ent populations of *Haemonchus* larvae show significant variations in the magnitude of the response (Table 1). The reasons for this are as yet unknown.

Larvae for these experiments were cultured and isolated by the method of Christie and Patterson (*7*) for *Haemonchus* and that of Wilson and Dick (*8*) for *Nippostrongylus*. *Haemonchus* were accumulated in water at 5° to 7°C for periods of 7 to 10 days. For a day before respirometry, and during the experiments themselves, they were contained in an antibiotic solution (penicillin and streptomycin, 50 mg/liter each; and actidione, 20 mg/liter), being washed 3 times initially and again immediately before distribution to the Warburg flasks. *Nippostrongylus* were kept on a shaker at 25°C in a balanced salt solution containing the same antibiotics at the above concentration ("¼ RLA+") (*9*) or at half strength ("¼ RLA") (*6*). Experiments with *Haemonchus* in water alone confirm that the response is confined to the larvae and is not a light-reversible inhibition of oxygen uptake by the antibiotics.

Table 1. Oxygen uptake at 30°C, per milliliter of larval suspension per hour, under different light conditions. Experimental design is same as described in legend of Fig. 1. Hc, *Haemonchus contortus*; Nb, *Nippostrongylus brasiliensis*.

Approx. light intensity (lu/m²)	Oxygen uptake (µl)			
	Exptl. flasks		Control flasks*	
	1 hr before†	1 hr after†	1 hr before†	1 hr after†
	Hc (experiment 1)			
3200	10.1	34.4	19.2	16.2
	10.5	39.6	18.5	17.3
	Hc (experiment 2)			
7000	21.7	47.7	30.9	30.2
	21.9	46.3	29.5	33.1
	Nb (experiment 1)			
	44.6	46.5	43.2	43.8
3200	44.8	45.7	39.9	41.4
	42.4	47.9	42.5	42.5
	Nb (experiment 2)			
	26.9	32.4	29.4	26.8
6700	25.7	28.8	29.6	27.3
	27.3	29.1	31.4	30.1

* Control flasks were in light throughout the experiments. † Before and after aluminum foil was removed from experimental flasks.

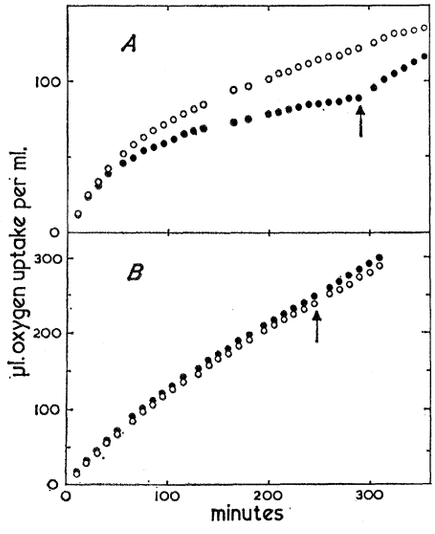

Fig. 1. Oxygen uptake at 30°C of *Haemonchus contortus* (*A*) and *Nippostrongylus brasiliensis* (*B*) infective larvae in daylight of approximately 3200 lu/m² (○) and in the dark (●). The arrows indicate where larvae in darkness were subjected to light by removal of the protecting aluminum foil. Each point is the mean of two (*A*) or three (*B*) 6-ml Warburg flasks containing 1 ml of larval suspension in simple antibiotic solution (*A*) and ¼ RLA/solution (*B*).

Warburg constant-volume manometric techniques were used to measure oxygen uptake at constant temperature (30°C in all cases; Braun system), and the larvae were subjected to darkness by enclosing the respirometer flasks and manometer arms in aluminum foil. The Warburg bath was constructed of transparent plastic and therefore allowed the maximum amount of light to reach larvae in uncovered flasks. The light source was diffuse daylight in a laboratory with windows facing northeast. Unless artificially diminished, the incident light falling on the apparatus varied between 2120 and 7600 lu/m² during the experiments.

