To the Graduate Council:

I am submitting herewith a thesis written by Robert Banks Simpson entitled "Some Aspects of the Persistence of Moraxella bovis on the External Surface of the Face Fly (Musca autumnalis) DeGeer." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Reid R. Gerhardt
Major Professor

We have read this thesis and recommend its acceptance:

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Accepted for the Council:

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Graduate Studies and Research

SOME ASPECTS OF THE PERSISTENCE OF *MORAXELLA BOVIS* ON THE EXTERNAL SURFACE OF THE FACE FLY (*MUSCA AUTUMNALIS*) DEGEER

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

Face flies were contaminated externally with Moraxella bovis. The maximum period of recovery of M. bovis from the flies was eight hours. Exposure to high humidity lengthened the time at which M. bovis was isolated from contaminated flies. Exposure to ultraviolet light reduced the amounts of M. bovis recovered from flies. M. bovis was found in the crops of face flies that were contaminated with a liquid layer of M. bovis and saline.

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I. INTRODUCTION

The face fly, Musca autumnalis DeGeer (Diptera: Muscidae), was first collected in North America at Middleton, Nova Scotia in 1952 (Vockeroth, 1953). By 1969, the fly was present in all of the continental United States except Florida, Louisiana, Texas, New Mexico and Arizona (USDA, 1969, 1975).

Since the discovery of the face fly in North America, much time and effort has been spent studying the biology and control of this insect. Review articles covering the major aspects of face fly biology, behavior and control were presented by Teskey (1960), Ode and Matthysse (1967) and Pickens and Miller (1980). Smith et al. assembled an annotated bibliography and supplements dealing with the face fly in North America (Smith et al., 1966; Smith and Linsdale, 1967, 1968, 1969, 1971; and Smith and Krancher, 1974).

Lacrimal secretions of cattle are believed to be a source of protein for face flies, which feed frequently in large numbers around the eyes and muzzles of cattle (Hammer, 1942; Matthew, 1960; Teskey, 1960; Treece, 1960; Turner and Hair, 1967). This feeding behavior enhances the fly's ability to transmit Moraxella bovis (Hauduroy), the causative organism of infectious bovine keratoconjunctivitis (IBK, Pinkeye).

IBK is a highly contagious disease which affects all ages and breeds of cattle (Baldwin, 1945). Blood et al. (1979) described the clinical findings associated with IBK:

> An incubation period of 2-3 days is usual although longer intervals, up to 3 weeks, have been observed after experimental introduction of the bacteria. Injection of the corneal vessels and oedema of the conjunctiva are the earliest signs and are accompanied by a copious, watery lacrimation, blepharospasm, photophobia and, in some cases, a slight to moderate fever with fall in milk yield and depression of appetite. In 1-2 days, a small opacity appears in the centre of the cornea and this may become elevated and ulcerated during the next 2 days although spontaneous recovery at this stage is quite common. This opacity becomes quite extensive and at the peak of the inflammation, about 6 days after signs first appear, it may cover the entire cornea. The colour of the opacity varies from white to deep yellow. As the acute inflammation subsides, the ocular discharge becomes purulent and the opacity begins to shrink, complete recovery occurring after a total course of 3-5 weeks.

The economic impact of IBK is quite considerable. In 1977, the cattle industry in Tennessee lost an estimated $10 million due to pinkeye (Hall, R., personal communication). Cattle showing signs of IBK have been reported to show reduced weight gains (Thrift and Overfield, 1974) and lowered milk production (Baldwin, 1945). Treatment of the disease and the possibility of injury due to temporary or permanent blindness add to the costs resulting from an episode of IBK.

Much debate has taken place concerning the etiology of IBK. Organisms which have been suggested as having some association with IBK include various bacteria, viruses,

rickettsia-like organisms and psittacosis agents (Wilcox, 1968). M. bovis, a bacterium, is now considered the causative organism of this costly cattle disease; it is the only organism for which the fulfillment of Koch's postulates has been demonstrated (Henson and Grumbles, 1960; Hughes et al., 1965).

Micromorphologically, M. bovis appears as a plump diplobacillus with rounded or almost square ends (Henriksen, 1973). Mitter (1915) was the first investigator to link IBK infection with an organism resembling M. bovis. Allen (1919) demonstrated a short, thick, gram-negative diplobacillus from infected eyes in an outbreak of infectious keratitis. Jones and Little (1923) recovered morphologically-similar bacteria from eyes of cattle during an outbreak of infectious ophthalmia. M. bovis was further implicated as the causative agent of IBK in later reports (Baldwin, 1945; Watt, 1951; Barner, 1952; Jackson, 1953; Faull and Hawksley, 1954; Gallagher, 1954; Wilcox, 1970).

While M. bovis is widely accepted as the causal agent of IBK, potentiating factors such as ultraviolet light, flies and dust are believed to play a key etiologic role. These factors irritate the eyes of cattle and appear to predispose them to bacterial invasion (Hughes et al., 1965; Hubbert and Hermann, 1976).

