Analysis of migration success of *Onchocerca lienalis* microfilariae in the haemocoel of *Simulium vittatum*

T. Lehmann[1]*, M.S. Cupp[2] and E.W. Cupp[2]

[1]Department of Entomology [2]Department of Veterinary Science, University of Arizona, Tucson, Arizona, USA

Abstract

Migration success (i.e. the proportion of worms that reach the thorax) of *Onchocerca lienalis* microfilariae (mf) in the haemocoel of *Simulium vittatum* was studied by inoculating mf into the posterior abdomen, and recording their distribution in the blackfly body at predetermined time points. Mf arrive into the thorax by active locomotion rather than by drifting in haemolymph currents. Migration into the thorax was completed by 12 h post inoculation (pi) but was not continuous throughout this period. Migration proceeded in two phases; the first occurred 0–2 h pi and the second at 6–12 h pi. Overall, migration success 12–24 h pi was only 36%, indicating that a substantial number of mf failed to reach the thorax, either because they were eliminated by the fly's defensive response or because they remained in the abdomen. Migration success was density independent. Mf that arrive into the thorax within 2 h pi did not differ in their migration potential from mf that remained in the abdomen at this time. In flies where more mf migrated successfully there was lower mf loss, indicating that migration success was linked to mf loss. Moreover, the proportion of mf in the thorax was not correlated with mf loss, suggesting that mf loss affected the number of mf that migrated successfully, rather than the reverse causal relationship.

Introduction

Filarial species that develop to the infective stage in the flight muscles of their dipteran host (e.g. *Onchocerca* spp. and *Brugia* spp.) follow a typical route in the body of their vector hosts, which include blackflies, biting midges, and mosquitoes. The microfilariae (mf) ingested with the blood meal penetrate through the wall of the posterior midgut into the abdominal haemocoel and move anteriorly into the thoracic muscles, where they invade individual cells (Laurence & Pester, 1961; Esslinger, 1962; Laurence, 1966; Eichler, 1973; Mellor, 1975). Migration success of mf migrating into the vector's thorax varies among different vector hosts of a given filariid species (Ewert, 1965; Mellor, 1975; Owen, 1978). This variation represents the presence of barriers the parasite faces prior to the beginning of the journey in the haemocoel, e.g. cibarial armature, and during the journey, e.g. immobilization of mf (Ham & Garms, 1988; Ham, 1992; Lehmann, 1994; Lehmann *et al.*, 1994b). Additionally, it represents the difficulty of navigation in the body of the living insect.

Migration success and vector defence may be linked in two ways: parasites that can migrate successfully fail to do so because they are attacked by the insect's defence system; alternatively, parasites are destroyed by the defence system because they do not reach the thorax fast enough (either because they had poor navigation skill or because random variation alone in the length of migration time). That mf are being eliminated (Lehmann, 1994; Lehmann *et al.*, 1994a,b) and immobilized (Ham & Garms, 1988; Lehmann *et al.*, 1994b) within a few hours pi suggests that the blackfly's defence system plays an important role in determining mf migration success.

The migration of *Onchocerca lienalis* mf toward the thoracic muscles of *Simulium vittatum*, a laboratory host, was investigated focusing on the following questions: 1) Can mf travel passively to the thorax by drifting in haemolymph currents? 2) What are the migration kinetics and how efficient is the migration of mf? 3) Does migration success depend on

*Present address: Entomology Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Chamblee GA, USA.

mf load? 4) Do mf differ in their migration potential? 5) What is the relationship between migration success and mf loss?

