EFFECT OF ENVIRONMENTAL TEMPERATURES ON INFECTION WITH *MYCOBACTERIUM MARINUM* (BALNEI) OF MICE AND A NUMBER OF POIKILOTHERMIC SPECIES

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ABSTRACT

CLARK, H FRED (Communicable Disease Center, Atlanta, Ga.), AND CHARLES C. SHEPARD. Effect of environmental temperatures on infection with *Mycobacterium marinum* (Balnei) of mice and a number of poikilothermic species. J. Bacteriol. **86**:1057–1069. 1963.—An exploration was made of the effect of environmental temperature on infections with *Mycobacterium marinum* of mice, young opossums, and bats, and of 50 species of poikilothermic animals. In artificial medium (7H9 broth) *M. marinum* grew most rapidly from 25 to 35 C, with generation times of 4 to 6 hr. At 37 C, the generation time was 14 hr; at 20 C, 20 hr; and, at 15 C and lower, little growth was observed. In mice, deep body temperatures were found to be 36.5 to 37.3 C at environmental temperatures of 4 to 30 C. At an environmental temperature of 34 C, they averaged 39.1 C; at 37 C they averaged 40.2 C. Foot-pad temperatures were within a few degrees of ambient temperatures from 10 to 34 C. In mouse foot-pad infections, the optimal environmental temperature for infection was 20 C, and the generation time of the infecting bacilli at this environmental temperature was about 15 hr. Intravenously inoculated mice developed peripheral infections of nose, feet, and tail at environmental temperatures of 4 to 30 C. At these temperatures, they had severe pneumonic involvement, and the mice at lower temperatures tended to succumb most rapidly to systemic infection. At 34 C, the intravenously infected mice did not develop peripheral infections and there was no pulmonary involvement. Young opossums, whose deep body temperatures are only 34 to 36 C, were inoculated in the foot-pad and intravenously. Foot-pad infection developed without systemic involvement. Bats, which assume environmental temperature when at rest, were inoculated in the foot-pad. Foot-pad infections were observed but no systemic disease. The bats could be maintained for only short periods, however. Poikilothermic animals were studied. Deep body temperatures were found to be nearly identical with ambient temperature. A total of 50 species of reptiles, amphibians, and fish were infected intraperitoneally in a number of experiments, as animals were available. Susceptibility to *M. marinum* was found throughout these species. There was no tendency to peripheral involvement. In experiments to determine the optimal environmental temperature for infection, cricket frogs (*Acris*), American chameleons (*Anolis*), young garter snakes (*Thamnophis*), and the young of three species of turtles were inoculated intraperitoneally. The optimal temperature for infection was found to be 30 C in each case, and infections at 20 C were definitely slower. The generation time of *M. marinum* in American chameleons at 30 C was about 19 hr; at 20 C, it was about 46 hr; and, at 10 C, the bacilli did not apparently multiply. Transmission studies revealed instances where infected animals shed *M. marinum* into the waters in which they were kept, and where animals became infected from water containing *M. marinum*.

Mycobacterium leprae (Shepard, 1960) and two cultivable species, *M. marinum* (*M. balnei*) and *M. ulcerans* (Fenner, 1956), grow preferentially in the mouse foot-pad. The selective susceptibility of the foot-pad to these organisms is presumably a result of its low temperature. The two cultivable species have temperature optima on artificial media of 31 to 35 C, which is less than the deep body temperature of mice.

The ability to grow *M. leprae* in the mouse foot-pad makes possible many studies, but the slow growth of the organism and the low yields

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TABLE 1. *Generation times of Mycobacterium marinum in 7H9 broth**

Temp	Generation time
C	*hr*
4	—†
10	—
15	>47‡
20	20.2
25	6.3
30	5.6
33	4.6
35	4.3
37	14.3

* Determined by the method of Youmans and Youmans (1949).

† No growth observed.

‡ Turbidity appeared only in the tube receiving the largest inoculum during the period of observation.

of bacilli have limited the range of experimentation. It seemed possible that altering the environmental temperature of the mouse might increase the rate of growth or yield of bacilli, or even allow generalized infection. It also seemed possible that other animal species with imperfect or absent temperature-regulating mechanisms, placed at proper temperatures, might allow a more favorable result.

To explore the validity of these hypotheses, we have used *M. marinum*, which apparently has a temperature optimum in the mouse similar to that of *M. leprae*, but is a much more rapid grower; we have inoculated it peripherally and systemically into mice and a variety of mammals and cold-blooded vertebrates held at different environmental temperatures.

MATERIALS AND METHODS

Environmental temperature control. Regular animal rooms were maintained at 20 and 25 C. A cold room (4 C) and a walk-in incubator (37 C) were utilized for those temperatures. Standard water-jacketed bacteriological incubators were used for temperatures of 10, 15, 30, and 34 C, the former two being placed in the cold room. All temperatures were maintained within ±1 C.