Flies, in general, have long been suspected of playing a possible role in the dissemination of IBK (Allen, 1919;

Jones and Little, 1923). Keratitis contagiosa was reported to occur in cattle of North America long before the presence of the face fly (Billings, 1889). However, as the face fly became more prevalent in the United States, an increase in the occurrence of eye disorders was noticed (Treece, 1960; Benson and Wingo, 1963). Steve and Lilly (1965) suggested that the face fly might be involved as a mechanical vector in the spread of IBK. Cheng (1967) reported a consistent increase in cases of IBK in pastures where cows were more heavily infested with face flies. Gerhardt et al. (1976) reported that a 65% reduction in face fly numbers on cattle resulted in a much lower incidence of IBK. Brown and Adkins (1972) demonstrated that face flies could transmit M. bovis from an artificial medium to the eyes of susceptible calves. Gerhardt et al. (in press) observed the number of M. bovis isolations from cattle to be highly correlated to the number of face flies on those animals. They also isolated M. bovis from face flies of a wild population that were aspirated from heads of restrained cattle.

Steve and Lilly (1965) reported that M. bovis remained viable on legs and wings of face flies up to three days after exposure to M. bovis cultures. They contaminated flies by replacing one end of a container used to hold the flies with a blood agar plate overlaid with M. bovis and saline in a thin liquid layer. As they held the plate over a light

source, they reported that the flies were attracted downward to the plate for feeding and tracking. Their results differ from an earlier study which reported recovery of diplobacilli from house flies no longer than three hours after exposure (Jones and Little, 1924). Both reports concluded that M. bovis was apparently destroyed upon ingestion, but Glass et al. (unpublished manuscript) recovered it from the crops of face flies up to 48 hours after ingestion.

Greenberg (1973) mentioned numerous factors considered to be detrimental to the viability and mechanical transport of microbes on the integument of the fly. These factors include dessication, insolation of the body surface, ultraviolet radiation and the constant self-cleaning activity of the fly. McGuire and Durant (1957) found amounts of internal bacteria to greatly outnumber counts of bacteria on the external surface of flies. Greenberg further suggests that some reports of pathogen persistence on insects may be exaggerated as a result of the failure to recognize the importance of fly-to-fly contamination via the surface of the insects' holding cages used during such a study. He points out other investigations that substantiate this theory (Ostrolenk and Welch, 1942; Gwatkin and Mitchell, 1944; Webb and Graham, 1956).

Most strains of the genus Moraxella are more or less fastidious (Bergey's Manual, 1974). This indicates that the

in vitro persistence of *M. bovis* is limited without the presence of certain vital environmental conditions. The length of time that *M. bovis* remains viable on the external surface of the face fly is an important factor in the determination of the fly's ability to disseminate this pathogen.

This study originated with the concept that *M. bovis* is only a moderately hardy organism, and may not be as persistent on the external surface of the face fly as was previously reported. The effect of such environmental conditions as relative humidity and ultraviolet light on the longevity of *M. bovis* was determined. Uninfected calves were exposed to externally contaminated flies in an effort to transmit *M. bovis* to the eyes. An attempt was made to evaluate the possibility of *M. bovis* ingestion during the external longevity trial of Steve and Lilly (1965).

II. MATERIALS AND METHODS

Laboratory-reared face flies were contaminated with M. bovis by placing them for five minutes onto potato dextrose agar plates (100 × 15 mm) overlaid with a suspension of a known concentration of M. bovis in distilled water. The flies were exposed at the rate of four flies per 0.5 milliliter of suspension per plate. The flies were CO_2-anesthetized for ease of handling. The persistence of M. bovis on the flies was measured by allowing the flies to track on sterile 5% bovine blood agar plates (100 × 15 mm) for five minutes at selected time intervals after contamination at the rate of four flies per plate. The resulting M. bovis colonies were counted after a 24-hour incubation at 37°C. At this time, Beta-hemolytic, flat, gray, semi-translucent colonies were considered to be M. bovis. With the absence of any other organisms resembling M. bovis, it was easily distinguished. On occasion, M. bovis determination was verified by the method described by Pugh et al. (1966). The data were tabulated on a basis of the number of M. bovis colonies per fly. The data were analyzed by simple linear regression and analysis of variance.

Precontamination Treatment

To ensure that this investigation dealt only with the external contamination of face flies, all flies mentioned,

unless otherwise specified, were given the following precontamination treatment: Approximately three hours before exposure to the bacterial suspension, food and water were removed from flies. Approximately one-half hour before exposure, flies were offered a water-soaked ball of cotton. This treatment encouraged repletion of the flies with water prior to exposure to the suspension.

To determine the success of this precontamination treatment in preventing ingestion of the suspension, 147 flies were exposed to potato dextrose agar plates overlaid with M. bovis suspensions containing nigrosin to facilitate detection of the suspension in the digestive tract should ingestion occur. Flies administered the treatment were dissected and digestive tracts were examined for the presence of nigrosin. Twenty-five additional untreated flies were offered a dyed 0.1 Molar sucrose solution, dissected and examined to ensure the efficacy of this technique.

Determination of Concentrations Used to Contaminate Flies

Concentrations of the bacteria in the suspensions used to contaminate flies were determined by making serial dilutions from those suspensions and plating known volumes of those dilutions onto blood agar. The resulting colonies were counted and the actual number of bacteria to which the flies were exposed was calculated. In the surface and fly-to fly contamination trials, face flies were contaminated

with a standard concentration suspension of M. bovis. This concentration was used so that the resulting M. bovis colonies on those blood agar plates exposed to flies immediately after contamination were discrete and could be counted. To consistently achieve this concentration, absorption readings were made with a Beckman Model 35 spectrophotometer for each bacterial suspension and compared with colony counts on blood agar plates resulting from previously prepared suspensions.