Materials and methods

Simulium vittatum rearing, *O. lienalis* mf source and preparation, injection procedure, and maintenance of infected flies were described previously (Lehmann, 1994; Lehmann *et al.*, 1994a,b); however, a brief description is given below with details of the method used to derive migration results. The migration assay consisted of parenteral inoculation of 33 *O. lienalis* mf in about 0.8 µl of incubation medium (unless otherwise stated) into the sixth abdominal segment of 1-day old *S. vittatum* adult females. Infected flies were frozen (–70°C) at predetermined times in vials containing 6% dimethyl sulphoxide. To determine the distribution of mf, vials were thawed at 37°C, and the abdomen was separated from the thorax using a razor blade under the dissecting microscope. Likewise, the head was separated from the thorax before each part was dissected separately in a drop of 0.85% NaCl. Mf from each part were counted under a light microscope (×40). Usually mf were intact and motile, but when parts of mf were observed they were counted based on their size and whether the part was an anterior or posterior end. For example, two small anterior parts were considered two mf, but two parts of the same size were considered as one mf if one part was anterior and the other posterior. Dead mf were also included in the total mf count. Mf count, rather than the proportion of mf, in the thorax was used to measure migration success if flies were inoculated with the same number of mf.

Results

Migration and haemolymph currents

To examine whether mf travel passively to the thorax by drifting in haemolymph currents, flies were inoculated with either freshly heat-killed (about 70°C for 5 min), or live mf (from the same preparation) into the posterior abdomen as described above. Freshly-killed mf remained in the abdomen even after 24 h pi, but live mf arrived into the thorax in considerable numbers (fig. 1). The results support the hypothesis that mf actively travel through the abdominal tissue of the fly. Freshly–killed mf are identical to live mf in their size and specific gravity, but differ in motility and, thus, the passive travel hypothesis, although less likely, could not be ruled out entirely.

Migration kinetics

Migration into the thorax was completed at 12 h pi (fig. 1). Most notably, migration occurred in two distinct phases: an early phase, 0–2 h pi, and a late phase, 6–12 h pi, while the number of mf in the thorax did not change significantly 2–6 h pi and 12–24 h pi (fig. 1, table 1). The three clusters of time group means, time zero, 1.5–5.5 h, and 9–24 h (fig. 2) established two migration phases that occurred between these periods. Homogenous migration behaviour among the three experiments was evident by the insignificant interaction ($P>0.9$, table 1) between experiment and time. Biphasic migration was also evident when mf that were inoculated into the thorax, moved first into the abdomen, but 'returned' into the thorax only after 6 h pi (fig. 1). The movement of mf inoculated into the thorax or the abdomen paralleled each other 1.5–20 h pi. Furthermore, the final migration success of mf inoculated into the thorax was apparently similar to that of mf inoculated into the abdomen. Because mf did not arrest their movement upon direct inoculation into the thorax and because a larger number of mf arrived into the thorax 6–12 h pi than 0–6 h pi, it is probable that the ability of mf to recognize the thorax changed during the 12 h pi.

At the end of the migration period (12–24 h pi), the mean number of mf in the thorax was 11.5. Thus, overall migration success in these three experiments was 35%. If the mf loss that occurred during the first 2 h pi (fig. 3, and Lehmann *et al.*, 1994a) is not considered, the comparable migration success was 63% out of the total number of mf recovered.

Migration success and mf load

Groups of flies were inoculated with doses of mf varying from 5 to 100 mf/fly. Mf were injected throughout this range in about 0.85 µl of complete medium using calibrated glass needles. The linear relationships between the number of mf that arrived into the thorax and mf load (fig. 3) indicated a constant rate (36% of the number inoculated) of migration success 24 h pi. The aptness of the linear model was verified using a lack of fit test ($P>0.45$).

Variation in mf migration potential

To test the hypothesis that mf differ in their migration potential, success of mf collected from thoraces of flies 2–3 h pi ('enriched competent migrators') was compared with that of mf collected from abdomens of the same flies ('enriched incompetent migrators'). Then flies were inoculated with 25 mf/fly from these groups or from a control group that was not previously injected into flies. Migration success was assessed 6 and 24 h pi. In contrast to the hypothesis, migration success was not different ($P>0.39$) between 'competent migrators' and 'incompetent migrators' (fig. 4). Both groups had lower success ($P=0.047$) than that of the uninjected control (fig. 4). These results suggest that mf do not differ in their migration potential; and assuming that the repeated injection itself did not affect the mf, exposure to the fly haemocoel reduces migration success of mf. This reduction in migration success represented a higher rate of elimination of mf previously exposed to the fly's haemocoel rather than a pure

Table 1. Migration kinetics of *O. lienalis* mf into the thorax of *S. vittatum*: ANOVA table.

