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Experimental introduction of a microsporidian into a wild population of *Culex pipiens fatigans* Wied.

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Chemical control of C. p. fatigans *frequently fails because the mosquito rapidly develops resistance to insecticides. A possible alternative or complementary method is biological control, including the introduction of pathogens. The microsporidian* Plistophora culicis *was known to infect readily and have an adverse effect on* C. p. fatigans *populations in the laboratory, so an attempt was made to introduce and establish this pathogen in a wild population of the mosquito on the Pacific island of Nauru. Two years after introduction the pathogen was still present in the wild population. However, the infection rate was similar to that found in naturally occurring infections in other mosquitos and is almost certainly not high enough to affect a natural population of* C. p. fatigans *adversely.*

Control of the mosquito *Culex pipiens fatigans* Wiedemann is complicated by its ability to develop resistance to many residual insecticides. A possible alternative method of control is the use of pathogens as biological control agents in an integrated control programme.

Laboratory experiments have shown that the microsporidian *Plistophora culicis* (Weiser) readily infects *C. p. fatigans* and causes a reduction in the net reproduction rate in experimental populations (Reynolds, 1970). This article describes a field experiment to determine whether *P. culicis* would become established in a wild population of *C. p. fatigans*. The requirements for an area in which to carry out the experiment were that the wild population of *C. p. fatigans* be numerically large and geographically isolated, that the area be of such a size that all breeding sites could be found, and that adequate facilities such as laboratory space and local assistance be available. After several areas had been considered, the island of Nauru was chosen as best meeting the requirements. In addition, a WHO consultant had recently carried out a survey of the mosquito population on the island with particular reference to parasites and pathogens.[2]

The Republic of Nauru is an island, 21.2 km² in area, oval in shape and about 19 km in circumference, situated in the Pacific Ocean about 48 km south of the equator at latitude 0°32′S and longitude 166°55′E. The nearest land is Ocean Island, 306 km to the east.

The climate is tropical, tempered by sea breezes. The mean minimum temperature ranges from 23° to 26°C and the mean maximum from 29° to 32°C. The average annual rainfall for the period 1950–67 was 201.2 cm, ranging from a high of 373.9 cm to a low of 31.2 cm. Rain falls mainly during the westerly monsoon season, which usually occurs from November to February.

The author visited Nauru from October 1967 to February 1968. This period was chosen to include the latter part of the dry season and the early part of the rains. This object was achieved, as very little rain fell until mid-December.

MATERIALS AND METHODS

Before the author left for Nauru, *P. culicis* was mass-propagated at the London School of Tropical Medicine and Hygiene, using *Anopheles stephensi* Liston, an excellent host. *A. stephensi* eggs were hatched in water to which *P. culicis* spores had been added to give a final concentration of 2 000 spores/ml. Most of the *A. stephensi* died with heavy microsporidial infections in the late larval or early pupal stages, so that the spores could be harvested by siev-

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[2] Chapman, H. C. (1967) Mimeographed document WHO/VBC/67.28.

ing off all larvae and pupae on the day the first pupae appeared and storing them in distilled water in plastic screw-top bottles at 4°C. This also killed any living larvae and pupae. To extract the spores, the larvae and pupae were broken up by adding glass beads to the plastic bottles and shaking gently; the larger pieces of debris were then removed by filtering the suspension through fine mesh cloth. In a 4-month period, 455 bowls of infected *A. stephensi* were raised, giving a total of 12.6×10^9 *P. culicis* spores, which were taken to Nauru.

On the island mosquito larvae were collected using a long-handled ladle with a capacity of approximately 300 ml or, when the water level was out of reach of the ladle or there were very few larvae, a 9-litre bucket on a length of rope. Adults were caught by means of an aspirator tube.