Bacteria. All studies were performed with strain Balnei X of *M. marinum* (ATCC 11564), originally isolated by Linell and Nordén (1954) from a human case of "swimming pool granuloma." Stock cultures were maintained on Loewenstein-Jensen medium at 33 C. For inoculation, bacteria were grown up for 3 to 6 days in Tween-albumin broth at 33 C, filtered through Whatman no. 1 filter paper, and diluted in Tryptose broth. Colony counts of each inoculum were obtained by plating tenfold dilutions on 7H9 agar with oleic acid-albumin supplement; results are expressed as "viable units."

Poikilothermic animals. Most of the reptiles, amphibians, and fish were collected by one of us (H F.C.) in a variety of locales in eastern United States and Canada. *Anolis* (American chameleons) and *Carassius* (goldfish) were purchased. To minimize nutritional variables, reptiles and amphibians were usually not fed. Most of these animals can survive for long periods without eating. Reptiles withstood higher temperatures than did amphibians; snakes survived temperatures as high as 33 C, and box-turtles (*Terrepene carolina*) tolerated 37 C. Frogs could not be maintained above 30 C, and salamanders not above 25 C. None of the animals used could withstand long exposure to 4 C, but all species tolerated 10 C.

The poikilotherms were all inoculated intraperitoneally. Snakes were inoculated midventrally about 70% of the distance from snout to vent; at this point, the only major underlying organ is intestine. Turtles were inoculated through a hind leg cavity. All other reptiles, amphibians, and fish were inoculated at a central midventral point of the abdomen. Because of the inelasticity of the skin of these animals, the smallest possible gauge needle was used to avoid loss of inoculum.

RESULTS

Artificial media. The temperature optimum of Balnei X in 7H9 with oleic acid-albumin supplement was determined by the method of Youmans and Youmans (1949). Tubes inoculated with tenfold dilutions of a filtered broth culture were placed at the temperatures given in Table 1, each tube was examined at 12-hr intervals, and the time of first detectable turbidity recorded. To minimize temperature deviation during turbidity readings requiring removal from the temperature chamber, tubes were placed in a water bath maintained in the chamber prior to removal. Tubes were kept in this water bath except during the actual moment of reading, but were removed from the water bath and wiped dry before being replaced in the chamber. The most rapid growth

was observed at 25 to 35 C. The similarity of the growth rates in this range corresponds with the observations of Linell and Nordén (1954) on the growth of Balnei X on Loewenstein-Jensen medium. They reported no growth at 36 to 38 C on Loewenstein-Jensen medium but did achieve relatively rapid growth at 36 to 38 C on glycerol agar medium.

Mice. Deep body temperatures of mice were determined by the rectal insertion of an 18-gauge blunt thermistor probe used with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). Temperatures were taken of groups of three to nine mice acclimatized for 3 weeks to environmental temperatures of 4 to 37 C. Each group at 4 to 30 C averaged between 36.5 and 37.3 C. Eight mice at 34 C averaged 39.1 C. Three mice at 37 C averaged 40.2 C.

Foot-pad temperatures were taken with a 22-gauge hypodermic thermistor probe. Foot-pads of mice maintained at 10 to 34 C all were within a few degrees of ambient temperature. There was more variation among these measurements than with the deep body measurements.

Groups of six mice were infected after being adapted for 14 days to temperatures of 4, 10, 15, 20, 25, 30, 34, and 37 C. In each group, three mice were inoculated into the right hind foot-pad with 2.2×10^4 viable units, and three mice were inoculated intravenously with 7.7×10^6 viable units. Beginning on the sixth day after inoculation, the mice inoculated in the foot-pad were examined at 2-day intervals. The amount of swelling was measured to the nearest 0.1 mm with a caliper. Redness and swelling were also graded 1+ to 3+; abscess formation was called 4+.

Results with the mice inoculated in the foot-pad are shown in Fig. 1, expressed in millimeters of swelling. (The responses graded from 1+ to 4+ were in complete agreement.) The optimal temperature was clearly 20 C. At this temperature, the onset of swelling was the most rapid and the maximal amount of swelling was the greatest. Temperatures above the optimum inhibited the response more than did temperatures below.

In the intravenously inoculated mice, the infection was allowed to progress until the animal died or became moribund, at which time visceral organs and peripheral lesions were smeared, or fixed and sectioned, and rated for relative numbers of acid-fast bacteria from 1+ to 5+. One

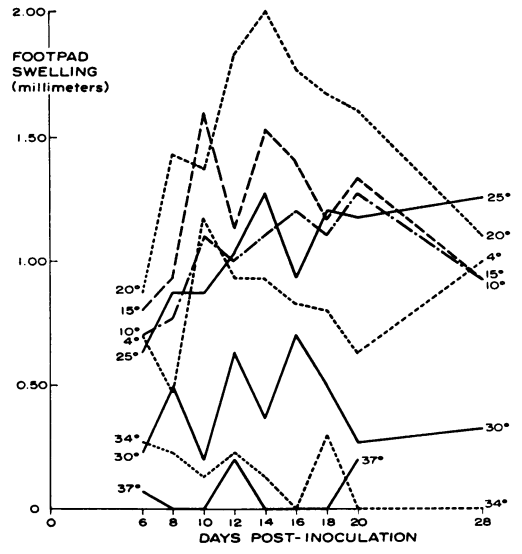

FIG. 1. *Foot-pad swelling in mice inoculated with Mycobacterium marinum and maintained at different environmental temperatures.*

mouse at 30 C and one at 34 C were killed and examined the same way on the twentieth day.