Source	df	MA[a]	F	P
Model	17	103.0	8.2	0.001
Error	111	12.6	—	—
Block (exp[b])	2	8.8	0.7	0.497
Time	8	184.0	14.7	0.001
Time * Block	7	1.8	0.14	0.995

[a]Calculations were based on type IV SS (SAS/STAT 1990) to consider that few cells were missing (not all time points were included in each experiment). When the interaction terms were removed or when time points were collapsed to five values such that each experiment included all time levels, ANOVA results were very similar.
[b]Results of experiments A (excluding dead mf and thorax injected flies), B, C, shown in figs 1 and 5 were analysed together, blocking by experiment.

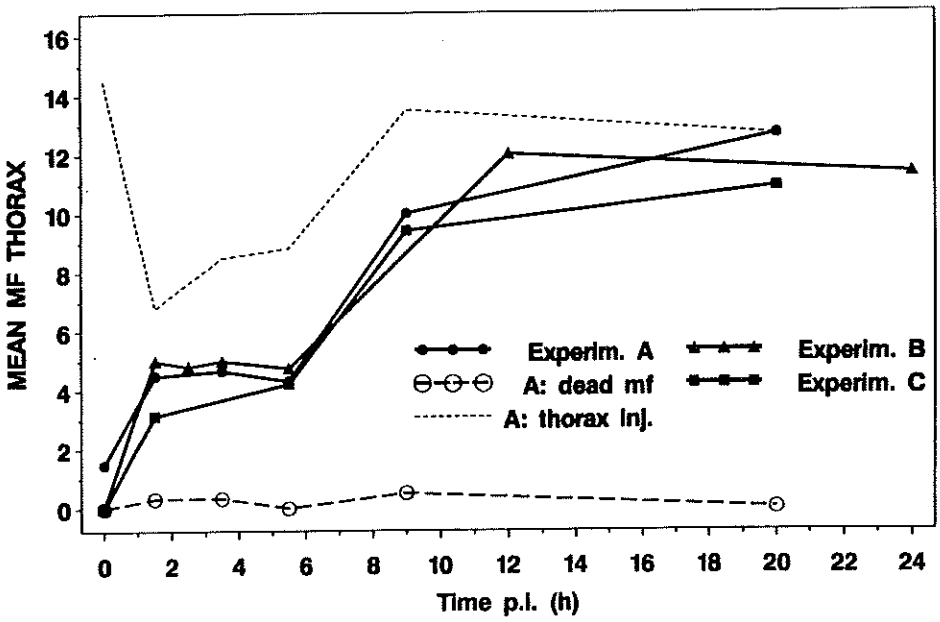

Fig. 1. Arrival of live and freshly-killed *O. lienalis* mf into the thorax over time. Flies were inoculated with live mf into the abdomen (Exp. A, B, C) or into the thorax (Thorax inj.), or with freshly-killed (ca. 70°C) mf into the abdomen (Dead mf). Groups of flies were dissected at different time points pi. The change in the mean number of mf in the thorax is plotted over time.