Absence of an effect of light on oxygen consumption does not, of course, rule out all types of photosensitivity. Photoklinokinesis, such as that reported for *Trichonema* larvae (*3*), need bear no relation to changes in metabolic rate. Thus *Haemonchus* and *Nippostrongylus* larvae may respond in some way to light below the threshold necessary to stimulate oxygen uptake. In the case of *Nippostrongylus*, however, the very high intensities of illumination necessary to bring about even small changes in oxygen uptake in my experiments, together with the conclusions of Parker and Haley (*5*) from behavioral studies, suggest that light sensitivity is of little consequence in its biology. Nevertheless, the demonstration of such a stimulus in both species under conditions of rigorous temperature control constitutes proof for the first time of a dermal light sense in nematodes.

PETER A. G. WILSON

Zoology Department,
University of Edinburgh, Scotland

References and Notes

1. D. M. Steven, *Biol. Rev.* **38**, 204 (1963).
2. F. Veglia, *Rep. Vet. Res. S. Afr.* **3** & **4**, 349 (1915).
3. N. A. Croll, *Parasitology* **55**, 579 (1965).
4. H. R. Wallace, *Helminthol. Abs.* **30**, 1 (1960).
5. J. C. Parker and A. J. Haley, *Exp. Parasitol.* **9**, 92 (1959).
6. P. A. G. Wilson, *ibid.* **17** (1965).
7. M. G. Christie and J. E. Patterson, *J. Helminthol.* **37**, 251 (1963).
8. P. A. G. Wilson and J. M. Dick, *ibid.* **38**, 399 (1964).
9. P. A. G. Wilson, *Exp. Parasitol.* **16**, 190 (1965).
10. I thank M. G. Christie and J. E. Patterson for the supply of clean *Haemonchus* larvae and Miss J. M. Dick for skilled technical assistance. MRC (U.K.) grant G964/364/B supports this work. Apparatus used was bought during the tenure of NIH grant AI-04683.

12 October 1965

Fluctuation Tests with Antibody-Forming Spleen Cell Populations

Abstract. *Shortly after immunization of mice, cells forming specific antibodies to one antigen, for example, sheep red blood cells, are nonrandomly distributed throughout the spleen. If the spleen donor has been immunized with two different antigens, for example, sheep red blood cells and chicken red blood cells, the nonrandom distribution of spleen cells forming antibody to one antigen differs significantly from that of cells forming antibody to the other antigen. These findings are in accord with a clonal distribution of antibody-forming cells.*

Jerne's plaque technique, which permits an estimation of the number of spleen cells forming specific antibodies (*1*), has made it possible to apply the fluctuation test, previously used in assays on bacterial populations (*2*), to populations of antibody-forming mouse cells. By comparison of the fluctuation due to sampling error, obtainable by plating several samples from one spleen piece, with the fluctuation in numbers of antibody-forming cells in different pieces of the same spleen, it was determined whether formation of antibodies to a given antigen was randomly distributed throughout the spleen or tended to occur in clones of antibody-forming plasma cells.

Mice (AKR) were immunized intravenously with 1×10^8 sheep red blood cells, and their spleens were removed 72 hours later. The spleen was cut into 24 pieces of approximately equal size by two longitudinal and seven crosswise cuts. Cell suspensions were prepared from each piece, counted in a hemocytometer, and assayed for number of

hemolysin-forming cells by localized hemolysis in agar (*1*). In addition, ten replicate samples of the cell suspension prepared from one single piece were assayed separately. As shown in the columns headed "Sheep RBC" of Tables 1 and 2, the variances among replicate samples from one piece were very low (*3*). In contrast, the variance among samples from different spleen pieces was very high, and the *P* (probability) value indicates that these samples were not derived from one homogeneous population. Also, the variance ratio, *F*, indicates that the variation from piece to piece is much larger than the variation due to sampling (*P* much less than 1 percent) (*4*). The magnitude of the variances decreased as the time between immunization and assay of spleen pieces increased.

