent increase in the percentage of *Salmonella* excreters (Table 1). Per cent multiplication was less at the highest inputs, as expected, but we were surprised to find that multiplication actually reached higher levels in flies given lower inputs. Thus, among 15 houseflies fed a relatively low dose of 1.2×10^3, there were five outputs which exceeded 10^6, whereas, among 21 houseflies fed the two highest doses (1.3×10^4 and 1.3×10^5), there was only one output which reached 10^6.

The fact that zero outputs occurred more frequently with lower dosage inputs and that many such flies consistently failed to excrete any salmonellae suggests some variability in the natural resistance of the fly which is completely overcome with doses above 10^4. Alternatively, it is possible that with small inputs the bacteria fail by chance to reach areas suitable for multiplication and, therefore, the variability is unrelated to resistance. Our data do not provide an answer as to which interpretation is correct, nor can we explain why a dose of about 20 *Salmonella* cells produced 60% excreters, whereas a dose 60 times greater produced only 87% excreters.

Three days after an infective meal, *Salmonella* cells are localized in the midgut of the housefly, with smaller populations in the hindgut, and

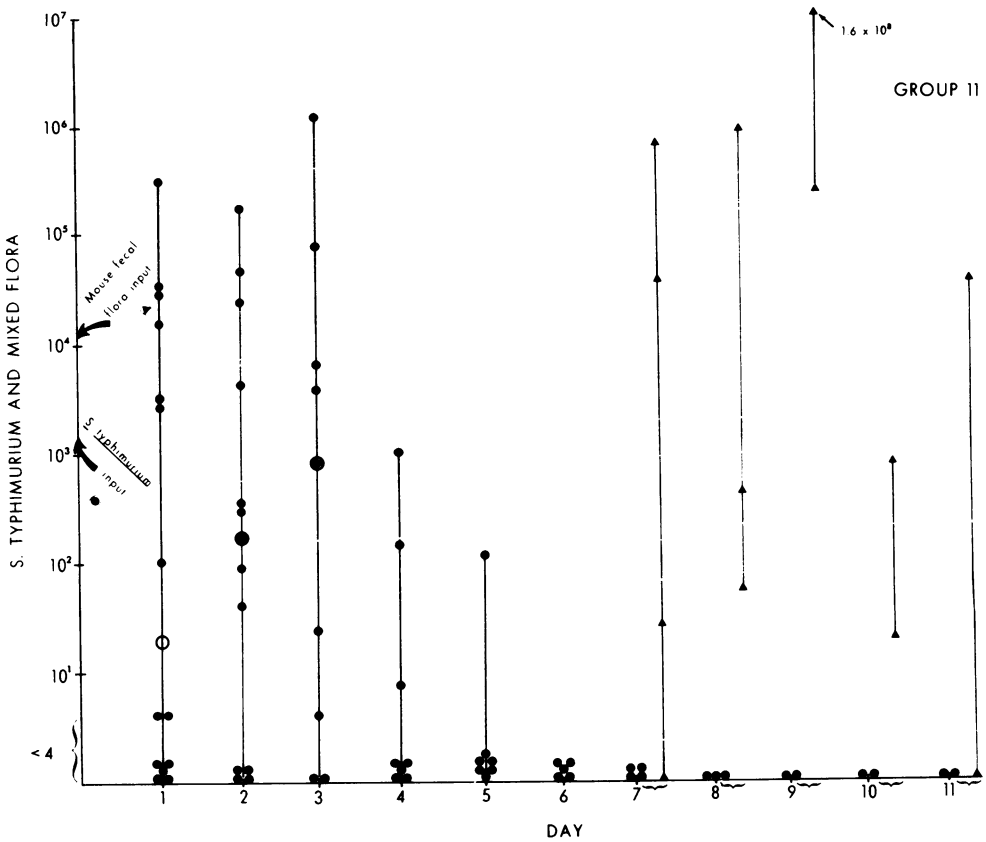

FIG. 5. *Daily 4-hr fecal output of S. typhimurium and mouse fecal flora in gnotobiotic houseflies. The mouse fecal flora was sampled from the seventh day on. Open circles are medians.*

few, if any, organisms left in the crop (Table 2). Weak sugar solutions (1%) pass directly to the midgut, whereas 5% solutions such as we used are shunted, by mediation of pharyngeal receptors, directly to the crop (12). It is noteworthy, therefore, that *Salmonella* cells did not remain in the crop after a few days, and this would minimize danger of transmission through vomit. We do not know why the magnitude of the output in the feces corresponded more closely to levels in the midgut than to those in the hindgut.

The graphs do not allow one to follow the daily outputs of an individual fly because of the complexities involved in graphing. Such data, however, disclose patterns which are otherwise not discernible. For example, flies that excreted no *Salmonella* cells during the first 2 or 3 days did not become excreters subsequently. Conversely, flies often excreted the organism during the initial few days, but not thereafter. There were also sampling periods with no recoverable *Salmonella* cells between periods with definite outputs. A frequently observed feature of implantation was an initial burst of multiplication followed by a gradual decline of the population which could be recovered up to 11 days (persistence was not correlated with dosage input). We do not know whether this decline was due to changes in the gut as the fly aged, which may have led to increasing resistance, or whether it was due to nutritional deficit, toxic accumulations, or something associated with the abnormality of immobilization.