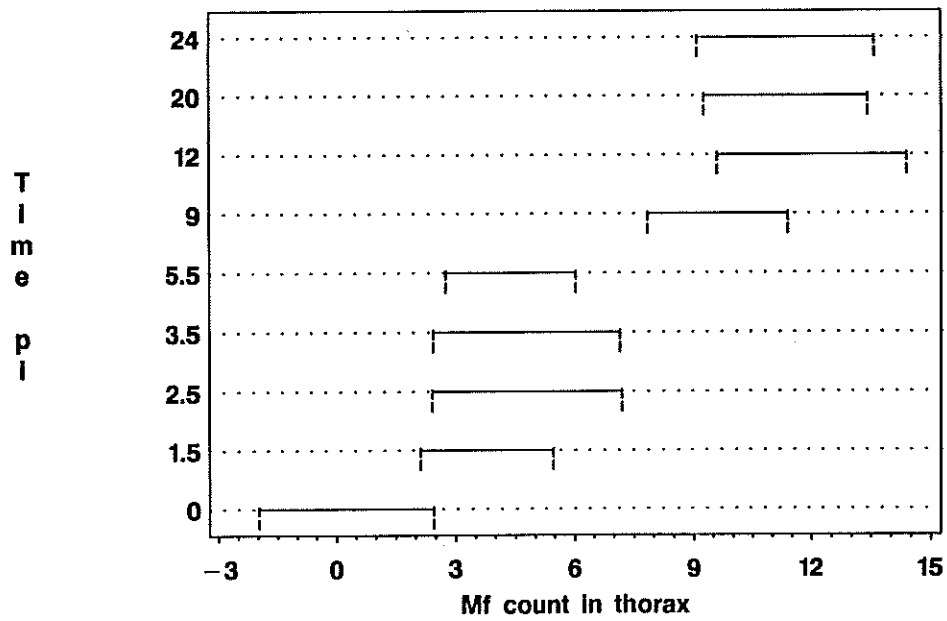

Fig. 2. Time dependent group means of *O. lienalis* mf count in the thorax and their 95% confidence intervals (CI) showing that migration occurred in two discrete phases. Calculation of CI is based on pooled variance estimated in table 1.

navigational effect, as comparable analysis of the proportion of mf in the thorax revealed no significant differences among the three groups ($P>0.13$; see also Lehmann *et al.*, 1994a).

The relationship between migration success and mf loss

Large variation in migration success (the number of mf in the thorax) was observed among flies dissected at

the same time point (excluding time 0) (Figs 3 and 5). Because migration of mf occurred in two phases (see above), the relationship between mf loss (expressed as 33–total mf in the fly) and migration success was examined separately in the following time periods: 1–2, 2.5–6, 8–12, and 20–24 h pi (flies were dissected only within these time intervals). Correlation coefficients were calculated between the number of mf in the thorax and mf loss for each period, and between the z scores of these variables to adjust for differences in the means and the variances among time points and among experiments. Using z scores rather than counts allows pooling of fly values from different time points and experiments without introducing bias into the correlation coefficient. Despite the inclusion of the number of mf in the thorax in the total mf of the fly, the correlation coefficient between these variables could, theoretically, take any negative or positive value (see also below).

The correlation coefficients were significantly negative regardless of whether the periods were pooled or considered separately (table 2). The negative association in the early periods was especially important because mf loss occurred during the first 2 h pi (fig. 5; Lehmann *et al.*, 1994a,b), and because the low numbers of mf in the thorax could not comprise the majority in the body. The negative correlation established a linkage between mf migration success and the intensity of mf loss in a fly, but not a causal relationship. This linkage may express that in a fly that mounted a strong defensive response, resulting in high mf loss, fewer mf remained to reach the thorax. Conversely, the correlation can mean that when more mf migrated into the thorax, mf loss was lower because successful migrators (in the thorax) gained protection from the fly's defence.

If the second interpretation is true, it implies that the proportion of mf in the thorax will also be negatively correlated with mf loss. This implication is derived from the assumption that mf in the thorax gain protection that is responsible for their overall higher rate of survival when more of them migrate successfully. The correlation coefficients between the proportion of mf in the thorax and mf loss were about zero (table 2), however, indicating that mf survive similarly in thorax and abdomen.

Discussion

It is difficult to measure migration of *O. lienalis* mf in *S. vittatum* because mf count in the thorax is a product of two or possibly three processes: the movement from the abdomen into the thorax; the loss of parasites from the body; and the movement backwards (indicated by movement into the abdomen of mf inoculated into the thorax). When considered alone, the proportion of mf in the thorax is misleading in describing migration success. According to this proportion, two thirds of the mf were in the thorax 20–24 h pi, while in fact, it represents only one third of the mf inoculated.

