Nature (Lond.) 251, 314-315 (1974).

I thank Drs R. A. Avery and M. Jarman and A. J. B. Mill for their criticism of the manuscript. This research forms part of a programme financed by a NERC contract to Professor J. S. Webb and Dr I. Thornton.

C. R. BOYDEN

Applied Geochemistry Research Group,
Imperial College,
London SW7 2BP UK

Received April 29; revised June 18, 1974.

1 Schulz-Baldes, M., *Mar. Biol.*, **2**, 98–102 (1973).
2 Schmidt-Nielsen, L., *Fedn Proc.* **29**, 1524–1532 (1970).
3 Davies, P. S., *J. mar. biol. Ass. UK*, **46**, 647–658 (1966).
4 Moore, P. G., and Edwards, D. E., *Standard statistical calculations*, (Pitmans, London, 1965).
5 Peden, J. D., Crothers, J. H., Waterfall, C. E., and Beasley, J., *Mar. Pollut. Bull.*, **4**, 7–9 (1973).
6 Romeril, M. G., *Rev. Int. Oceanogr. Med.*, **33**, 31–47 (1974).
7 Nickless, G., Stenner, R., and Terrile, N., *Mar. Poll. Bull.*, **3**, 188–190 (1972).
8 Cross, F. A., Hardy, L. H., Jones, N. Y., and Barber, R. T., *J. Fish Res. Bd Can.*, **30**, 1287–1291 (1973).
9 Hemmingsen, A. M., *Rep. Steno Mem. Hosp., Copenhagen*, **9**, 7–110 (1960).

VUILLAUME

LSD treatment of *Pieris brassicae* and consequences on the progeny

AT 20° C, under white light illumination and circadian rhythm, nymphal diapause of *Pieris brassicae* is dependent on a short 9-h photophase. Some psychodysleptics (mescaline sulphate and LSD), when injected during the photosentitive larval period, suppress diapause induction as if the larvae were subjected to a long 16-h photophase[1]. Abnormalities in the behaviour of the progeny of treated animals were observed. We studied them for three generations after the treatment.

Three couples of *P. brassicae*, progeny of a single original pair, were isolated. The progeny of each of these pairs were divided into two groups, one served as control (normal stock) and the other was treated with LSD. Control and treated animals of each progeny, respectively, were pooled at the end of each experiment after assessment of the fluctuation. Both stocks were subjected to a 9-h white light photophase treatment at 20° C (14,600 erg cm^{-2} s^{-1} with a Mazda TFR/40/BBL lamp).

LSD was diluted in Ringer solution to different concentrations. Animals were injected with 5 μl of the solution 24 h after the fourth moult.

As previously described[1], injection of 20 μg of LSD per animal results in about 80% of continuous development and 20% diapause induction. In the parental generation the LD_{10} was 35 μg per animal.

Among non-diapausing animals, different crosses were performed (Table 1). The normal partners were taken from the normal stock defined earlier.

The first striking observation was that all these F_1 larvae were highly resistant to LSD with a LD_{10} higher than 60 μg per animal. Second, 100% of the diapausing progeny of cross B, whether or not injected with 20 μg of LSD, died either at the end of the chrysalid stage or as malformed imagos (Table 1, B_1 and B_2, respectively).

Among diapausing animals, new crosses were performed (Table 1). Normal animals were taken from the progeny of the normal stock defined earlier. The sensitivity of the F_2 larvae to LSD in the three types of crosses seemed very high: the LD_{10} fluctuated between 5 and 10 μg per animal.

Different effects resulting from a treatment by LSD are apparent in the progeny of treated animals: an altered response to a 9-h photophase and the sensitivity to toxic effects of the drug. Two types of toxic effects have been detected. One is a short term effect measured through LD_{10}, the second is a long term effect apparent at the imaginal moult in diapausing animals.

The progeny of treated males and females (Table 1, B_1 and B_2) die at the imaginal moult although the egg laying ability of parents and hatching percentage, which were measured during all these experiments were found to be normal.

The early toxic effect of LSD follows a curious pattern with a LD_{10} of 35 μg for the parents, of more than 60 μg for the F_1 and 5–10 μg for the F_2 and the F_3.

The high resistance to LSD of the second generation may be explained by a detoxification mechanism, consequence of the parental treatment, enhanced by a further LSD treatment which could explain the slight improvement of viability in column B_2. Analysis of the effect of LSD on diapause induction must take the existence of such a resistance mechanism in account.

