caught by the VF-or synthetic lure-traps (KLUN et al., 1973).

We do not know the mechanisms whereby repression of attraction and suppression of mating results from exposure of males to a single component of sex pheromones. The single component released artificially must reach the receptors of the male in combination with the natural sex pheromone released by the females. It is tempting to speculate, therefore, that an imbalance created in the ratio of the pheromonal components can possibly result in reception of a different signal by males. This signal fails to cause the males to orient toward the source or to be sexually stimulated. Pheromonal components in an imbalanced ratio apparently do not evoke any specific behavioral reactions in males. Perhaps such ratios are equivalent to signs from a different species. Hence use of components, especially minor ones, of sex pheromones to disrupt the chemical communication of insects may become an important

aspect of insect control in the near future.

REFERENCES

- BEROZA, M., G. M. MUSCHIK and C. R. GENTRY (1973) *Nature, New Biol.* **244**: 149-150.
 HIRAI, Y., Y. TAMAKI and T. YUSHIMA (1974) *Nature* **247**: 231-232.
 JACOBSON, M. (1972) "*Insect sex pheromones*" Academic Press, N. Y. and Lond., 382 pp.
 KLUN, A., O. L. CHAPMAN, K. C. MATTES, P. W. WOJTKOWSKI and M. BEROZA (1973) *Science* **181**: 661-663.
 OYAMA, M. (1974) *Jap. appl. Ent. Zool.* **18**: 9-13.
 TAMAKI, Y. (1972) *Seibutsu-Kagaku* **24**: 119-129.
 TAMAKI, Y. and T. YUSHIMA (1974) *Appl. Ent. Zool.* **9**: 73-79.
 TAMAKI, Y., H. NOGUCHI and T. YUSHIMA (1973) *Appl. Ent. Zool.* **8**: 200-203.
 YUSHIMA, T., Y. TAMAKI, S. KAMANO and M. OYAMA (1974) *Appl. Ent. Zool.* **7**: 147-152.

Bacterial Production of the Ovipositional Attractants for Mosquitoes on Fatty Acid Substrates¹

Toshiaki IKESHOJI

Department of Agricultural Biology, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Katzumi SARTO and Akira YANO

Department of Parasitology, School of Medicine Yokohama City University, Minamiku, Yokohama 232, Japan

(Received May 7, 1975)

The investigation of kairomones inducing mosquito oviposition in the field water is of importance for understanding of the ecology, and the eventual identification of a powerful attractant provides a novel means for assessment and control of mosquito populations.

There has been an ample evidence for the fact that microorganisms, bacteria in particular,

produce the ovipositional attractants and stimulants for mosquito oviposition in the mosquito-breeding waters. IKESHOJI, UMINO and HIRAKOSO (1975) isolated from the mosquito-breeding waters a few *Pseudomonas* spp. which produced the ovipositional stimulants for *Culex pipiens fatigans* Coquillett on a gelatin medium.

While, HAZARD, MAYER and SAVAGE (1967) isolated from hay infusion *Aerobacter aerogens* which, in distilled water, was quite attractive for *Culex pipiens quinquefasciatus* Say and *Aedes aegypti* (L.). Later, MAW (1970) found that n-capric acid, when treated into artificial pools, made the water quite attractive for oviposition of *Culex restuans* Theobald within several days after the treatment. He also proved the water to be attractive for *A. aegypti*, *Culex pipiens* L. and *Culex tarsalis* Coq. in the laboratory and incriminated a few unidentified bacteria of the family Pseudomadaceae as the possible agents producing the attractive kairomones. Although MAW thought n-capric acid to be mere carbon supply for the rapid growth of the bacteria, we suspect that some of the bacterial metabolites become the precursors in the biosynthesis of the ovipositional at-

¹ Appl. Ent. Zool. **10** (3): 239-242 (1975)

tractants.

To isolate and identify the attractive principles in future, we confirmed the MAW's finding, isolated the responsible bacterium and defined the conditions of the *in vitro* production of the attractant.