The responses of intravenously inoculated mice are given in Table 2. All mice at 37 C died within 3 days, apparently from the combined stress of heat and inoculation. All other deaths occurring in the first 4 months were probably specific, as indicated by gross pathology and uniformly extensive lung infection. Gross peripheral lesions consisted typically of redness and swelling, frequently progressing to abscess formation and drainage. Smears and sections of these lesions always contained very great numbers of acid-fast bacteria. The lungs of all the mice dying in the first 4 months were grossly pneumonic, and they contained very great numbers of mycobacteria. (The single exception was a mouse kept at 37 C whose death on day three was probably nonspecific.)

Gross testicular infections developed in only three mice, TM-27, TM-32, and TM-33; these progressed to abscess formation with 5+ numbers of organisms. A single mouse, TM-27, had an extensive kidney infection, which was located primarily in the tubules, although a few glomeruli also contained large numbers of mycobacteria. No animal developed an extensive infection of liver or spleen.

The time of specific death bore an inverse rela-

TABLE 2. *Results of intravenous inoculation of Mycobacterium marinum in mice maintained at different environmental temperatures*[a]

Environmental temp (C)	Animal	Day of death	Sites of gross peripheral lesions at death	Rating of no. of acid-fast bacilli in smear or section[b]		
				Lung	Liver	Nose[c]
4	TM-1	14	Nose	3+	2+	5+
4	TM-2	15	Cannibalized	—	—	—
4	TM-3	22	Nose, feet, tail	5+	2+	5+
10	TM-7	18	Nose	4+	3+	4+
10	TM-8	19	Nose	5+	2+	5+
10	TM-9	26	Nose, feet, tail	5+	2+	4+
15	TM-13	19	No data	5+	2+	5+
15	TM-14	37	Nose, feet, tail	5+	2+	4+
15	TM-15	90	Cannibalized	—	—	—
20	TM-19	20	Nose	5+	2+	4+
20	TM-20	29	Nose, feet, tail	4+	2+	5+
25	TM-25	29	Nose, feet, tail	5+	2+	5+
25	TM-26	35	Nose, feet, tail, scrotum	5+	2+	5+
25	TM-27	87	Nose, feet, tail, ears, testis	5+	2+	3+
30	TM-32	21	Nose, foot, tail, ears, testis	4+	1+	0+
34	TM-38	146		0+	1+	0+
34	TM-39	165		0+	2+	1+
37	TM-43	3		0+	2+	
30	TM-31[d]	20		0+	0+	0+
30	TM-33[d]	194	Foot, ear, testis	0+	0+	
34	TM-37[d]	20		0+	0+	0+

[a] Each animal received a dose of 7.7×10^6 viable organisms in a volume of 0.1 ml.

[b] Ratings represent tenfold differences in bacterial numbers. A "5+" slide had at least 100 bacilli per field. A "4+" slide had at least ten bacilli per field, etc.

[c] A shallow section through the nostrils, near the tip of the nose, was examined.

[d] Mice sacrificed. (One survivor at 20 C was not sacrificed.)

tionship to environmental temperature. Of 12 animals below 25 C, 9 died from infection within 30 days, whereas only 2 of 9 mice at 25 C or above died from infection within 30 days. No mouse at 34 C developed definite peripheral lesions or extensive lung infections. It was at this temperature that deep body temperatures were found to be elevated.

The rate of growth of the bacilli in the mouse foot-pad was determined by the method of Fenner (1956). A temperature of 20 C was selected for this determination on the basis of the results obtained in the preceding experiment. Groups of five mice were inoculated into the foot-pad with *M. marinum* in serial tenfold dilutions from an original suspension with 1.9×10^5 viable units per 0.03 ml. The mice were examined daily, and the first day of definite redness or swelling was recorded.

The results of the generation time experiment are shown in Fig. 2. The line drawn to best possible fit gives a generation time of about 15 hr. This is roughly three times the generation time of *M. marinum* observed in bacteriological medium in its optimal temperature range.

Mice from this and from another foot-pad experiment on temperature were killed 150 to 200 days after inoculation. Acid-fast bacilli were usu-

ally detectable in smears and sections of mice with gross lesions. *M. marinum* was successfully cultured from several of the foot-pad lesions of mice kept for this length of time at 4, 10, 20, and 30 C.

Other mammals. Young opossums (*Didelphus virginiana*) have a lower deep body temperature, 34 to 36 C, than any common laboratory animal. An experiment was performed to see if opossums would, therefore, allow generalization of peripheral infection with *M. marinum*. Four young animals were inoculated, two in the right hind foot-pad with 4.5×10^6, and two in the ventral tail muscles with 3.0×10^6 viable units. One opossum inoculated by each route was kept at 20 C, and the other at 4 C. Swelling of the inoculated sites was pronounced, especially at 20 C, but no systemic disease was produced. In a second experiment, four young opossums were inoculated intravenously with 3.7×10^7 organisms and kept at 20 C. Three developed lesions of one or more foot-pads, but generalized infections did not develop in any.