During a preliminary survey all mosquito larvae collected were examined at a magnification of 16× using a bright light against a black background. All larvae that looked in any way unusual, together with a random 10% of the collection, were dissected and examined under a compound microscope. Any larva that still gave rise to doubt was treated with Giemsa stain and reexamined. All adults were examined under a compound microscope, as were all larvae and adults collected after the introduction of the parasites.

Adults were bred out from as many larval collections as possible so that the identification of the mosquito could be checked and any infection more readily found.

PRELIMINARY SURVEY

Chapman (*op. cit.*) carried out a survey for pathogens and parasites of mosquitos on Nauru in November/December 1966. He found no parasites or pathogens in approximately 400 000 larvae and 500 adults examined.

Breeding was at a low ebb on the author's arrival because of lack of rain. The rainfall for the 7 months from May to November 1967 was 17.5 cm, compared with 66.9 and 210.1 cm in the same period in 1966 and 1965. The breeding sites were abandoned water tanks, which were usually highly polluted, bomb craters, ground pools, and pools polluted by drainage from pig-pens. After the rainy season started in December the number and size of the ground pools increased and more bomb craters held water. Breeding occurred in all these sites, and also to a limited extent in tins, coconut husks, and discarded tires.

Table 1. *C. p. fatigans* specimens examined during preliminary survey

Source	Larvae		Adults	
	16 × magnification	by microscope	reared	wild-caught
random sampling	2 603	401	241	84
possible sites	3 431	345	135	—
Total	6 034	746	376	84

All breeding sites were given a number. Six of the sites were later selected for the introduction of *P. culicis* spores. The numbers of *C. p. fatigans* examined for evidence of pre-existing infections during the preliminary survey are shown in Table 1. No pathogens or parasites were found in a total of 7 240 specimens examined from various sites. A total of 615 *Aedes aegypti* (L.) larvae and 310 adults were also examined, and again no parasites or pathogens were found. The wild-caught *C. p. fatigans* adults were checked especially for *Wuchereria bancrofti*, but no infections were found.

Mosquitos did not breed in Buada lagoon, which is stocked with an edible fish known locally as *ibia*. Certain ground pools and bomb craters have been stocked with *Gambusia affinis* and/or *Tilapia* sp. Where either of these fish occurs no mosquito larvae were found.

Only two species of mosquito were found on the island, *C. p. fatigans* and *A. aegypti*. This is in agreement with Chapman's findings, although Belkin (1962) records, in addition to these two species, *Culex sitiens* Wiedemann and *C. annulirostris* Skuse.

As a result of this preliminary survey it was decided to carry out most of the experimental work at the southern end of the island, where most of the *C. p. fatigans* breeding was taking place.

EXPERIMENTS AND RESULTS

Introduction of parasites

In the first instance, on 13 November, *P. culicis* was introduced into four sites, numbered 15, 18, 19, and 22. Site 15 was a well containing reasonably clean water that was occasionally used for washing clothes but never drunk. The well was 90 cm in diameter at water level, and the water 60 cm deep. The final spore concentration in the well water was

5 800 spores/ml. Site 18 was an abandoned cistern. The water was polluted with coconuts, coconut fronds, bits of wood, and a little household rubbish. Its diameter was 1.4 m and the depth of water 30 cm. The final spore concentration was 6 100/ml. Site 19, another abandoned cistern, was heavily polluted with coconuts, fronds, and household rubbish and surfaced with a thick algal scum. The cistern measured 4.9 by 4 m, the depth of water being 0.3 m. Spores were introduced into one corner only of this site, the area treated being approximately 1.2 by 0.6 m. The final concentration of spores in the treated area was about 6 000/ml. Site 22 was an abandoned cistern partially covered with corrugated iron sheets tilted to catch the rain. The water was polluted with leaves, coconuts, and green algae. The cistern measured 1.4 × 1.4 m, and the depth of water was 15 cm. The final spore concentration was 5 800/ml.

These sites all lay in the coastal belt at the southern end of the island, distributed over a distance of about 3 km.