Attractancy of the waters treated with 5 fatty acids

We placed humic soil collected from a tree base at the school yard into a glass container, $15 \times 25 \times 30 \text{ cm}^3$, in 2- or 3-cm depth, added 4 l of tap water, and left it indoor at a temperature of 25° to 32°C . At 2 days after flooding, the container was treated with one of the following 5 fatty acids at either 100 ppm or 300 ppm; n-caprylic, n-pelargonic, capric, n-lauric and n-myristic acids. The untreated water was kept as a control under the same condition. The ovipositional attractancy of the treated water was tested by taking the surfacial water into a small petridish, 5 cm in diam, and giving the choice of oviposition to 30 to 40 gravid females of *Culex pipiens molestus* FORSKAL in a cage against the untreated water. The petridishes were set in a completely dark room in the evening and the numbers of egg-rafts laid by the next morning were counted. The attractancy are expressed in ratios of the numbers of egg-rafts laid on the treated waters against those laid on the untreated. This *in vitro* experiment was repeated 4 times with 2 tests for each water sample.

Fig. 1. Increase of the ovipositional attractancy of the waters treated with 300-ppm fatty acids.

The remarkable attractancy, 7 ± 6.7 times at the 95% conf. limit against the untreated water, appeared with the water treated with 300-ppm capric acid on the 7th day, and reached the maximum, 19.5 ± 6.2 times, on the 10th day, and 20.1 ± 2.8 times on the 13th day (Fig. 1). The treatment of pelargonic and lauric acids, which are closer to capric acid in the molecular size, showed good attractancy, 3 to 6 times, until the 16th day. The treatment of caprylic acid also showed some attractancy throughout the experimental period. The treatment of myristic acid, on the contrary, showed repellency till the 13th day and became attractive to some extent, later. The 100-ppm treatments of the 4 fatty acids excluding myristic acid, made the waters attractive earlier than the corresponding 300-ppm treatments, but in less intensities; they showed 2- to 6-time attractancy against the untreated water.

The waters, when treated with the 4 shorter fatty acids at 300 ppm, became muddy gradually in appearance. By the 7th day the waters turned to yellow, and by the 10th day to brown. Heavy scum was formed on the water surface by the 13th day. This color change of the treated waters, which synchronized with the increase of attractancy, progressed earlier by two days with the 100-ppm treatments. The myristic acid treatment, however, made the water less muddy than the other acids did. All treated waters became clear on the 15th day when the attractancy was almost lost.

The bacterial numbers at 2- or 3-cm depth from the surface, which were counted with a blood-cell counter by staining with acid fuchsin, increased from $3 \times 10^6/\text{ml}$ at the beginning to $2 \times 10^8/\text{ml}$ in the end in the capric acid-treated water at 300 ppm. The latter count was approximately 33 times more than that in the untreated water (Fig. 2). This change of the bacterial count was naturally reflected on the dissolved oxygen in both treated and untreated waters. The DO at 2- or 3-cm depth in the treated water decreased rapidly from 10 ppm on the 2nd day to 2 ppm on the 5th day and remained there even after its complete loss of the attractancy, whereas it decreased slightly from 14.5 ppm to 11 ppm in the untreated water.

In the end of the experiment, the 16th day, the attractancy was almost lost in spite of the maximum level of the bacterial count in the treated water. This is possibly due to the deple-

Fig. 2. Increase of the bacterial numbers and decrease of the dissolved oxygen in the waters treated with 300-ppm capric acid.

Table 1. ATTRACTANCY OF THE BACTERIAL CULTURES WITH 1% FATTY ACIDS TO *A. aegypti* AND *C. p. molestus*^a

Fatty acids	Attractancy in times to	
	<i>aegypti</i>	<i>molestus</i>
Caprylic	5.5	6.1
Pelargonic	16.8	8.5
Capric	4.9	14.4
Lauric	1.5	3.1
Myristic	1.8	1.2

^a The figures italicized are significantly different from 1.0 at the 5% level of t-tests.

tion of capric acid substrate, and the change of the bacterial flora. This also suggests that the attractive principles are the intermediate metabolites of the bacterium specific on the capric acid substrate.

In vitro production of the attractants on the capric acid substrate

Several bacteria were isolated on agar-plates (Meat infusion agar of Difco lab., Michigan) by inoculating with the capric acid-treated water which was showing the maximum attractancy. When each bacterial colony was subcultured aerobically at 37°C in test-tubes with nutrient broth (Difco lab.) containing 1% capric acid, only

a *Pseudomonas* grew well and produced the good attractancy. This bacterium was identified as *Pseudomonas aeruginosa*.