Bats assume the environmental temperature when at rest. Thus, it was thought they might be especially prone to systemic infection with *M. marinum*. Small groups of Eastern pipistrels (*Pipistrellus subflavus*) and Florida freetail bats (*Tadarida cynocephala*) were inoculated in the foot-pad and placed at temperatures from 15 to 34 C. Several bats developed foot-pad lesions similar to those in mice, but none developed significant visceral infections within the period they could be kept alive, usually 5 to 14 days after inoculation.

Poikilothermic animals. The deep body temperatures of a limited number of our snakes, turtles, lizards, and frogs were determined by intraperitoneal insertion of a hypodermic thermistor probe and found to be almost exactly the same as environmental temperature. Although some poikilotherms can reportedly maintain body temperatures considerably below environmental temperature by evaporative cooling, this was limited in these experiments by the maintenance of the maximal practicable humidity at each temperature.

The animals were inoculated intraperitoneally with varying doses, in the range of 10^4 to 10^7, and placed usually at 20 or 25 C. Infection was usually allowed to progress until death. Gross lesions and bacilli were never concentrated in peripheral areas. The great majority of acid-fast organisms were seen in liver, lung, intestine, and spleen. The degree of infection was evaluated by rating smears or sections of these organs for relative numbers of mycobacteria.

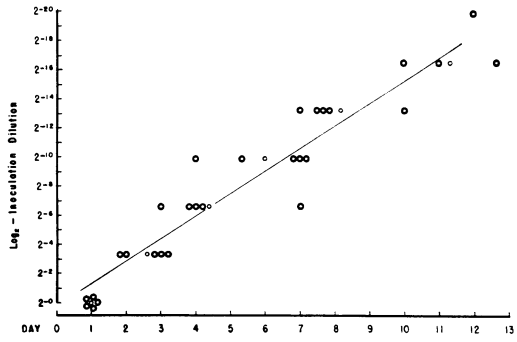

FIG. 2. *Time of appearance of redness or swelling of foot-pad after inoculation of Mycobacterium marinum in serial tenfold dilutions at 20 C. The small circles refer to the average time for that dilution. Generation time = 15 hr.*

The results of a number of individual experiments are presented in Table 3. Because the animals were available in limited numbers at irregular times, comparisons between species were frequently not possible in one experiment. Furthermore, great differences in the size of the animals, from frogs weighing less than 1 g to alligators weighing several kilograms, made per kilogram dosage comparisons difficult. It is difficult to say from these data what rating represents multiplication of bacteria, but experience with animals kept at bacteriostatic temperatures and with animals dying very soon after inoculation indicates that probably all 4+ ratings and most 3+ ratings represent definite increase in numbers of mycobacteria. The few failures to obtain this number of mycobacteria in smear or section may have resulted from failure to sample a localized infection in a large animal.

It is evident from the results that susceptibility to *M. marinum* is very widespread among the poikilotherms. Dosage seemed a critical factor, in that bacillary multiplication could be obtained very consistently in small species and in the young of larger species.

Results of experiments to determine the optimal temperature for growth of *M. marinum* in species representing four different orders of reptiles and amphibians are given in Table 4. The cricket frog (*Acris*), the American chameleon (*Anolis*), young garter snakes (*Thamnophis*), and

TABLE 3. *Maximal infections obtained in poikilothermic animals inoculated with Mycobacterium marinum*

Species	No. inoculated	Conditions of maximally infected individual(s) of species						
		Dose in viable units	Environmental temp (C)	Day of death	Rating of no. of acid-fast bacilli in smear or section			
					Liver	Lung	Gut	Other
Reptilia								
Crocodilia								
Alligator mississippiensis (American alligator)	4	5.6×10^6	25	108	2+	1+	2+	
Chelonia (turtles)								
Chelydra serpentina (snapping turtle)	3[a]	—[b]	25	—[b]	3+	3+	4+	
Sternothaerus odoratus (stinkpot turtle)	2[a]	1.10×10^6	30	9	5+	5+	5+	
Kinosternon subrubrum subrubrum (Eastern mud turtle)	5[a]	3.4×10^6	25	14	4+	3+	2+	
Terrapene carolina carolina (Eastern box turtle)	11[a]	3.4×10^6	30	22	3+	4+		
Chrysemys picta picta (Eastern painted turtle)	7[a]	7.5×10^5	25	16	4+	4+	3+	
Pseudemys scripta scripta (yellow-bellied turtle)	6[a]	7.5×10^5, 1.1×10^6	25, 30	24, 11	5+	5+	4+	
Gopherus polyphemus (gopher tortoise)	1	5.1×10^6	25	52	0+	0+	1+	Bladder 3+
Trionyx spinifer asper (Gulf Coast softshell)	1[a]	2.0×10^5	20	55	3+	3+	3+	Heart 4+
Squamata (lizards, snakes)								
Anolis carolinensis carolinensis (green anole)	73	5.1×10^3 to 2.2×10^7	20, 25, 30	2 to 23	5+	5+		
Sceloporus undulatus undulatus (Southern fence lizard)	4	2.0×10^4 to 2.0×10^6	20, 30	5 to 26	5+	5+		
Cnemidopherus sexlineatus (six-lined racerunner)	1	1.7×10^6	25	7	4+	4+	3+	
Lygosoma laterale (ground skink)	1	2.0×10^4	25	14	4+	5+		
Natrix taxispilota (brown water snake)	2	9.0×10^2	33	66[c]	2+	3+		
Natrix sipedon confluens (broad-banded water snake)	2	2.0×10^6	25	173	0+	3+		
Natrix septemvittata (queen snake)	1	5.6×10^6	20	91	0+	0+		
Storeria dekayi wrightorum (midland brown snake)	1	8.1×10^6	25	15	3+	3+	3+	