Flies Exposed to Varying Laboratory Conditions

In the four initial laboratory experiments, a total of 104 face flies were contaminated with concentrations of M. bovis ranging from 10.6×10^6 Colony Forming Units (CFU)/ml to 475×10^6 CFU/ml. Blood agar plates were exposed to flies at 1, 2, 5, 6 and 24 hours after contamination. After contamination, the flies were put into perforated 6 cc. Monoject® disposable syringe sleeves which were screened at the open end. Between blood agar exposures, the flies were placed into clean sleeves and held in the laboratory at 26-27°C and variable relative humidity.

In a separate test, flies were contaminated with a M. bovis concentration of 450×10^6 CFU/ml and exposed to three different conditions in the laboratory. Thirty-two flies were exposed to 56% relative humidity and ultraviolet

light, 28 flies were exposed to 56% relative humidity with no ultraviolet exposure, and 32 additional flies were exposed to 80% relative humidity with no exposure to ultraviolet light. The flies exposed to 56% RH and no ultraviolet light served as a control group for the flies exposed to the other two conditions. The source of ultraviolet light was a Westinghouse® FS 40 sunlamp. The flies were placed 21-42 cm from the lamp for five minutes after contamination. The persistence of M. bovis on the flies was monitored at 5, 35, 95, 155, 300, 480 and 1,440 minutes after contamination.

Slide smears containing 0.1 ml of the same concentration of M. bovis were prepared and exposed to the same three conditions. The persistence of M. bovis on the slides (75 × 25 mm) was monitored by rubbing a sterile cotton applicator across each slide and streaking the applicator onto blood agar plates at 35, 95, 155, 300, 480 and 1,440 minutes after the preparation of the slide. Sixty slide smears were exposed to each condition with samples taken from ten slides at each time interval. The temperature was 27°C in both the fly and slide smear experiments.

A comparison was made to determine the difference, in M. bovis persistence, between 76 contaminated flies that were returned to the same cages after blood agar exposure and 76 contaminated flies that were placed in clean cages between exposures. To investigate any possible cross-contamination resulting from the handling of flies in pools

of four, 24 additional flies were placed into individual cages after contamination and remained separated from any other flies throughout the trial. They were placed in clean individual cages after each blood agar plate exposure. The persistence of M. bovis on these individually-caged flies was compared to that of flies caged in groups of four that were placed in clean cages between blood agar exposure. The concentration of M. bovis used to contaminate the flies grouped in fours was 9,428 CFU/ml. Three slightly different concentrations, ranging from 9,428 CFU/ml to 11,785 CFU/ml, were used during the individual fly trials. In both comparisons, the persistence of M. bovis was monitored at 5, 35, 95 and 155 minutes after contamination.

Exposure of Uninfected Calves to Contaminated Flies

On three successive days, externally contaminated face flies were placed in 7 × 9 cm screen wire cages covering both eyes of two uninfected Angus calves on the Grasslands Farm Unit of the Plateau Experiment Station in Crossville, Tennessee.

To obtain an estimate of the number of bacteria actually being picked up by the flies, each day 20 additional flies were contaminated with the suspension used that day and blood agar plates were immediately exposed to them. The average number of bacteria per fly immediately after contamination was then determined.

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Prior to each day's fly exposure, the eyes of both calves were swabbed with a sterile cotton applicator for isolation of M. bovis. The amount of lacrimation was rated both prior to and following the fly exposure with the system used by Shugart et al. (1979).

The following number of flies were exposed to an assigned eye each day for one hour:

Eye 1—10 flies
Eye 2—20 flies
Eye 3—30 flies
Eye 4—40 flies (only 30 flies on day two)

Following each day's exposure, 20 flies were randomly selected and allowed to track on blood agar plates in order to detect any M. bovis remaining on the flies.

Additional swab samples were taken from each eye three days after the last exposure. At this time, eyes were also evaluated for clinical signs of IBK.

Ingestion of M. bovis in Saline

An attempt was made to repeat the method of Steve and Lilly (1965). They contaminated face flies with M. bovis in a saline suspension and attempted to recover the bacteria from the exoskeleton and digestive tract, respectively. Twenty flies (one day old) were placed in a pint cardboard ice-cream container with aluminum screening used to enclose each end of the container. A few drops of physiological

saline were applied to a blood agar plate with a 24-hour, streaked culture of M. bovis. The surface of the plate was then smeared and spread in order to produce a thin liquid layer. The concentration of M. bovis in this layer was estimated to be $1,218 \times 10^6$ CFU/ml. Without a precontamination treatment, the flies were placed directly on these plates for approximately ten minutes. They were then cold-anesthetized, and their crops were removed and streaked onto blood agar plates.

Twenty additional flies were allowed to track on blood agar at 5, 35, 95, 155 and 1,440 minutes following contamination. These flies were held in clean containers between tracking exposures. After the last tracking exposure, their crops were removed and plated as previously described.

III. RESULTS AND DISCUSSION

The use of the precontamination treatment was highly effective in preventing the ingestion of the bacterial suspension. In the tests carried out to determine the efficacy of this treatment, none of the 147 face flies exposed to the dyed suspension showed any dye in their digestive tracts. The dye was detected in the digestive tracts of 13 out of the 25 flies exposed to the sucrose solution. The precontamination treatment was necessary to avoid any complications that may have resulted from the flies' ingestion of M. bovis and regurgitation of bacteria onto the blood agar during the CO_2 anesthesia.