The question now arose whether the nonrandom distribution of hemolysin-forming cells within a given spleen shortly after immunization was the reflection of an anatomical heterogeneity (some parts of the spleen being poorer in plasma cells than others) or a reflection of a clonal distribution of antibody-forming cells. To answer this, spleen donors were immunized with two antigens, devoid of cross-reactivity, namely either sheep and chicken red blood cells (Table 1), or sheep and horse red blood cells (Table 2). Chicken red blood cells and sheep red blood cells were injected simultaneously, but since horse red blood cells are less immunogenic in the mice used than sheep red blood cells are, the horse cells were injected 96 hours, and the sheep cells 72 hours, prior to the removal of the spleen. If the nonrandom distribution of hemolysin-forming cells was the result of an anatomical feature, it would be expected that pieces high in number of cells forming antibody to one antigen, would also be high in cells forming antibody to the other antigen. Similarly, pieces with low counts should be low in tests with either antigen. However, the results (Tables 1 and 2) showed that there was no such parallelism (coefficient of correlation 0.161 in the case of sheep red blood cell versus chicken red blood cell; 0.238 in the case of sheep red blood cell versus horse red blood cell). Hence, it may be concluded that the differential nonrandom distribution of responses to two different antigens could be a reflection of a clonal distribution of cells forming antibodies to a given antigen. The data do not reveal whether such a distribution results from the division of antibody-forming cells or, possibly, from a gradual spread of

Table 1. Number of hemolysin-forming cells in samples taken from different pieces of one spleen and in a series of similar samples taken from one piece of this spleen. Spleen donor was immunized 72 hours earlier with 1×10^8 sheep red blood cells (RBC) and 1×10^8 chicken RBC. Results presented here remained essentially the same when plaque counts were adjusted in terms of equal number of cells per piece. After such adjustment the variances for samples from different pieces were 2424.68 and 350.55; the chi-squares values were 607.77 and 224.64, respectively. The cell count for the repeatedly sampled single piece was 2.2×10^7 per piece.

Samples from different pieces				Samples from a single piece		
		Plaque count			Plaque count	
Piece No.	No. cells ($\times 2 \times 10^{-5}$/piece)	Chicken RBC ($\times 10^{-1}$/piece)	Sheep RBC ($\times 10^{-1}$/piece)	Sample No.	Chicken RBC ($\times 2 \times 10^{-1}$/piece)	Sheep RBC ($\times 10^{-1}$/piece)
1	110	147	19	1	51	39
2	105	31	12	2	56	39
3	142	61	51	3	60	38
4	90	83	10	4	51	36
5	138	109	12	5	49	30
6	116	41	14	6	46	38
7	125	63	19	7	47	34
8	116	33	20	8	56	38
9	133	92	62	9	52	34
10	105	152	32	10	62	37
11	157	50	48			
12	114	81	62			
13	130	61	18			
14	103	38	35			
15	80	130	48			
16	112	35	18			
17	125	37	8			
18	96	97	13			
19	114	88	47			
20	91	21	37			
Average	115.1	72.5	29.3	Average	53.0	36.3
Variance		1555.63	326.32	Variance	28.67	8.22
Chi-square		407.68	211.97	Chi-Square	4.87	2.04
P much less than		.005	.005	*P*	.839	.991

F (for chicken red blood cells) = 54.26
F (for sheep red blood cells) = 39.70

Table 2. Number of hemolysin-forming cells in samples taken from different pieces of one spleen and in a series of similar samples taken from one piece of this spleen. The spleen donor was immunized with 1×10^9 horse red blood cells (RBC) and 24 hours later with 1×10^8 sheep RBC. Then 72 hours later the donor was killed. After adjustment of the plaque counts, the variances for samples from different pieces were 335.68 and 83.92, the chi-square values were 269.11 and 170.5, respectively. The cell count for the repeatedly sampled single piece was 2.6×10^7 per piece.