To repeat the good attractancy of capric acid metabolites *in vitro*, the nutrient broth added with one of the 5 fatty acids was inoculated with the *Pseudomonas* and incubated at 37°C for 2 to 4 days. The bacterial cultures diluted to 25-fold with tap water was tested against the similar diluent of the un-inoculated medium. The bacterial culture with capric acid was quite attractive to *C. p. molestus* as it was proved in the preceeding *in vivo* experiment, showing 14.4-time attractancy against the control, and 4.9-time to *A. aegypti* (Table 1). The culture with pelargonic acid, on the contrary, was 16.8 times attractive to *A. aegypti*, and 8.5 times to *C. p. molestus*. However, all bacterial cultures, which involved the undigested deterrent fatty acids, were less attractive than the control tap water. Therefore, hereafter, the ether-extract of the bacterial culture or its neutral fraction was bioassayed by dropping on a glass-slide, and covering a water-filled petridish with it treated-side-down, so that only odor characteristic could be tested.

The extraction and fractionation scheme is shown in Figure 3. Five or 50 μ l of the ether-extract, which was yielded from 25 or 250 mg of capric acid, showed 11.5-time attractancy to *A. aegypti*, and 0.2-time attractancy or the high repellency to *C. p. molestus*. The aqueous fractions after eliminating the ether solvent by stirring on a hot plate for few hours, also showed some attractancy to both species. The complete disap-

Fig. 3. Preliminary isolation scheme of the ovipositional attractants from the bacterial culture containing 1% capric acid, and the attractancy of the corresponding fractions to *A. aegypti* (upper figures) and *C. p. molestus* (lower figures).

Table 2. ATTRACTANCY TO THE MOSQUITOES OF THE BACTERIAL CULTURES CONTAINING WITH VARIOUS QUANTITIES OF NUTRIENT BROTH AND IN VARIOUS INCUBATING PERIODS

Broth (g/l)	Fraction	3 days		5 days		7 days	
		<i>aeg.</i>	<i>mol.</i>	<i>aeg.</i>	<i>mol.</i>	<i>aeg.</i>	<i>mol.</i>
8	Ether	2.0	0.2	11.5	0.2	2.9 ^a	0.4 ^a
	Aqueous	0.4	5.2	2.0	2.9	1.0	1.6
2	Ether	1.1	0.3	2.1	0.1	5.8 ^a	0.3 ^a
	Aqueous	1.0	1.2	2.1	3.0		
0.8	Ether	0.8	0.2	3.9	0.1		
	Aqueous	1.6	2.5	0.9	4.6		
0	Ether	0.5		1.0	0.3		
	Aqueous	0.8	1.4	0.8	1.3		

^a Neutral fraction of the ether-extract was tested.

pearance of the attractancy to *C. p. molestus* from the ether-phase is due to the separation from the possible co-attractant or stimulant left in the aqueous phase. Washing the ether-extract with an alkali salted out the excessive fatty acid as the soap. The resulted neutral fraction showed 7.3-time attractancy to *A. aegypti*, but 0.5-time attractancy to *C. p. molestus*.

The culturing condition for the maximum attractancy was sought by changing the quantity of the nutrient broth and the incubating period. The 5-day culture with 8-g nutrient broth per litre of water turned out the best to *A. aegypti*, showing 11.5-time attractancy in the ether-extract (Table 2). Regardless the culturing condition, the good attractancy was always observed in the ether-extracts to *A. aegypti*. The aqueous phases, on the contrary, induced more oviposition of *C. p. molestus* as previously observed in Figure 3.

Meanwhile, the nutrient broth without capric acid, when inoculated with the same bacterium, showed no appreciable attractancy to these mosquitoes.

Therefore, we conclude that the *Pseudomonas* produces the ovipositional attractants for both species of mosquitoes on capric and pelargonic acid substrates, and the attractants would be the intermediate metabolites of these fatty acids.

This work was done with the grant from the Ministry of Education, No. 957063. We express our gratitude to Prof. Shigeo HAYASHI, Department of Parasitology, Yokohama City University, and Prof. Yoshiharu MATSUMOTO, Department of Agricultural Biology, University of Tokyo, for their cooperation and encouragement in conducting this research. We are also grateful to Prof. TADOKORO, Department of Microbiology, Yokohama City University, for his identification of the bacterium.

REFERENCES

- HAZARD, E. I., M. S. MAYER and K. E. SAVAGE (1967) *Mosquito News* **27**: 133-136.
 IKESHOJI, T., T. UMINO and S. HIRAKOSO (1967) *Jap. J. Exp. Med.* **37**: 61-69.
 MAW, M. C. (1970) *Nature* **227**: 1154-1155.