The use of potato dextrose agar as a substrate, as well as the use of distilled water as the medium for the bacterial suspension apparently further eliminated the possibility of flies ingesting M. bovis during contamination. When flies are placed on blood agar containing M. bovis cultures, they appear to be highly attracted to the blood for feeding (personal observation).

In the initial laboratory experiments using four different concentrations, linear regression analysis indicates the rapid decrease in bacteria numbers (Figure 1, Appendix). Six hours was the maximum period of time at which M. bovis was recovered from the flies. Elapsed time

had a significant effect ($P < .01$) on the amount of M. bovis recovered from flies in all concentrations except 475×10^6 colony forming units (CFU)/ml. The concentration of bacteria used to contaminate the flies had no significant effect ($P < .01$) on the rate at which the M. bovis colonies declined in number.

The results from exposing contaminated flies to three different laboratory conditions are presented in Figures 2 and 3, Appendix. There were no significant differences ($P < .05$) between the rates at which M. bovis was recovered from flies exposed to any of the three conditions. However, M. bovis was isolated eight hours after contamination from flies exposed to high humidity and no ultraviolet light. This was the maximum length of time at which M. bovis was detected during the entire investigation. These results are comparable to those from the slide smears. When the slides were exposed to 56% humidity, with or without ultraviolet light exposure, the maximum time of M. bovis recovery was five hours. Eight hours was the maximum recovery time from the slides exposed to the high humidity.

Time had a significant effect on the survival of M. bovis on flies that were always returned to the same cages ($P < .01$), as well as on flies that were placed in clean cages ($P < .05$) (Figure 4, Appendix). No significant difference ($P < .05$) was noted between the rates at which M. bovis numbers declined

in number. The longest interval between contamination and blood agar exposure in these trials was 155 minutes. The number of M. bovis colonies generally approached zero by this time.

In the earlier trials in which higher concentrations of bacteria were used, the number of colonies did not approach zero this rapidly. However, when the slope and intercept of a higher concentration was compared with that of a lower concentration, no significant difference ($P < .05$) was noted.

Caging individual flies separately throughout the period of the trial had no significant effect ($P < .05$) on the duration of M. bovis persistence in comparison to that of flies grouped in fours (Figure 5, Appendix). The use of three slightly different concentrations during the individual fly trials had no effect ($P < .05$) on M. bovis survival, and the effect of time on M. bovis persistence was highly significant ($P < .01$).

No M. bovis was recovered from the 60 flies selected to track on blood agar after the one hour calf exposure (Table 1, Appendix). No M. bovis was recovered by swabbing the eyes of the calves exposed to contaminated flies for three consecutive days, and no clinical signs of IBK were observed in these animals three days after exposure to the flies. The flies were observed to feed readily on and around the eyes

and substantial lacrimation was noted after each day's exposure.

In the experiment undertaken to determine if M. bovis was ingested with saline on blood agar, the flies were readily attracted to the light when one of the screened ends was held above the light source. When one of the ends was replaced with a blood agar plate containing a thin liquid layer of M. bovis and saline, and that plate held over the light, the flies did not respond to the light. For this reason, flies had to be placed directly on the plates to achieve contamination.

M. bovis was recovered from 15 out of the 20 crops that were dissected and plated immediately after contamination. When 20 additional flies were contaminated in the same manner and allowed to track on sterile blood agar plates, M. bovis colonies were too numerous to count on these plates at 5, 35, 95 and 155 minutes after contamination. When sterile blood agar plates were exposed to the flies for tracking at 1,440 minutes after contamination, each plate was positive for M. bovis. The number of M. bovis colonies/fly on these plates ranged from 22 to 137, with a mean of 95 colonies/fly. M. bovis was recovered from three of the 20 crops when dissected and plated.

IV. SUMMARY

The results of this study indicate that the survival of M. bovis on the external surface of the face fly is not as extended as was previously reported. The rapid decline in the rate at which M. bovis was recovered from face flies was the obvious, consistent trend in each experiment. M. bovis was never recovered from externally contaminated face flies at 24 hours after contamination; and the maximum recovery time of eight hours was from flies and slide smears that were exposed to what was considered to be favorable conditions for extended survival. Exposure of contaminated flies to ultraviolet light did shorten the survival of M. bovis on those flies, but this shortened survival was not significantly different from that of flies exposed to no ultraviolet light.

The somewhat brief persistence of M. bovis on the external surface of the face fly indicates that the fly may not be able to transmit M. bovis from their external surface between herds of cattle that are separated by substantial distances. Instead, any such external transmission would probably occur among animals of the same herd.

The recovery of M. bovis from the crops of face flies which were contaminated with M. bovis and saline indicates that previously reported work concerning the persistence of

M. bovis on the external surface of the fly may have not considered the possibility of complications resulting from the ingestion of bacteria during contamination, especially in view of the fact that CO_2-anesthesia causes flies to regurgitate.

The rapid rate of decline of M. bovis on the external surface of face flies and the recovery of M. bovis from the crops of face flies indicate an increased likelihood that the transmission of M. bovis by the face fly may involve an internal, as well as external, avenue.