Probably, most mf are actively propelling themselves into this area rather than passively drifting through haemo-lymph currents, as freshly-killed mf did not arrive in the thorax. Furthermore, because most of the live mf that were inoculated into the thorax were found in the abdomen 1 h pi, it is unlikely that such haemolymph currents will also transfer mf in the opposite direction i.e. from the abdomen into the thorax. Finally, a haemolymph current alone cannot explain the fact that migration occurred in two separate phases (fig. 1). In histological sections of infected flies, mf were found penetrating

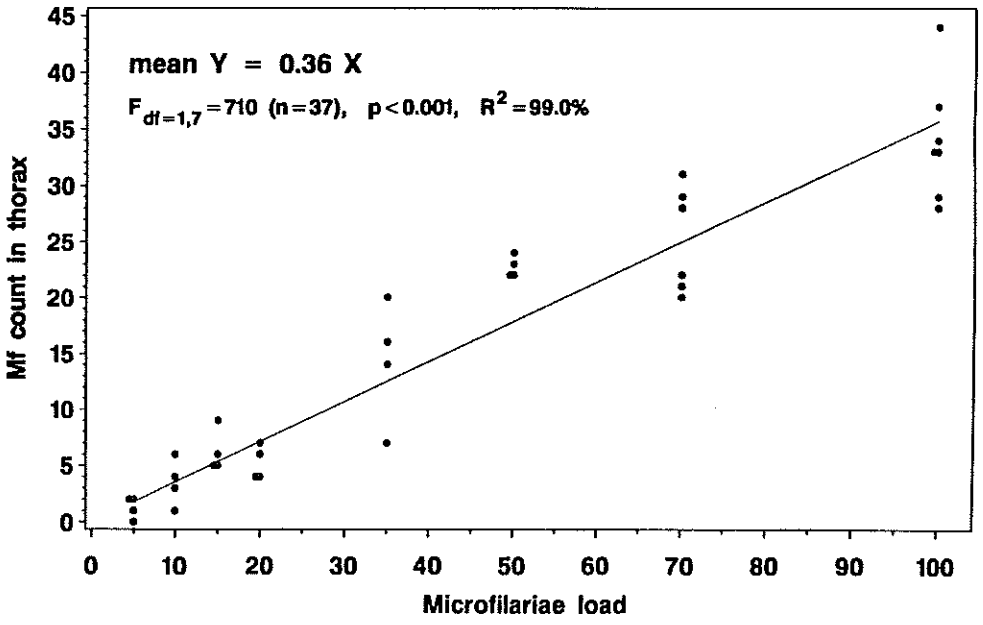

Fig. 3. The number of *O. lienalis* mf that arrived into the thorax in relation to mf dose 24 h pi. The linear relationship between the number of mf that arrived into the thorax and mf load indicates a constant rate of migration success (36%). Regression was forced through the origin and carried out on fly group means; dots represent individual fly counts, duplicated values were jittered horizontally.

every tissue in the abdomen (Esslinger, 1962). Their presence in cells of most abdominal tissues during their migration, together with our experimental data, indicate that mf arrival in the thorax results primarily from their active locomotion.

Migration of mf into the thorax was completed at 12 h pi (fig. 1), despite the fact that many motile mf remained in the abdomen. Apparently, after 12 h pi, these mf have a minimal chance to locate the thorax. Migration of *O. lienalis* mf to the thorax of *S. ornatum*, a natural vector, was completed at 6 h post feeding, when 25% of the mf arrived into the thorax (Eichler, 1973 [reported as *O. gutturosa*, but see Anderson, 1992]). Migration of *O. volvulus* mf in *Simulium damnosum* sensu lato was completed at 6 h post feeding, when 75% of the mf arrived into the thorax (Laurence, 1966). Migration of *O. cervicalis* mf in *Culicoides nubeculosus*, a natural vector, and in *C. variipennis*, a laboratory vector, was completed at 16 h post feeding (Mellor, 1975). Migration success in that system was 60% and 40% respectively. In these studies, however, mf that failed to migrate into the thorax remained in the midgut rather than in the abdominal haemocoel. The common explanation, that mf were arrested by the peritrophic membrane, cannot explain why parenterally inoculated *O. lienalis* mf had a similar migration success (or failure) when the gut was bypassed.