At each site the required number of *P. culicis* spores was mixed with 9 litres of water in a bucket, and the suspension was poured as evenly as possible over the area to be treated. Only a part of site 19 was treated so that the effectiveness of treating a section of a larger body of water could be investigated.

At the first check, 15 days after the spores were introduced, parasitized larvae were recovered from sites 15, 18, and 22. Site 22 yielded parasitized larvae at regular examinations up to day 66, the last check before the author's departure from Nauru. At sites 15 and 18 mosquito breeding was drastically reduced, ceasing completely by day 66 and day 49 respectively. No parasitized larvae were recovered from site 19. The results are shown in detail in Table 2.

When the rainfall increased in December it was hoped that more *C. p. fatigans* breeding sites suitable for the introduction of spores might be formed. Since this did not happen, it was decided to use artificial containers.

Three 200-litre metal drums were cut in half horizontally and steam-cleaned. This gave 6 containers, each 60 cm in diameter and 45 cm deep, which were sunk about 30 cm into the ground to prevent pigs uprooting them. The half-drums were placed in 2 rows of 3, the rows being about 70 m away from, and on either side of, 2 occupied houses.

The 3 containers to the west of the houses were half-filled with water from site 22, which by this time was known to be producing parasitized larvae. The other 3 half-drums, to the east of the houses, were half-filled with water from a water tank used by the occupants of the two houses. *A. aegypti* was breeding in large numbers in this tank. Care was taken to ensure that no larvae were added to any of the containers with the water. The rows of containers were designated E (those east of the houses) or W (those west of the houses). Within the rows, the containers were numbered 1 to 3 from south to north. They were left until breeding occurred naturally.

P. culicis spores were added 10 days later to drums E2, E3, and W3 to give final concentrations of 6 800, 6 800, and 6 200 spores per millilitre, respectively. These 3 drums all had *C. p. fatigans* larval counts of 35 per dip or more. Larval counts in the other 3 drums were less than 1 per dip.

Table 2. Examination of *C. p. fatigans* larvae from treated sites

Location	Days after introduction					
	15	22	34	49	57	66
Site 15						
No. examined	25	3	47	6	1	[a]
No. infected	3	0	14	2	0	
% infected	12.0	0.0	29.8	3.33	0.0	
Site 18						
No. examined	15	47	9	[a]	[a]	[a]
No. infected	1	5	1			
% infected	6.7	10.6	11.1			
Site 19						
No. examined	34	85	124	[b]	[b]	[b]
No. infected	0	0	0			
% infected	0.0	0.0	0.0			
Site 22						
No. examined	11	68	95	82	76	78
No. infected	2	7	8	6	12	11
% infected	18.2	10.3	8.4	7.3	15.8	14.1
Site 49 [c]						
No. examined	57					
No. infected	0					
% infected	0.0					

[a] No larvae found in site.
[b] Not checked.
[c] Site treated 51 days after other sites.

Table 3. Examination of *C. p. fatigans* larvae from 6 experimental drums

Drum	Number examined	Number parasitized	% parasitized
E1	4	0	0.0
E2[a]	68	20	29.4
E3[a]	65	14	21.5
W1	2	0	0.0
W2	23	0	0.0
W3[a]	59	0	0.0

[a] Spores introduced 14 days before examination.

Two weeks after the introduction of spores larvae were collected from all 6 drums, kept in the insectary, and examined on death. Parasitized larvae were recovered from drums E2 and E3 only (see Table 3).

Later two further sites were treated. These were site 49, an abandoned cistern with some organic pollution situated near Buada lagoon, and site 8, a bomb crater about 200 m west of site 15. Site 49 was treated with the remainder of the mass-propagated spore material to give a final concentration of 1 000 spores/ml. Only one check was carried out, at which no parasitized larvae were found. Site 8 was treated with all the spores obtained in laboratory experimental work on the island. The author left Nauru shortly afterwards, and no checks were carried out on this site.