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APPENDIX

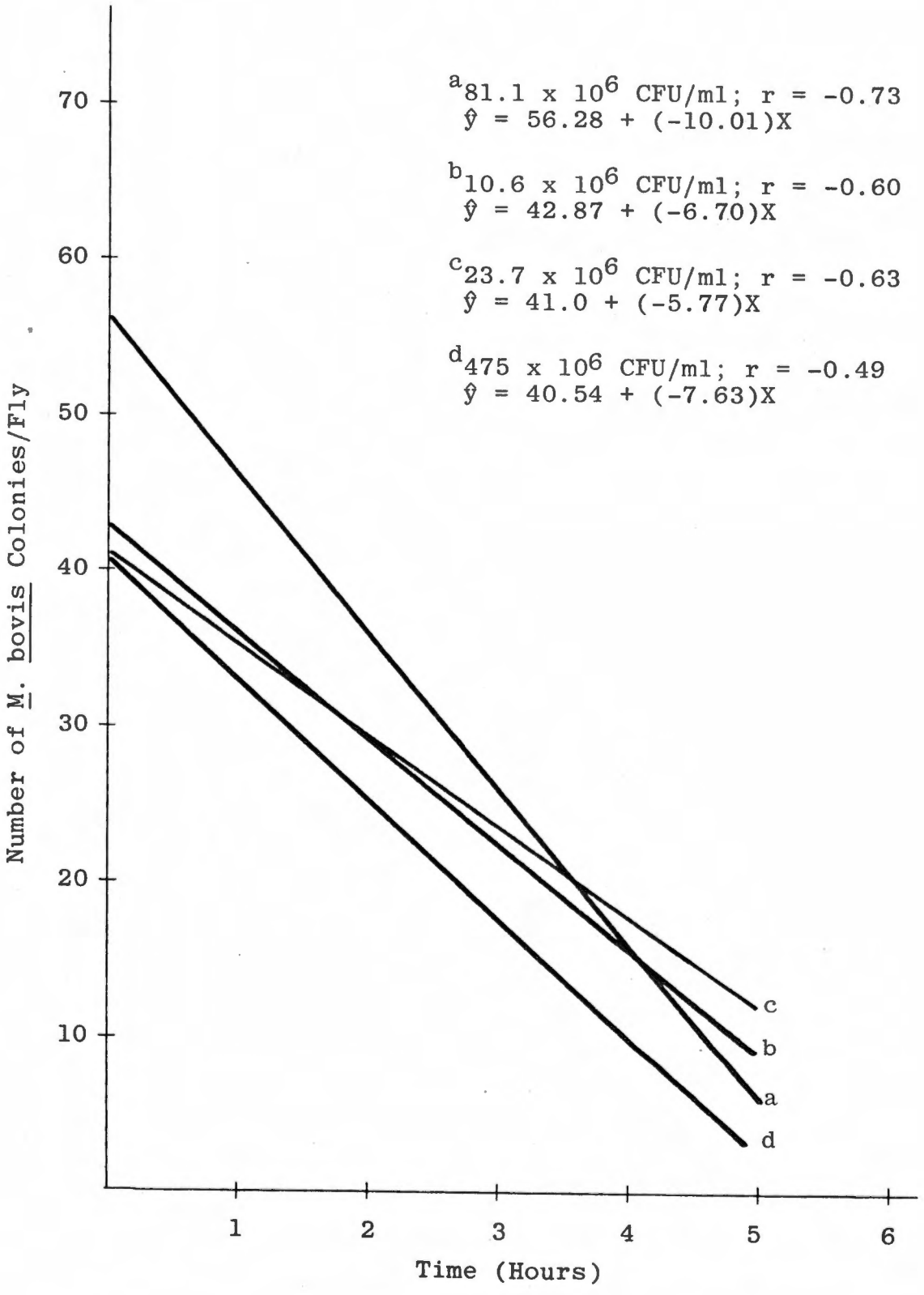

Figure 1. Recovery of M. bovis from face flies contaminated with different concentrations.