Table 1 Effect of LSD on diapause induction by a 9-h photophase

Parents	Treated			Not treated			Control (Ringer)
Total number	378			312			59
% diapausing	18			78			86
F_1	A: treated ♂ × normal ♀		B: treated ♂ × treated ♀		C: normal ♂ × treated ♀		Control
	A_1	A_2	B_1	B_2	C_1	C_2	
Total number	77	111*	60	101*	91	128*	312
% diapausing	99	100*	25	97*	79	48*	78
F_2	A_1 ♂ × normal ♀		C_1 ♀ × normal ♂		C_2 ♀ × normal ♂		
Total number	107		133		110		
% diapausing	45		57		65		

* When some animals were treated again, their total number and percentage of diapause are given (F_1 column 2).
Amount of LSD injected per animal was 20 μg. The control value in F_1 is taken from the parents.

The untreated progeny of treated males and females (B_1) show 25% of diapause induction only. This 'parental effect' is only observed if both parents were treated and completely disappears after a 20 μg LSD treatment (B_2). Such an induced resistance to LSD is asymmetric. If only one of the parents is treated and if this parent is the male, then treatment of the progeny has no effect (A_1/A_2). If this parent is the female, then a slight shift toward photophase resistance after LSD treatment can be observed (C_1/C_2).

We must then conclude that the resistance mechanism observed in the F_1 is very efficient when transmitted by males (A_1) and less efficient when transmitted by females (C_1). The LSD triggered enhancement of the resistance to the drug seems to be transmitted only by the males (A_2 and B_2) and is only apparent when a parental effect is present (B_1/B_2).

This parental effect can be observed in the F_2 generation even if only the grandfather or the grandmother was treated with LSD (results are statistically different from the normal 80% of diapause induction with less than 1% chance of error). We are apparently able to observe this transmission because the resistance mechanism, which in the F_1 masked the parental effect, is absent in the F_2.

The most probable target for LSD is the genetic material present in the germinal cells of the parental generation. Successive crosses with normal animals lead to a dilution of treated genetic material and a progressive shift toward a normal situation.

Damage to chromosome structure after LSD treatment has been described in different animals such as mice,

humans and amphibians[2-6]. Unfortunately such damage is difficult to detect in Lepidoptera.

If what we have found in an insect is also true in mammals then we should observe a high resistance to LSD in the progeny of treated parents and physiological effects detectable even several generations after treatment could be predicted as these are common features of the different crosses we have performed. This could be confirmed in mice.

MONIQUE VUILLAUME

Laboratoire de Zoologie (ERA 227)

ANDRÉ BERKALOFF

Institut de Microbiologie (LA 136),
Université de Paris Sud,
91405 Orsay, France

Received March 12; revised April 22, 1974.

[1] Vuillaume, M., and Seugé, J., *Bull. biol.*, **116**, (3) 285 (1972).
[2] Jagiello, G., and Polani, P. E., *Cytogenetics*, **8**, 136 (1969).
[3] Skakkebaek, N. E., and Philip, J., *Science*, **160**, 1246 (1968).
[4] Skakkebaek, N. E., *J. Reprod. Fert.*, **22**, 141 (1970).
[5] Hulten, M., Lindsten, J., Lidberg, L., and Ekelund, H., *Ann. Genet.*, **1**, 201 (1968).
[6] Chibon, P., and Belanger, M., *C. r. Acad. Sci. série D, Paris*, **74**, 80 (1972).

Regions of the freezing curve causing changes in structure and viability of ram sperm

WHEN ram spermatozoa are chilled and frozen, their acrosomes are damaged even in the presence of protective substances in the diluent. It has been suggested[1] that these structural changes contribute to the reduced fertility of frozen ram semen. Although the motility and structure of spermatozoa are affected to different extents by freezing it is not known if these changes occur simultaneously or are caused at different stages of the freezing process.

The present work was designed to identify the critical regions of the freezing curve by assessing the motility of and acrosomal damage to spermatozoa after cooling to various temperatures below zero. The experimental design was a 4^2 factorial array of temperature to which the sample was cooled (−5° C, −15° C, −45° C and −196° C) and level of egg yolk in the diluent (0%, 0.15%, 0.75% and 3.75% v/v). Samples of ram semen were collected in a buffered glucose solution similar to that described previously[1] except that the glucose content was increased from 185 mM to 247 mM. The semen was diluted at 30° C and then cooled at a constant rate of 0.21° C min^{-1} to 5° C. An equal volume of diluent containing glycerol was added at 5° C and the diluted semen was held at this temperature, for not less than 5 h. The final dilution rate was 20-fold. Semen was frozen in plastic straws (0.25 ml, Cassou, l'Aigle) at a constant rate of 12° C fall min^{-1} to −55° C in a controlled freezing device. For lower temperatures the straws were transferred at −55° C to liquid nitrogen (−196° C). The order of the freezing treatments in successive replications of the experiment was determined according to a Latin square design. By this means, the main effect of freezing treatments was separated from the possible influence of differences in the equilibration time at 5° C.