TABLE 3.—*Continued*

Species	No. inoculated	Conditions of maximally infected individual(s) of species						
		Dose in viable units	Environmental temp (C)	Day of death	Rating of no. of acid-fast bacilli in smear or section			
					Liver	Lung	Gut	Other
Thamnophis sirtalis sirtalis (Eastern garter snake)	22[a]	3.4×10^6 to 5.1×10^6	20, 30	4 to 15	5+	4+		
Thamnophis sauritus proximus (Western ribbon snake)	4	2.0×10^5	34	7	2+	2+		Kidney 3+
Haldea valeriae valeruae (Eastern earth snake)	1	1.0×10^4	25	20[c]	4+	1+	3+	
Heterodon platyrhinos (Eastern hognose snake)	1	2.0×10^6	20	183	0+	0+		Kidney 3+
Diadophis punctatus strictogenys (Mississippi ringneck snake)	1	2.0×10^4	25	23	4+	3+		
Coluber constrictor priapus (Southern black racer)	1	5.6×10^6	20	104	4+	3+		
Drymarchon corais couperi (Eastern indigo snake)	1	5.1×10^6	25	157	0+	0+	0+	
Elaphe guttata guttata (corn snake)	1	1.0×10^7	25	22[c]	2+	2+	1+	
Elaphe obsoleta obsoleta (black rat snake)	1	5.1×10^6	25	56	4+	5+	3+	
Elaphe obsoleta quadrivittata (yellow rat snake)	1	1.0×10^7	25	11	5+	5+	4+	
Micruvus fulvius fulvius (Eastern coral snake)	1	1.0×10^7	25	12	4+	3+	4+	
Agkistrodon piscivorus piscivorus (Eastern cottonmouth)	2	2.0×10^6	10[d]	168	1+	2+	0+	
Amphibia								
Urodela (salamanders)								
Amphiuma means tridactylum (three-toed amphiuma)	4	2.0×10^6	20	17	3+	3+		
Desmognathus fuscus fuscus (Northern dusky salamander)	2	1.9×10^6	25	7	2+		4+	Spleen 5+
Desmognathus ochrophaeus carolinensis (Blue Ridge Mountain salamander)	12	1.1×10^2	20	39	5+			Spleen 4+
Desmognathus monticola (seal salamander)	16	3.4×10^4, 3.4×10^6	20	9, 24	4+		4+	Spleen 5+
Desmognathus quadramaculatus (black-bellied salamander)	2	3.4×10^6	20	12, 13	2+		4+	Spleen 4+

TABLE 3.—*Continued*

Species	No. inoculated	Conditions of maximally infected individual(s) of species						
		Dose in viable units	Environmental temp (C)	Day of death	Rating of no. of acid-fast bacilli in smear or section			
					Liver	Lung	Gut	Other
Leurognathus marmoratus intermedius (Southern shovel-nosed salamander)	1	3.4×10^6	20	12	3+		5+	Spleen 4+
Plethodon cinereus polycentratus (Georgia red-backed salamander)	1	1.3×10^4	20	21	5+			
Plethodon glutinosus (slimy salamander)	2*	1.1×10^6	25	9	4+		5+	
Plethodon jordani shermani (red-legged salamander)	54	1.1×10^2 to 3.4×10^6	20	5 to 163	4+		5+	Spleen 5+
Gyrinophilus danielsi dunni (Carolina spring salamander)	1	3.4×10^6	20	36	3+		3+	
Pseudotriton ruber ruber (Northern red salamander)	2	1.3×10^4	25	19	4+		5+	
Anura (frogs, toads)								
Bufo woodhousei fowleri (Fowler's toad)	2	3.4×10^6	25	19	3+	3+		
Bufo valliceps (Gulf Coast toad)	1	2.0×10^5	25	16	1+	0+		
Acris sp. (cricket frog)	64	3.7×10^5 to 2.2×10^7	15 to 30	4 to 17	5+	5+		
Hyla ocularis (little grass frog)	1	1.9×10^5	25	13				Viscera 4+
Pseudacris triseriata feriarum (upland chorus frog)	1	1.3×10^4	20	69	3+	3+		
Rana catesbiana (bullfrog)	1	2.3×10^6	25	38	2+	1+		
Rana clamitans clamitans (bronze frog)	2	1.3×10^4	20	46	1+	1+		Kidney 2+
Rana pipiens sphenocephala (Southern leopard frog)	2	1.9×10^6	20	20	3+	3+		
Teleostomi								
Cypriniformes								
Carassius auratus (goldfish)	4	7.0×10^5	25	37, 48	5+		5+	
Cyprinodontiformes								
Fundulus notti (starhead top minnow)	3	1.9×10^6	20	12	4+		5+	
Gambusia affnis (Gambusia)	59	2.0×10^3 to 2.3×10^5	25	8 to 25	5+		5+	