A sample was designated < 4 when no organisms were recovered. This could mean absence of organisms in the feces or failure to recover them, or that no defecation occurred during the 4-hr sampling period. The graph for group 11 supports the contention that, in the majority of cases, counts of < 4 *Salmonella* cells probably mean absence of the organism rather than no defecation. Here, it is clearly seen that, although *Salmonella* was absent from flies between days 5 and 11, the mixed flora was recovered throughout this period. Nevertheless, there is little question that some samples

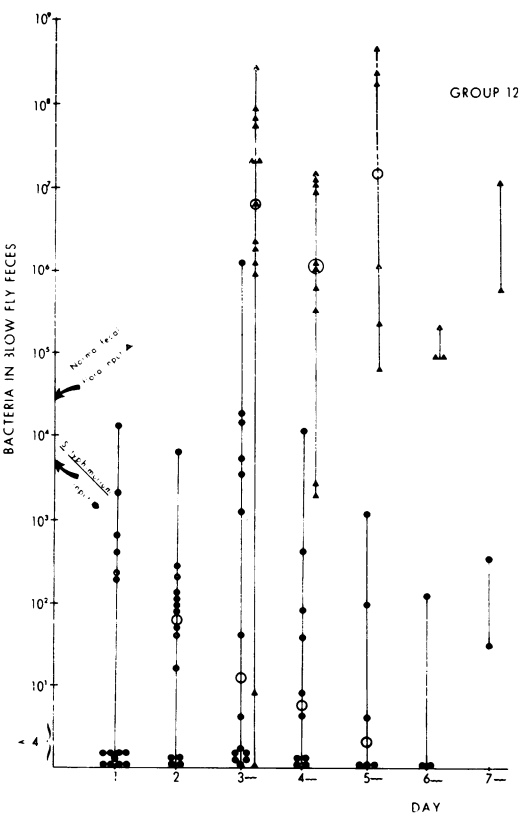

FIG. 6. *Daily 4-hr fecal output of S. typhimurium and mouse fecal flora from gnotobiotic green bottle flies. Open circles are medians.*

designated < 4 meant the flies did not defecate on cue. This would account for inconsistent outputs in some individuals.

The possibility that fly defecations cease before death, thus diminishing the transmission potential of the fly, was also investigated. Among 177 flies which shed organisms in their feces 2 days before death, only 9 failed to do so 1 day before their demise. Flies may therefore continue to disseminate pathogens until close to the time of death.

P. mirabilis in the housefly gut markedly antagonized *Salmonella*, and this is most evident when we contrast the latter's persistence as a monoflora. When houseflies were fed 1,200 *Salmonella* cells alone (group 4), 13 of 15 flies (87%) excreted the organism on the first day, and 8 of 13 (62%) did so on the second day. In the presence of *Proteus*, however, *Salmonella* excreters were reduced to 27% on the first day and to 0% on the second day. The accelerated extinction of *Salmonella* was highly significant ($P < 0.001$, Wilcoxon test; 11). On the other hand, *Proteus* excretion continued for the life of the flies (Fig. 4). This antagonism was previously demonstrated in the blowfly maggot gut where the suppression ratio between the two organisms reached 11,500:1 (*Proteus to Salmonella*), compared with 20:1 in vitro (6). Because of its wide distribution and natural occurrence in flies, *P. mirabilis* may be a factor of considerable importance in influencing the survival of pathogens in the digestive tracts of these medically important insects.

The specificity of the above antagonism is suggested by the results of substituting a mouse fecal flora for *Proteus*. In the presence of such a mixed flora, elimination of *Salmonella* was not as rapid as in the presence of *P. mirabilis*. In fact, during the first 3 days, *Salmonella* output, as measured by percentages of flies showing multiplication and excretion, was the same as in flies given a comparable dose of *Salmonella* alone. In the presence of mixed flora, *Salmonella* output was inhibited for the first 2 days, as shown by comparison of median cumulative outputs ($P = 0.05$, Wilcoxon test). Significant suppression of *Salmonella* occurred on days 4 and 5, whereas in monobiotic flies *Salmonella* survived for 8 or 9 days. In this context, it is noteworthy that the mouse fecal flora at input contained no demonstrable *P. mirabilis* and few *P. vulgaris* cells; cells of *E. coli* and *Micrococcus* sp. were numerically dominant. *E. coli* has been shown to exert no suppressive effect on *S. typhimurium* in the blowfly life cycle (6). This is not to say that other organisms besides *Proteus* may not, singly or in combination, antagonize *Salmonella*. It would have been particularly interesting to determine whether the decline of *Salmonella* cells on the fourth and fifth days was correlated with the rise of one or another element of the mixed flora.