Concurrently with the field experiments, infection experiments were started in the laboratory on Nauru, the primary objects being to establish potential infection rates and to obtain more spores for further introduction in the field. In these experiments an inoculum of 6 000 *P. culicis* spores per millilitre of larval breeding water was used. An inoculum of this size had been found to produce infection rates of 80–100% in England (Reynolds, 1970). Water and egg rafts were obtained from various natural breeding sites.

Infection rates in these experiments were found to range from 17.1% to 39.0%, except that when water from site 19 was used, no infections were obtained in 3 trials. Apparently the water from that site contained some factor inimical to *P. culicis* while not affecting the growth of the mosquito larvae. The other infection rates were lower than those obtained in England. This may have been because the spores lost a certain degree of viability between harvesting and use, a period of up to 3 months, or, more probably, because the strain of *C. p. fatigans* on the island was less susceptible to infection than the Rangoon laboratory strain used in England. An attempt to send *C. p. fatigans* adults from Nauru to England failed owing to poor communications.

Follow-up survey

As a return visit to Nauru was not possible, the two health inspectors in Nauru were asked to collect *C. p. fatigans* larvae from the sites that had been " seeded " and send them to the author for examination. The collections were made between May and October 1969. The larvae were killed immediately after collection by immersion in water at 60°C and placed on blotting paper, which was then well wetted with a 10% formaldehyde solution. The blotting paper and larvae were put in a sealed container and sent by air mail to England. On receipt the larvae were dissected individually and the resulting dissections treated with Giemsa stain and examined under a compound microscope.

Seventy-nine of the larvae recovered from site 22, the only site that consistently produced parasitized larvae during the author's visit to Nauru, were found to be parasitized with *P. culicis*. As Table 4 shows, the only other site that yielded parasitized larvae was site 15. Shortly after the collections started the drums dried out owing to lack of rain and *Gambusia* were found in site 49. In both cases no further larvae were found during the survey.

Table 4. Follow-up survey of *C. p. fatigans* larvae, May–October 1969

Site	Number examined	Number parasitized	% parasitized
15	4 139	3	0.07
18	3 141	0	0.0
22	3 965	79	1.99
49	485	0	0.0
drums	227	0	0.0
Total	11 957	82	0.69

Introduction of a mermithid nematode

An attempt was also made to introduce a mermithid nematode parasitic in mosquito larvae in its larval stages. This mermithid, obtained from Mr J. Muspratt, was originally found in tree holes in

Livingstone, Zambia (Muspratt, 1945). The mermithid was cultured in England using the method advocated by Muspratt (1965). Eight culture jars were taken to Nauru and, when shown to be infective, were introduced one in each of 4 tree holes and 4 in a bomb crater. In the tree holes both *C p. fatigans* and *A. aegypti* were breeding, while only *C. p. fatigans* was found in the bomb crater. A check 10 days after introduction showed both *C. p. fatigans* and *A. aegypti* larvae to be infected in the tree holes. No mermithids were found in 460 larvae from the bomb crater examined over a period of 30 days after introduction.

DISCUSSION

P. culicis, which was present in site 22 18–24 months after introduction, can be said to have become established in this one site, and possibly also in site 15. The infection rates found in the follow-up survey are considered to be minimum figures owing to the methods used, especially the fixation of larvae before dissection, which meant that light and early infections would probably not be detected.

The infection rate of 1.99% obtained at site 22 is comparable to the natural infection rates found by Kellen & Wills (1962) in Californian mosquitos infected with various species of *Thelohania*. It appears from the follow-up survey that there has been no reduction in mosquito numbers on Nauru; this supposition is supported by correspondents on the island. The presence of a naturally occurring microsporidian infection has not been shown to diminish a mosquito population, although it may have deleterious effects on individuals. These effects may well be masked by the high natural mortality that keeps mosquito populations relatively constant.

No evidence of dispersion of *P. culicis* to unseeded sites was found. Dispersion would occur principally by infected adult mosquitos dying over water, the spores being liberated on disintegration of the mosquito's body.