[a] Maximal infection in immature indivual(s).

[b] A young turtle of this species was never inoculated, but was accidentally dropped into water at 25 C containing fish inoculated with *M. marinum*. It died several months later with mycobacterial infection of the liver, lung, and gut rated 3+, 3+, and 4+, respectively.

[c] Sacrificed.

[d] Another *Agkistrodon* died after 8 days at 30 C but was too decomposed for bacterial examination.

TABLE 4. *Results with different poikilothermic species held at different environmental temperatures**

Species	No. in group	Temp (C)	Inoculum in viable units	Visceral site smeared	Grade of visceral smear and day of death†								
					2–4	5–8	9–12	13–16	17–20	25–28	29–36	65–88	113–116
Acris (cricket frogs)	5	30	3.7×10^6	Liver	5+	5+							
					5+	5+							
						5+							
	5	20					5+	5+					
								5+					
								5+					
	5	10					3+		3+				
									3+				
									3+				
									3+				
Thamnophis (garter snakes)	5	30	3.4×10^6	Liver	5+	5+	3+						
					4+								
					4+								
	5	20					4+	4+			3+		
							4+	4+					
	5	10										3+	
												3+	
												2+	
												1+	
												1+	
Anolis (American chameleon)	4	30	5.1×10^5	Lung	5+	5+							
					5+	5+							
	4	25				5+	5+						
						5+							
						5+							
	4	20					5+	5+	5+				
								5+					
Chrysemys (C), *Pseudemys* (P), and *Sternothaerus* (S), (all turtles)	3	30	3.4×10^6	Liver			5+(P)						
	3	20					5+(S)	3+(C)					
	2	10						4+(S)	1+ (C)	4+(P)		0+(C)	2+(P)

* All individuals in each group were inoculated with the same dosage of *Mycobacterium marinum*.
† No deaths occurred on days 37 to 64.

the young of three species of turtles were utilized in four separate experiments with inocula ranging from 5.1×10^5 to 3.7×10^6.

The time of death was inversely proportional to temperatures in each experiment, and death with maximal numbers of bacteria was produced first at 30 C in each case. Maximal numbers of mycobacteria were not produced at 10 C.

To determine the rate of growth of *M. marinum* as affected by environmental temperature, a uniform group of *Anolis carolinensis* (American chameleons) was inoculated with 4.5×10^6 viable units and placed at 10, 20, and 30 C. An animal at each temperature was killed at regular intervals, and homogenized in a Waring Blendor for a direct microscopic count of the acid-fast bacteria by a method in use in the laboratory (Shepard, 1960).

The results are given in Fig. 3. There was an apparent initial decrease in numbers of bacteria at 20 and 30 C. Bacterial growth then proceeded obviously more rapidly at 30 C. The line of best possible fit to the values at 30 C gives a generation time of about 19 hr, roughly equivalent to

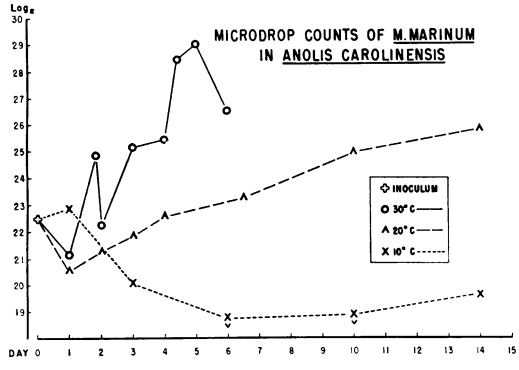

FIG. 3. *Number of acid-fast bacteria in Anolis carolinensis according to time after infection and temperature. The values for 10 C at 6 and 10 days refer to counts in which no acid-fast bacteria were observed.*

the rate observed in the mouse foot-pad at its optimal environmental temperature of 20 C. The line fitted to the values for *Anolis* at 20 C indicates a generation time of about 46 hr. At 10 C, there was no apparent multiplication of the inoculated bacilli.

The comparative histological appearance of infections in *Anolis* at different temperatures from the same experiment is given in Fig. 4.