Migration did not occur continuously, but occurred in two distinct phases (figs 1, 2). The second migration phase is apparently of greater migratory importance because during this phase more mf arrived in the thorax despite the fact that only two thirds of the mf remained in the body as a whole and a sixth of those were already in the thorax. Thus, the second migration phase involved a considerably larger proportion of mf. Increased ability to

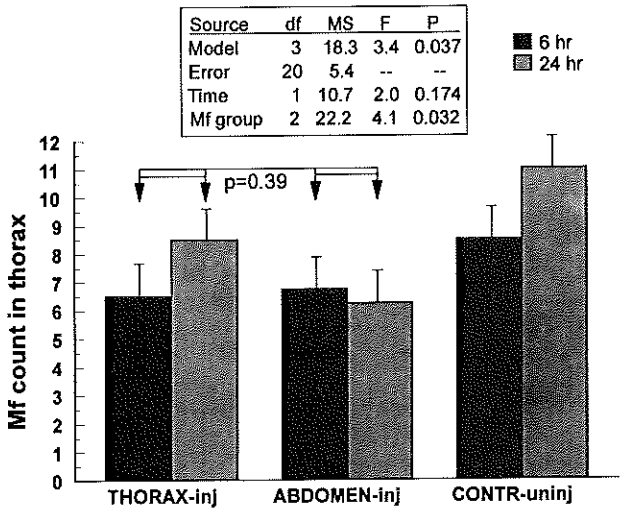

Fig. 4. Migration success of *O. lienalis* mf that were recovered from thorax vs. abdomen of previously inoculated flies. Mf were collected either from thoraces (THORAX-inj) or abdomens (ABDOMEN-inj) of previously injected flies (2–3 h pi), or from the original mf preparation (CONTR-uninj), then inoculated into flies. Their migration success was determined 6 and 24 h pi. Group means (4 flies/group) and SEM based on the pooled variance (inset) are plotted. The difference between mf recovered from thorax and abdomen was not significant as indicated by arrows. Table summarizes model structure and analysis results.

Table 2. Relationship between *O. lienalis* mf migration success and mf loss.

Period pi (N)	Correlation between mf loss and mf count in thorax		mf proportion in thorax	
	r[a]	P[a]	r[a]	P[a]
1–2 h (24)	–0.53	0.008	–0.14	0.50
	(–0.57)	(0.004)	(–0.15)	(0.48)
2.5–6 h (46)	–0.35	0.017	0.02	0.88
	(–0.30)	(0.041)	(0.16)	(0.27)
8–12 h (26)	–0.57	0.002	0.01	0.96
	(–0.49)	(0.011)	(0.07)	(0.73)
20–24 h (21)	–0.80	0.001	–0.09	0.68
	(–0.94)	(0.001)	(–0.35)	(0.13)
1–24 (117)	–0.51	0.001	–0.03	0.72

[a]Values without parenthesis refer to correlation between the z-scores of the counts and values within parenthesis refer to correlation between the counts. Z-scores were calculated based on the mean and the standard deviation of each time point separately for each experiment as follows: (individual count – mean)/standard deviation.