At site 19 the failure to produce any infection was supported by the laboratory findings. It was thought that the water contained some inimical factor, but its nature could not be investigated on the island. The unexpected cessation of mosquito breeding in sites 15 and 18 could not be satisfactorily explained; it may have been due to contamination of the site by local inhabitants, although no evidence of this could be obtained. It was not thought to be a consequence of the experimental work carried out in the site. Breeding had begun again by the time the follow-up survey started.

The dosage of spores applied to the larval breeding sites, resulting in a final concentration of about 6 000 spores per ml of water in the site, was based entirely on laboratory studies carried out in England, where infection rates of 80–100% were obtained at this concentration (Reynolds, 1970). It was possibly too low to produce high infection rates in natural water with an abundant and varied food supply. Another point to be established before similar introductions are attempted is that the local strain of mosquito is readily susceptible to infection. This may not have been so in Nauru.

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Sections of the work described formed part of a thesis submitted for the degree of Doctor of Philosophy in the University of London.

RÉSUMÉ

INTRODUCTION EXPÉRIMENTALE D'UNE MICROSPORIDIE DANS UNE POPULATION SAUVAGE DE *CULEX PIPIENS FATIGANS* WIED.

Au laboratoire, la microsporidie *Plistophora culicis* infecte aisément *Culex pipiens fatigans* et abaisse le taux de reproduction du moustique. Pour étudier les possibilités d'introduction du parasite dans une population naturelle, on a choisi l'île de Nauru, petit atoll isolé de la Micronésie. *C. p. fatigans* y prolifère abondamment

pendant toute l'année et on a pu repérer ses gîtes. Une enquête préliminaire, portant sur 7240 *C. p. fatigans*, adultes et larves, et sur 925 *Aedes aegypti*, adultes et larves, n'avait décelé aucun parasite ou autre agent pathogène.

Dans un premier temps, des spores de *P. culicis* ont été introduites dans 4 gîtes artificiels de *C. p. fatigans*. Dans 3 d'entre eux, des larves infectées ont été trouvées après 15 jours: dans l'un, 14,1% des larves examinées après 66 jours étaient parasitées; dans les deux autres, la reproduction du moustique a été fortement entravée et a cessé complètement après 66 et 49 jours respectivement, mais le phénomène ne paraît pas attribuable à l'action du parasite. Dans le 4e gîte, en dépit d'une intense pullulation de *C. p. fatigans*, aucune larve infectée n'a été découverte; une étude de laboratoire a fait soupçonner l'existence dans l'eau du gîte d'un facteur inconnu empêchant l'infection de s'établir.

Après ensemencement de 3 gîtes artificiels occupés par *C. p. fatigans* par des spores de *P. culicis*, des larves parasitées ont été récoltées dans 2 d'entre eux après 14 jours.

Au laboratoire, on a obtenu par introduction de spores de *P. culicis* des taux d'infection de *C. p. fatigans* de 17,1 à 39,0%.

Une enquête effectuée 18 à 24 mois après l'introduction de *P. culicis* dans les gîtes a permis de trouver des larves infectées dans un gîte, le taux d'infection étant de 1,99% sur 3965 larves.

On a aussi tenté d'infecter *C. p. fatigans* et *Ae. aegypti* par un parasite mirmithidé isolé à partir de larves de moustiques en Zambie: 10 jours après le début de l'essai, des larves parasitées ont été récoltées dans 3 gîtes sur 4.

Ces expériences montrent que la pénétration et le maintien de *P. culicis* dans une population naturelle de *C. p. fatigans* sont réalisables. Cependant, après 18-24 mois, les taux d'infection ainsi provoqués ne diffèrent pas de ceux observés dans l'infection naturelle d'insectes par des microsporidies et ils ne sont pas assez élevés pour modifier de façon sensible la densité des populations de moustiques.

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