Samples from different pieces				Samples from a single piece		
		Plaque count			Plaque count	
Piece No.	No. cells ($\times 2 \times 10^{-5}$/piece)	Horse RBC ($\times 0.25 \times 10^{-1}$/piece)	Sheep RBC ($\times 10^{-1}$/piece)	Sample No.	Horse RBC ($\times 10^{-1}$/piece)	Sheep RBC ($\times 10^{-1}$/piece)
1	154	11	1	1	4	8
2	121	61	6	2	4	10
3	120	40	5	3	3	14
4	152	6	15	4	4	10
5	101	11	2	5	2	14
6	120	8	5	6	6	11
7	146	5	13	7	5	10
8	138	24	22	8	2	9
9	104	34	12	9	5	9
10	130	56	34	10	3	11
11	148	7	3			
12	156	15	0			
13	143	47	11			
14	137	10	29			
15	137	34	5			
16	125	31	2			
17	102	11	4			
18	107	17	1			
19	115	6	7			
20	135	26	10			
Average	129.6	23.0	9.4	Average	3.8	10.6
Variance		305.16	88.79	Variance	1.73	4.04
Chi-square		252.09	180.43	Chi-square	4.11	3.43
P much less than		.005	.005	*P*	.904	.944

F (for horse red blood cells) = 189.54
F (for sheep red blood cells) = 21.98

specific information through existing cells. In any event, a clonal distribution of antibody-forming cells is not necessarily a confirmation of the clonal theory of antibody formation (*5*) since the events reflected in the present tests are probably only the response of individual cells to information produced within, and transmitted from, a different cell; the latter may even be of an entirely different type (*6*).

MASAYASU NAKANO
WERNER BRAUN
Institute of Microbiology, Rutgers University, New Brunswick, New Jersey

References and Notes

1. N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963); N. K. Jerne, A. A. Nordin, C. Henry, in *Cell-bound Antibodies* (Wistar Institute Press, Philadelphia, 1963).
2. S. E. Luria and M. Delbrück, *Genetics* **28**, 491 (1943).
3. In fact, the variances are so low that a word about this is in order. It should be noted that the replicate samples from a single piece are derived from a thoroughly homogenized cell suspension and that only a very minor proportion of the many cells plated are scored. A Poisson distribution, therefore, cannot be expected for these samples.
4. Since the distribution of plaque counts of individual pieces was not normal, it was necessary to transform the counts, at $\log_{10}$, to normalize the distributions. In spite of such transformations the statistical conclusions remained similar.
5. F. M. Burnet, *The Clonal Selection Theory of Acquired Immunity* (Vanderbilt Univ. Press, Nashville, 1959); J. Lederberg, *Science* **129**, 1649 (1959).
6. M. Fishman, J. J. van Rood, F. L. Adler, in *Molecular and Cellular Basis of Antibody Formation*, J. Sterzl, Ed. (Publishing House of the Czechoslovak Academy of Sciences, Prague, 1965).
7. We thank Drs. H. P. Andrews and C. E. Cress for aid in statistical considerations, and Dr. D. W. Talmage for stimulating discussions.
8. Supported by NIH grant AM 08742 and NSF grant 19974.