Transmission studies. Visceral smears from freshly caught poikilothermic animals were frequently examined, and no mycobacteria were ever found. However, there were several cases in which *M. marinum* apparently spread in the laboratory to uninoculated or Tryptose broth-inoculated control animals. This occurred most often with fish and amphibians kept in water in close proximity to infected aquatic animals. Considerable numbers of acid-fast bacteria could often be demonstrated in the water in which *M. marinum*-inoculated animals were kept. A small snapping turtle (*Chelydra serpentina*) which was accidentally dropped into water containing infected fish died several months later with extensive mycobacterial infection.

Several experiments were performed to demonstrate infection of tadpoles (*Pseudacris*) by exposure to *M. marinum* in water. Groups of 20 tadpoles were placed in flasks containing about 250 ml of water to which had been added viable doses of 9.2×10^6, 9.2×10^3, and 9.2×10^1 *M. marinum*, respectively. The tadpoles were kept at 20 C. After 6 days, acid-fast bacilli could be seen in visceral smears from four of four tadpoles in the first flask, three of four from the second, and zero of four in the third. In a second experiment, tadpoles were placed for 12 hr in water at 25 C containing about 3×10^4 viable *M. marinum* per ml and then removed to fresh water. Acid-fast bacilli were later found in visceral smears from 12 of 24 tadpoles examined. Two uninoculated tadpoles were placed in about 1 liter of water at 20 C containing three top minnows (*Fundulus notti*), each inoculated intraperitoneally with a dose of 1.8×10^6. The fish died on days 1, 2, and 12, and were removed. The last to die was extensively infected. Both tadpoles died on day 22, each with extensive acid-fast infection.

DISCUSSION

Susceptibility to *M. marinum* was seen to be very widespread among animal species. Mice, young opossums, bats, and 50 cold-blooded species were studied. Chick embryos are susceptible if they are kept at 33 C (Fenner, 1956). Humans are susceptible to natural infections of the peripheral, cooler skin.

Temperature is the common factor affecting the distribution of susceptibility among species, as well as the distribution of the disease in a particular animal. Systemic disease was produced readily here only in the poikilotherms. In mice that had been inoculated intravenously, involvement of the peripheral, hairless tissues was obvious and severe when the mice were kept at 4 to 30 C. The lungs, which probably received the greatest portion of the bacilli given intravenously, were also involved at these temperatures. Pulmonary involvement of mice inoculated intravenously or intraperitoneally with large doses of *M. marinum* was not observed by Fenner (1956), but it was seen by Linell and Nordén (1954) after intraperitoneal injection of similarly large doses.

The rates of growth of the organism were estimated in three systems (mouse foot-pad, *Anolis*, and bacteriological medium) at different temperatures. The optimal rate of growth in the bacteriological medium was about 5 hr. Leach and Fenner (1954) reported a value of 11 hr at 33 C based on the increase in opacity of a culture growing in Tween-albumin medium. The method used here (Youmans and Youmans, 1949) measures the logarithmic rate of growth during earlier stages of the growth curve, and also allows the use of more favorable media. With *M. tuberculo-*