The samples were thawed 10 min after reaching the final temperature. All samples were examined under the microscope within 5 min of thawing, and motility was estimated on a subjective scale of 0–4 (ref. 2) and the percentage of motile spermatozoa was estimated to the nearest 10%. Smears of frozen-thawed semen were fixed, stained and scored as described by Watson and Martin[1], who scored acrosomal damage on a 0–3 scale (0 for an undamaged acrosome and 3 indicating loss of the acrosome).

The results and statistical analyses are shown in Table 1. A significant decline in the motility and percentage of motile cells after cooling and warming was seen in those samples cooled to −15° C or below, compared with those cooled only to −5° C ($P < 0.001$). Crystallisation of the external medium occurred at −12° C. Samples cooled to −5° C were therefore supercooled but not frozen; all other samples were frozen. This probably accounts for the reduction in survival of frozen samples since no difference in survival rate was detected between samples cooled to −15° C, −45° C and −196° C. Spermatozoa frozen to −15° C or below showed a greater mean acrosomal deterioration than those supercooled to −5° C ($P < 0.01$), but freezing to −45° C or −196° C was less detrimental than freezing to −15° C ($P < 0.001$). The greatest changes in the acrosomes were seen in those spermatozoa frozen to −15° C. Damage to acrosomes did not result from crystallisation of the suspending medium, however, since the degree of damage was similar in the samples cooled to −5° C and −196° C. Dehydration of the cells with consequent salt concentration, and intracellular ice formation and recrystallisation could all be factors contributing to the damage since these events could occur during the 10 min pause before rewarming. They are less likely to occur at lower temperatures because of the lower energy state of the sample.

The presence of egg yolk in the diluent improved the survival of spermatozoa after cooling and warming ($P < 0.001$). Survival with 0.75% egg yolk was significantly greater than with either the higher or the lower level of egg yolk ($P < 0.05$—motility score; $P < 0.001$—percent motile score). Acrosomal damage was decreased by increasing levels of egg yolk ($P < 0.05$). More egg yolk was thus required to protect the acrosomes, than to preserve motility. It may be that the stresses induced by chilling and freezing in the large surface area of the membranes surrounding the head of the spermatozoa are greater than those

Table 1 Mean survival and acrosome scores for ram spermatozoa cooled to different temperatures in diluents containing various levels of egg yolk

	Treatment	Motility (Scale, 0–4)	% Motile	Acrosome score (Scale, 0–3)
A	Temperature to which cooled (° C)			
1	−5	2.44	45.94	1.11
2	−15	1.86	30.94	1.51
3	−45	1.63	27.34	1.23
4	−196	1.80	30.47	1.12
B	Egg yolk level (%)			
0		1.42	22.19	1.32
0.15		1.92	32.50	1.30
0.75		2.28	43.44	1.22
3.75		2.09	36.56	1.13

Summary of analyses of variance

	Source of variation	Degrees of freedom	Variance ratios: Motility	% Motile*	Acrosome score
A	Cooling treatments				
	1 against 2, 3 and 4	1	37.93†	58.04†	8.75‡
	2 against 3 and 4	1	1.62	1.20	29.13†
	3 against 4	1	1.62	2.20	2.63
B	Egg yolk				
	Egg yolk against none	1	37.93†	54.77†	3.00
	Levels linear	1	1.62	2.37	5.50§
	quadratic	1	5.52§	15.86†	<0.01
	Equilibration	3	1.03	0.36	2.13
C	Ejaculates¶	7	4.14†	10.43†	3.13‡
	Pooled first-order interactions of treatments	51	0.49	0.80	1.19
	Residual (error) variance	60‖	*0.29*	*43.27*	*0.08*

* Data transformed to angles for analysis.
† $P < 0.001$; ‡ $P < 0.01$; § $P < 0.05$.
¶ 2 ejaculates were used within each replication row of the Latin square.
‖ 63-3 (for equilibration effect) degrees of freedom for 'error'.