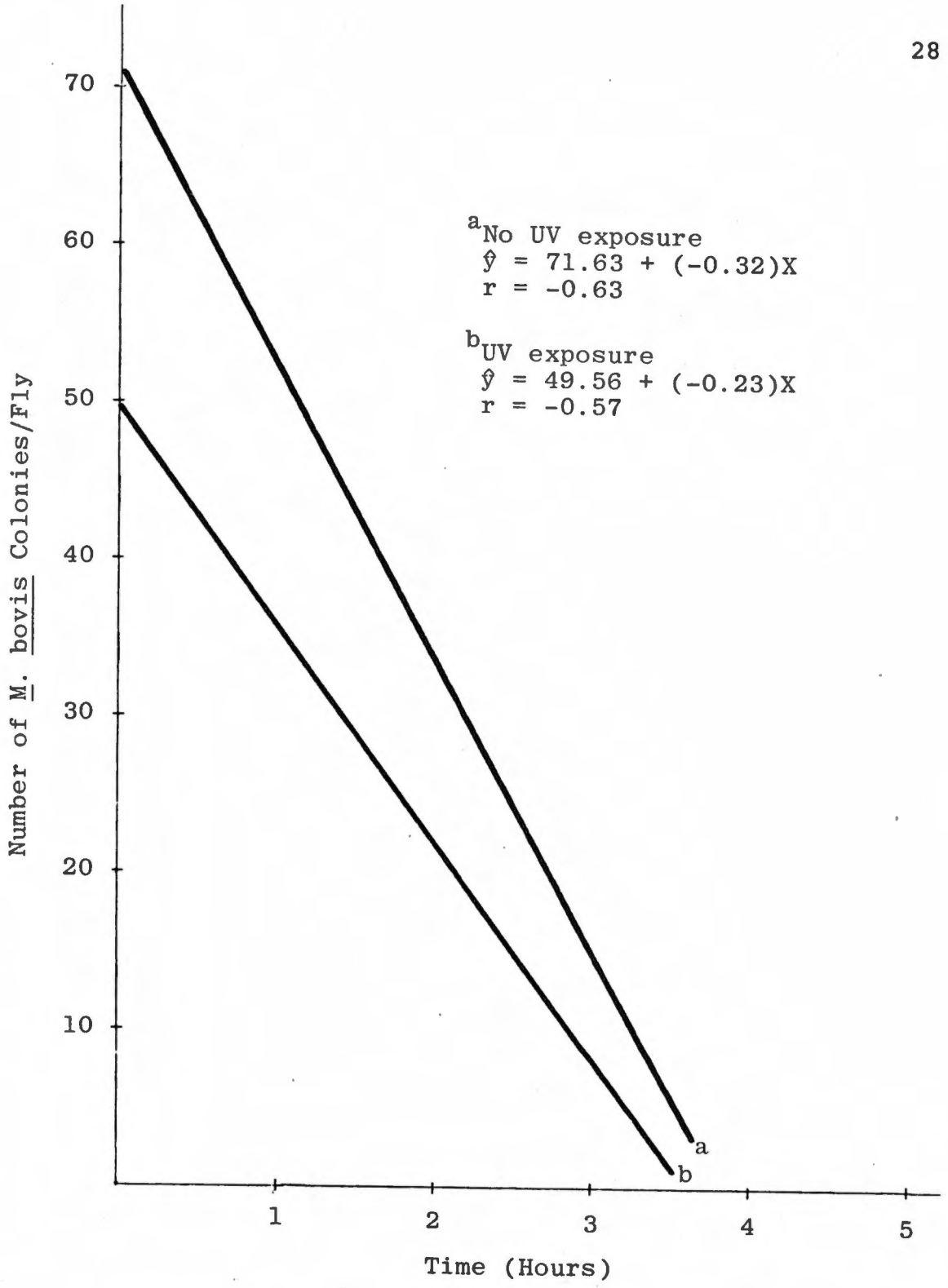

Figure 2. The effect of ultraviolet light on the recovery of M. bovis from contaminated face flies.

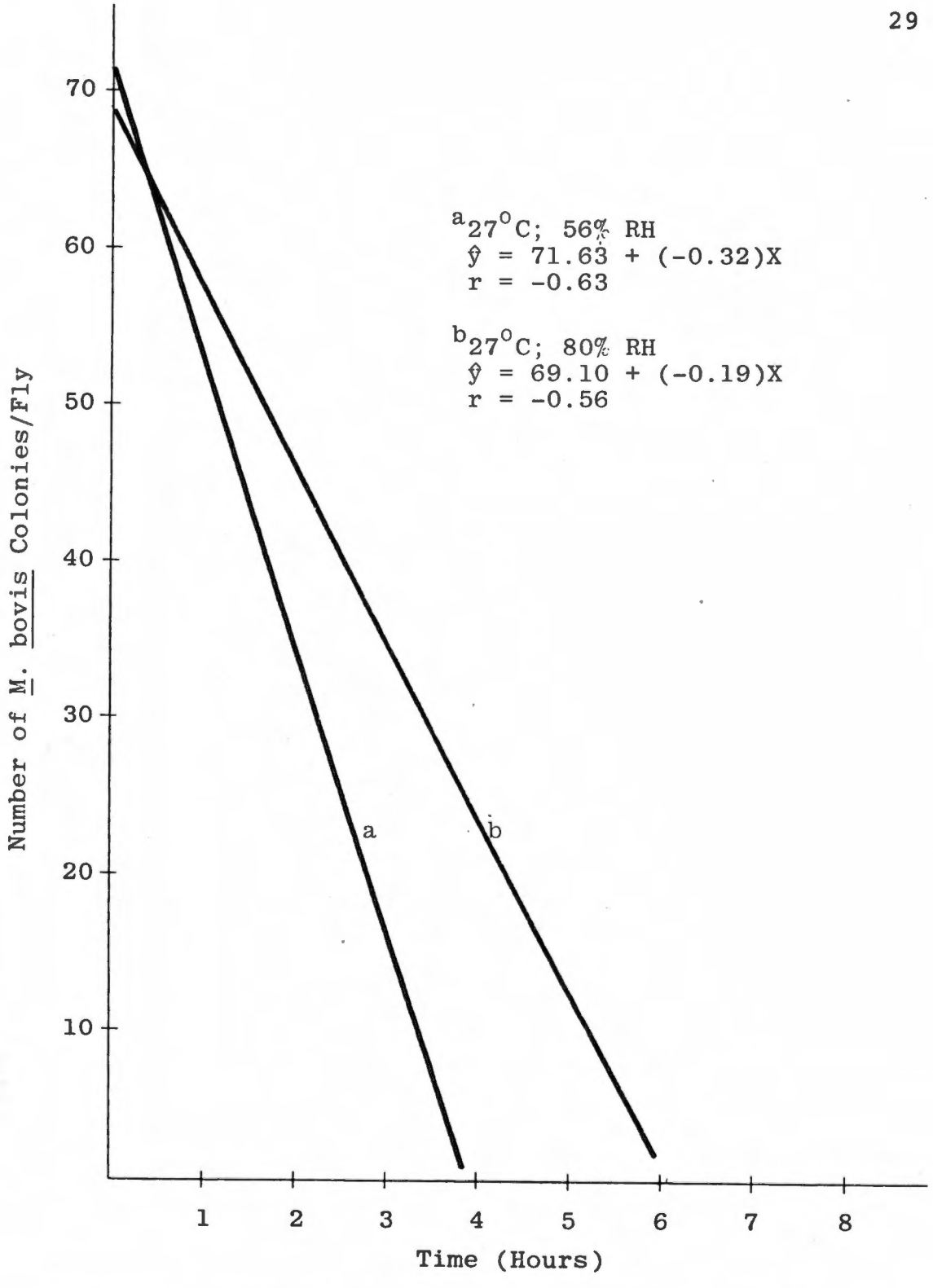

Figure 3. The effect of relative humidity on the recovery of M. bovis from contaminated face flies.