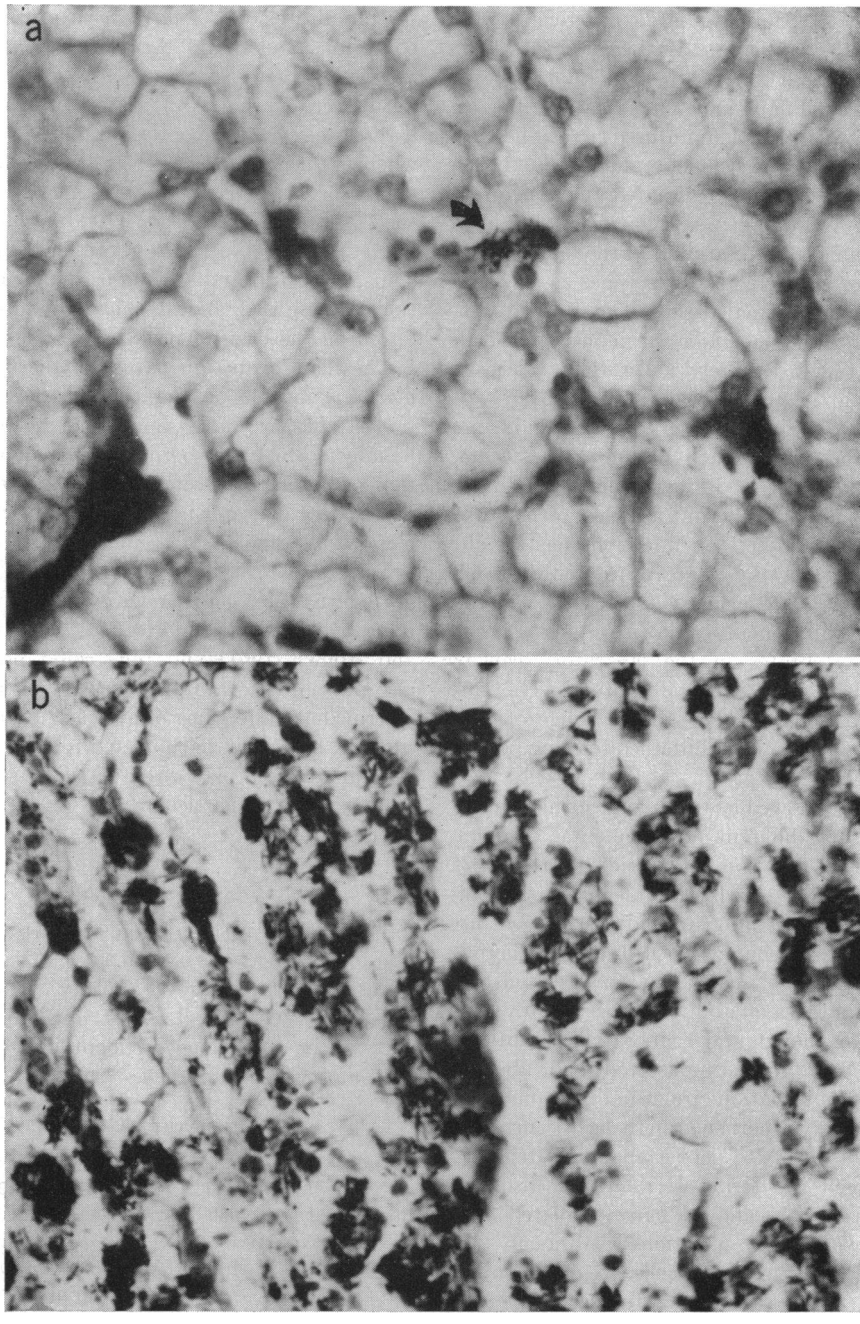

FIG. 4. *Liver of Anolis 5 days after inoculation. × 1840. (a) In the animal at 10 C, mycobacteria were thinly scattered in individual phagocytes (arrow), or in very small infiltrates. (b) In the animal at 30 C, there was massive intra- and extracellular infection.*

sis (at 37 C), similarly large differences were found by the two methods; Leach and Fenner (1954) reported 18 hr by their method, and the method of Youmans and Youmans (1949) gave 9 to 10 hr with the medium used here (Shepard, *unpublished data*). For mouse foot-pads, Leach and Fenner (1954) had estimated the generation time at 11 hr, compared with our value of 15 hr.

The optimal rate of growth in the bacteriological medium was roughly three times as fast as it was in the two animal tissues. The optimal temperature for *M. marinum* in mouse foot-pads appeared to be definitely lower than it was in *Anolis* or in bacteriological medium. Many explanations for this difference come to mind, since the bacterial environment in the three systems presumably was very different biochemically.

The use of poikilothermic animals in the study of temperature effects on infections has definite advantages, since the temperature of the tissues is as easily adjustable as it is for in vitro systems. Many of the difficulties in maintaining cold-blooded vertebrates in the laboratory can be eliminated by the use of cell cultures. They are easily prepared, thrive at a wide range of temperatures, and can be maintained for long periods without transfer.

This study allowed us to explore the husbandry of animals at different environmental temperatures, as a preparation for a study of *M. leprae*. Preliminary results with mice infected in the foot-pad with *M. leprae* indicate that the temperature optimum is also about 20 C. Of the poikilothermic animals used here, many can be ruled out because of limited availability, or because they do not live long enough under laboratory conditions. With *M. leprae* in the mouse foot-pad, about 5 months of observation are usually required to judge definitely whether growth has occurred.

Confusion seems to persist about the relationship between the terms *M. marinum* (isolated from diseased fish) and *M. balnei* (isolated from humans with skin infections), although the growth patterns in HeLa cells are very similar (Shepard, 1957); taxonomic studies have failed to reveal differences (McMillen and Kushner, 1959; Bojalil, 1959), and the immunoelectrophoretic patterns are nearly identical (Castelnuovo and Morellini, 1962). *M. balnei* was first separated from *M. marinum* because the latter was said to be noninfectious for mice (Linell and Nordén, 1954). However, we have studied a number of cultures of *M. marinum* from type culture collections and found them all capable of producing characteristic foot-pad lesions in mice. Zettergren et al. (1952) said that *M. marinum* had a temperature optimum at 18 to 20 C, whereas that of *M. balnei* was at 26 to 30 C. The figure for *M. marinum* is presumably based on the description of Aronson (1926), who did not, in fact, study temperatures between 20 and 37 C. Thus, there appears to be no basis for differentiation of these two, and *M. marinum* must be preferred on the basis of priority.

The maintenance in nature of the species *M. marinum* merits consideration. In our transmission studies, we have shown that tadpoles may be infected by water-borne *M. marinum* and also that parenterally infected fish can apparently act as a source of water-borne organisms for the infection of tadpoles. It seems likely that similar cycles could operate in nature. It has been reported that *M. marinum* grows in some swimming pools (Linell and Nordén, 1954; Mollohan and Romer, 1961) and thus could presumably also multiply in natural bodies of water without intermediate infections of animals. It seems reasonable, however, that infected reptiles, amphibians, and fish do play a role in the dissemination of the bacteria, particularly to previously non-infected waters.

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