26 July 1965

Pheromone: Evidence in a Decapod Crustacean

Abstract. *Males of the species* Portunus sanguinolentus *display a behavioral response to the presence of premolt females which is the same as their behavior when they are exposed to water in which premolt females have been kept. Release of a sex-attractant pheromone is indicated. When females are prevented from releasing urine, there is no evidence of the attractant.*

Copulation in many crustaceans is associated with the molting period (*1*). In the Brachyura, or true crabs, copulation in some species occurs immediately after molt of the female. For several days, the male carries the premolt female, and the male is in attendance at ecdysis. Copulation occurs 2 to 5 minutes after the female extracts herself from the exuvia (*2*). The way the male crab detects the premolt condition of the female is unknown. Certain workers conclude from their experiments that only the submissive behavior of the female indicates her condition to the male (*3*), whereas others suggest that detection of the premolt condition is chemosensory in crabs and other crustaceans (*4*). Occurrence of sex attractants in insects, in contrast with crustaceans, is well documented (*5*).

My experiments demonstrate release of a sex-attractant pheromone in *Portunus sanguinolentus* (Herbst), a large edible crab of the Indo-West Pacific, which closely resembles the blue crab of the United States. Evidence is also presented that suggests where the locus of release of the substance is in the female.

In 114 mating pairs of this species from Kaneohe Bay, Hawaii, males carried females up to 6 days before molt of the female, which occurred in any month. As Agassiz noticed (*6*), whenever a premolt female was placed in a holding cage containing several males, the latter began a peculiar display and search behavior. Each male became active, walked about the cage on the tips of its dactyls with its body elevated at maximum height above the substratum, extended its oxblood red chelae (female chelae are white), and attempted to pull whatever crab it came in contact with, male or female, into the precopulatory holding position. Crabs other than premolt females usually escaped.

During a 4-year period, 850 molting crabs were kept with adult males in holding cages which held up to 40 crabs at one time. In absence of premolt females, adult males never exhibited the search behavior or attempted to grasp premolt males or premolt juvenile females. The molting crabs had to be removed at the time of ecdysis to prevent them from being devoured. Adult males of *P. sanguinolentus* displayed no search behavior nor did they copulate with premolt or soft females of two other species of Portunidae, *Thalamita crenata* and *Podophthalmus vigil*, which also copulate when they molt. Nor did males of these other species make any attempt to copulate with *Portunus sanguinolentus* females which molted in their presence.

Evidence that a pheromone was present was sought by siphoning water in which females had been kept into aquariums containing adult males. Subject males were in intermolt and had been in captivity without exposure to premolt females for at least 48 hours prior to the experiments. Each female to be tested was kept in an 8-liter polyethylene bucket of clean sea water for 2 hours and then removed. The premolt females had been tested during the premolt stage before either of the two sexually mature instars (*2*). Hard, intermolt females served as controls. A positive response to the introduction of water was display and search behavior.

Table 1. Release of sex attractant by *Portunus* females as indicated by the response of males to water in which either premolt or intermolt females had been kept. Positive response (+) is search behavior of one male within 5 minutes after the siphon flow ended; negative response (−) is no reaction in 5 minutes by one male.

Trial	Crabs (No.)			Response	
	Premolt ♀(A)	Intermolt ♀(B)	♂	A	B
1	2	1	6	++++	−−
2	2	1	6	++−−	−−
3	2	1	6	++++	−−

Males were tested with water from the premolt and control females in an adjacent pair of wooden tanks. Dimensions of the tanks varied in different replicates, but identical conditions were maintained in the paired tanks in each replicate. Two males were placed in each tank, with running sea water, 1 hour before testing. The sea water supply was turned off, and the water level was lowered to allow test water to be added. Equal flow and pressure were maintained by placing each of the identical 8-liter buckets of water at the same height above the water level of the aquariums. Siphon tubes were of

Table 2. Release of sex attractant by *Portunus* females as indicated by the response of males to water in which premolt females had been kept before (C) and after (D) the excretory pores had been capped and after cap removal (E). Positive response (+) is search behavior of one male 5 minutes after the siphon flow ended; negative response (−) is no reaction in 5 minutes for one male.

Trial	Crabs (No.)		Response		
	♀	♂	C	D	E
1	1	4	++	−−	Not done
2	1	6	+−	−−	++
3	1	6	++	−−	++
4	1	6	++	−−	++