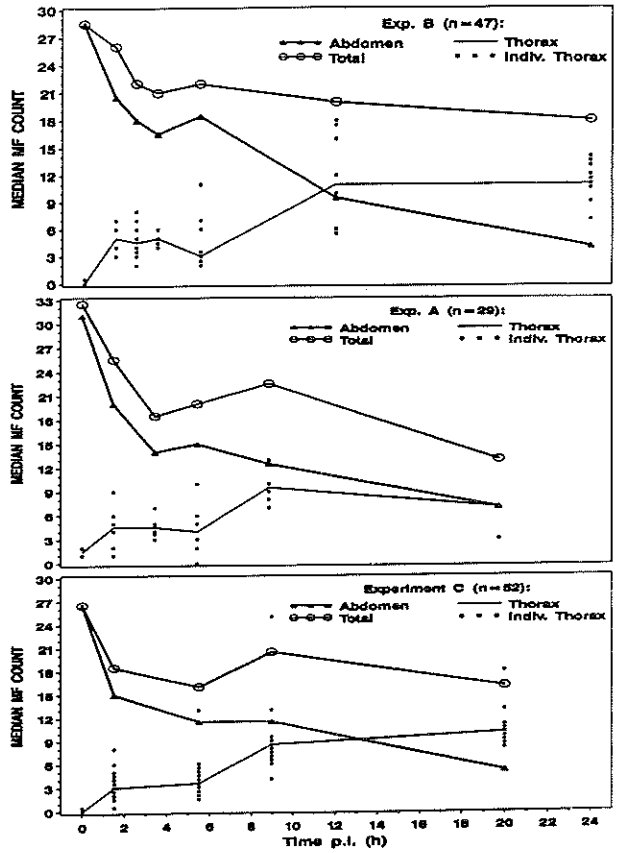

Fig. 5. The simultaneous change in the median *O. lienalis* mf count in the whole fly (indicating loss), in the abdomen, and in the thorax for each experiment. Individual thoracic counts are shown as dots (jittering allowed separation of no more than two duplicated values). Abdominal counts include infrequent mf that were counted in the head.

locate the thorax during the second migration phase was also indicated by the behaviour of mf inoculated directly into the thorax (fig. 1). The capacity of mf to respond to thoracic cues could be changed internally. External effects also could change mf ability to locate the thorax; for example, target molecules or mf sensory organs could be temporarily masked by compounds from the injection medium (20% foetal bovine serum, 79% RPMI 1640, and 1% antibiotics). Biphasic migration pattern was indicated in *O. lienalis* mf migrating in *S. ornatum* (Eichler, 1973). The first migration phase occurred 15 min to 1 h post feeding, and the second phase 4–6 h post feeding, although mf escaped the midgut in less than 1 h post feeding. This similarity suggests that the change in the mf ability to locate the thorax within the haemocoel was controlled internally. *Onchocerca volvulus* mf migrating in *Simulium damnosum* (Laurence, 1966) and *O. cervicalis* in *C. nubeculosus* and in *C. variipennis* (Mellor, 1975), however, had a continuous 1-phase migration.

Migration success was density-independent (fig. 3), and no facilitation nor limitation (Dye, 1992) seemed to be involved. The constant proportion of mf found in the thorax 24 h pi, while a substantial number of motile mf were in the abdomen, raised the question of what limits migration success. The hypothesis that about one third of mf represent competent migrators, while two thirds represent incompetent migrators agrees with other studies indicating that while passing from the uterus to the skin, mf 'mature', morphologically, antigenically, and in terms of development potential in the vector (Kartman, 1953; Mellor, 1974; Hollanda *et al.*, 1982; Fuhrman *et al.*, 1987; Holdsworth, 1987). Although we used only skin-inhabiting mf, some heterogeneity could remain in this subpopulation. This hypothesis was rejected, however, because similar migration success was observed between supposedly competent and incompetent migrators (fig. 4, table 2). Nevertheless, mf were recovered from flies at 2 h pi, hence, could not represent 'competent' migrators of the second migration phase. Thus, this hypothesis deserves further investigation.

The negative correlation between mf count in the thorax and mf loss (table 2) suggested that increased loss reduces migration success of mf (because less of them survive), or that increased migration success reduces mf loss, supposedly due to protection that mf gain in the thorax. The zero correlation coefficient between the proportion of mf in the thorax and mf loss (table 2), disproved the second explanation. The possibility that a 'third factor' is involved should also be expressed in a negative correlation between the proportion of mf in the thorax and mf loss. Such a relationship was evidently not present (table 2). Thus, the immune defence has a considerable effect on mf migration success (12–28% of the variation in mf migration success was accounted for by the variation in mf loss during the first and the second migration phases).

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