Hawley et al. (7) fed *S. schottmuelleri* to houseflies, the microbial content of whose digestive tracts was not known. They obtained no multiplication with an input as high as 900 organisms, but multiplication was reported with a dose of 1.8×10^4. A comparison can be made with our mixed-flora study, but it must be noted that there were significant qualitative and possibly quantitative differences. With an input one log lower, our *Salmonella* outputs were generally higher (10^3 to 10^4) for the first 2 days, decreasing steadily to zero in all flies by day 6. The results of Hawley et al., however, showed a gradual increase of recoverable *Salmonella* cells to as high as 10^8 by the sixth day, with the first days being the lowest (10^1 to 10^2).

Monoflora experiments with the green bottle fly, *P. sericata*, showed once again a positive

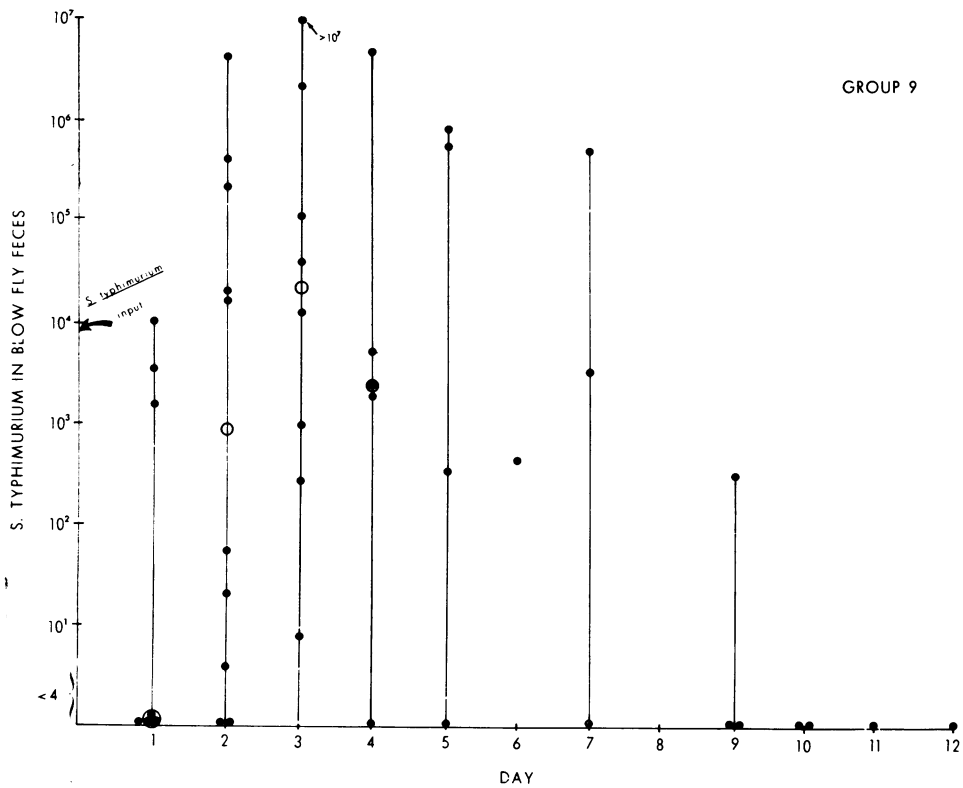

FIG. 7. *Daily 4-hr fecal output of S. typhimurium in monobiotic green bottle flies. Open circles are medians.*

correlation between size of input and percentage of excreters, but this fly appears to be a poorer host for *Salmonella* than the housefly. Given an input of about 100 *Salmonella* cells, the percentage of green bottle flies which excreted the organism was 45% compared with 81% in the housefly; also, the percentage of blow flies showing multiplication of the organism was slightly less than half that of the housefly. At the next higher input (730 organisms in the green bottle fly and 1,200 in the housefly), the percentages of excreters were close (88 and 87%), but there was decidedly less multiplication in the green bottle fly. If we compare outputs on days 2 and 3 when numbers of samples were maximal, we find that the green bottle flies produced no outputs above 10^4 in 16 samples, whereas the housefly produced 10 outputs above 10^4 in 20 samples.

Microbial competition in the green bottle fly was studied only between *S. typhimurium* and the mouse fecal flora. The damping of *Salmonella* by the fecal flora is suggested by a comparison of groups 9 and 12 (Fig. 6 and 7). Group 12, fed *Salmonella* plus fecal flora, showed multiplication of the pathogen in 4 of 15 flies (27%); group 9, fed *S. typhimurium* alone, showed multiplication in 6 of 10 flies (60%). However, since *Salmonella* inputs were not identical (difference of 1.4 times), demonstration of suppression by the fecal flora remains inconclusive.

Our results have demonstrated that the fate of *Salmonella* in an adult fly is significantly influenced by the following factors: (i) species of fly—both housefly and green bottle fly are synanthropic (closely associated with man) species and natural carriers of salmonellae and other pathogens, but the housefly is a distinctly superior host; (ii) size of input—low inputs can result in massive multiplication, but the percentage of successful implantations increases with dose up to 10^4; (iii) microbial condition of fly gut—interspecies antagonism leading to rapid elimination of *Salmonella*, or more gradual suppression by a mixed flora, may markedly effect the natural vector capacity of the fly.

ACKNOWLEDGMENT

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