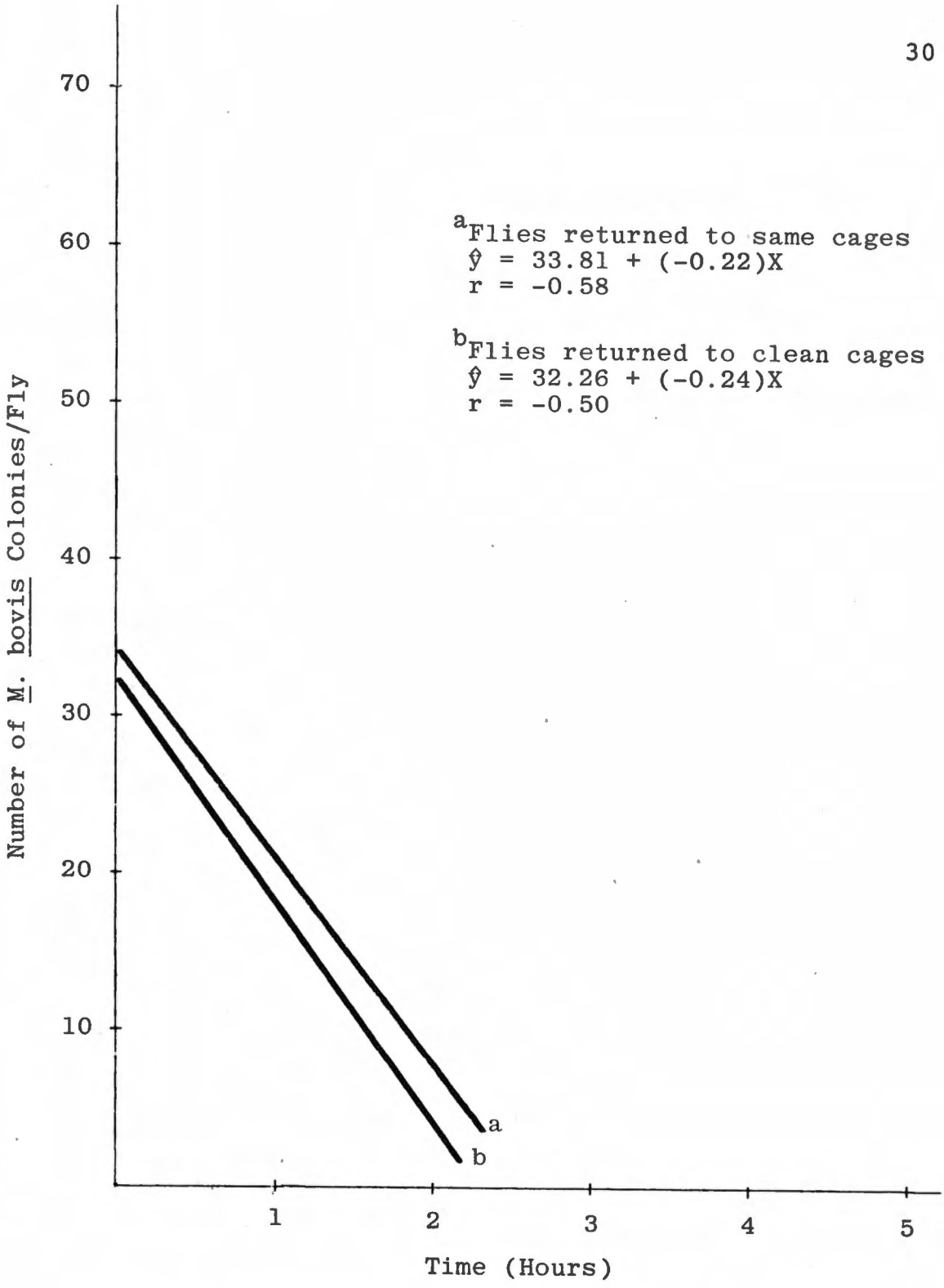

Figure 4. The effect of the cleanliness of holding cages on the recovery of M. bovis from contaminated face flies.

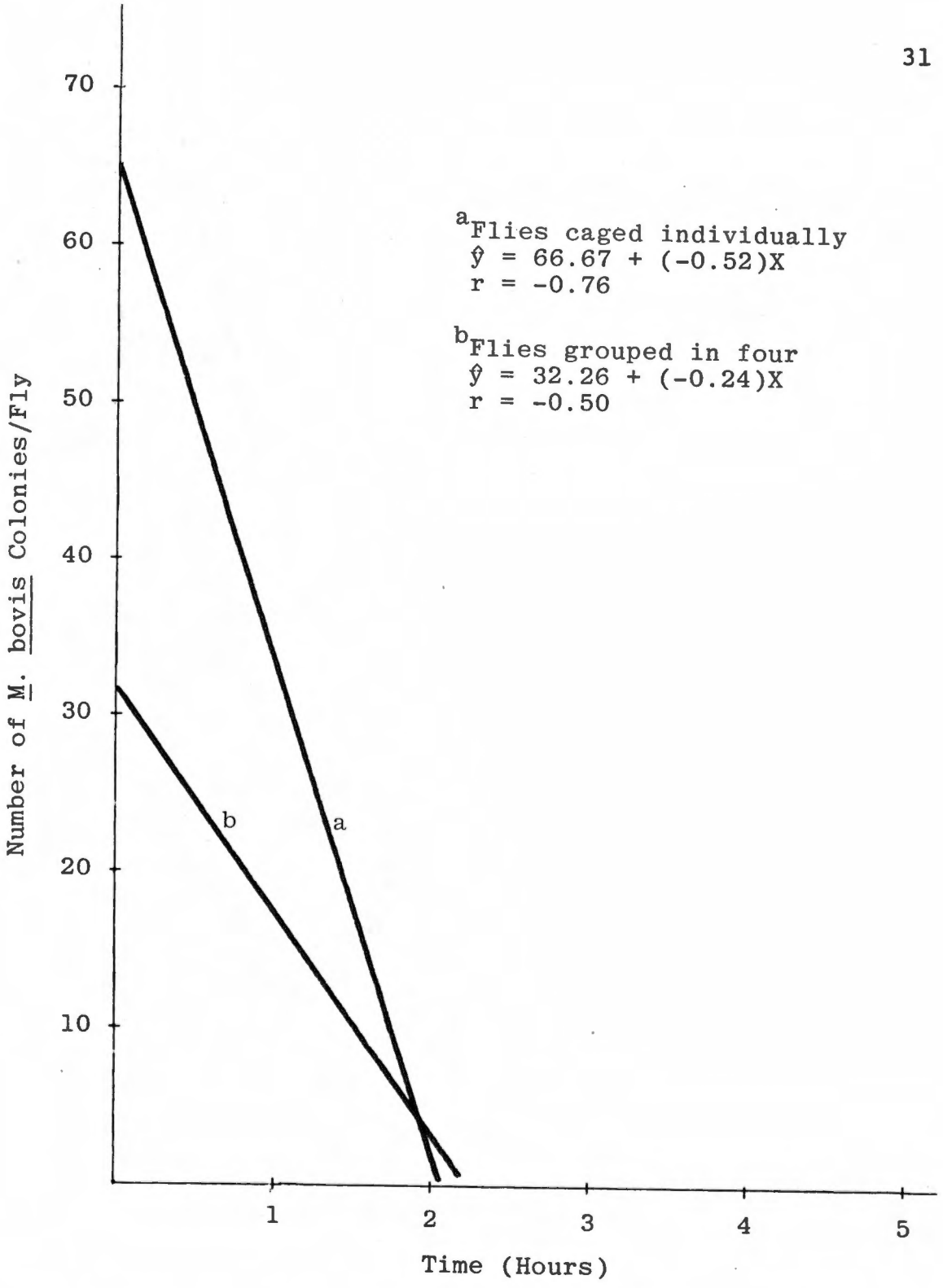

Figure 5. The effect of caging contaminated flies individually on the recovery of M. bovis.

Table 1. Results from Three Days Exposure of Uninfected Calves to Externally Contaminated Face Flies

Day	Estimated Concentration of Suspension Used to Contaminate Flies	Estimation of No. of Bacteria Picked Up by Each Fly During Contamination	Estimation of No. of Bacteria to Which Calves Were Exposed		Severity of Lacrimation	
					Pre-exp.	Post-exp.
1	45×10^6 CFU/ml	174	Eye 1—1,740	Eye 1	0	+1
			Eye 2—3,480	Eye 2	0	+2
			Eye 3—5,220	Eye 3	0	+1
			Eye 4—6,960	Eye 4	0	+1
2	1.9×10^6 CFU/ml	113	Eye 1—1,130	Eye 1	0	+2
			Eye 2—2,260	Eye 2	+1	+2
			Eye 3—3,390	Eye 3	0	+1
			Eye 4—3,390	Eye 4	0	+1
3	0.39×10^6 CFU/ml	27	Eye 1— 270	Eye 1	+1	+3
			Eye 2— 540	Eye 2	+2	+3
			Eye 3— 810	Eye 3	+2	0
			Eye 4—1,080	Eye 4	0	+1

VITA

Robert Banks Simpson was born in Alexandria, Tennessee on July 5, 1957. He graduated from Central High School in Columbia, Tennessee in 1975. After one year at Middle Tennessee State University, he transferred to The University of Tennessee, Knoxville where he majored in Animal Science and received his Bachelor of Science degree in Agriculture in June 1979. Later that month, he began as a graduate research assistant in the Department of Entomology and Plant Pathology and received his Master of Science degree in Entomology and Plant Pathology in December 1981.

While at The University of Tennessee, Knoxville, he became a member of the Alpha Gamma Rho social-professional fraternity, the Alpha Zeta fraternity, the Gamma Sigma Delta honor and service society, the Entomological Society of America and the Tennessee Entomological Society. He was a member of the 1977 University of Tennessee Dairy Products Judging Team, the 1978 University of Tennessee Livestock Judging Team, the 1978 College of Agriculture Student-Faculty Council and the Block and Bridle Club.

He is married to the former Kimberly Millsaps of Friendsville, Tennessee.