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28 February 1974

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Sodium: Stimulus for Puddling Behavior by Tiger Swallowtail Butterflies, Papilio glaucus

Abstract. *Male* Papilio glaucus *butterflies are attracted to sand soaked with dilute aqueous solutions of sodium salts. A sodium ion concentration of* 10^{-3} *molar is sufficient to stimulate puddling behavior. Acquisition of sodium may be the main ecological function of puddling behavior.*

Adult Lepidoptera of several families are frequently seen feeding at the margins of puddles or on animal feces or carrion, sometimes in large groups (*1*, *2*). The insects extend their proboscises and imbibe fluid from the surface of the mud. On dry mud, fluid may be released from the proboscis onto the surface and then reimbibed (*2*).

Puddling behavior probably permits the butterflies to take in nutrients above those provided by larval nutrition or available from nectar; evaporation of water from the puddle and from the surrounding wet mud concentrates the desirable nutrients in the surface layer (*2*). Sugars and probably also amino acids (*3*) are available from nectar, and it has been suggested that puddling permits the insects to obtain an adequate supply of salt (*2*, *4*). Downes (*2*) has suggested that nutrients needed for probably greater flight activity by male butterflies may explain the common observation that males of most species are seen puddling much more often than are females. Here we show that puddling behavior by males of the eastern tiger swallowtail butterfly, *Papilio glaucus* L., is stimulated by sodium ions.

In June 1973, male tiger swallowtail butterflies were found puddling on mud along the northwest shore of the upper Six Mile Creek reservoir, 60 m from Burns Road and 4 km southeast of Ithaca, New York. On 7 June, at this site, ten plastic trays (44 by 30 by 6 cm) were set out in the form of a grid consisting of two rows of five trays (Fig. 1). Each tray was filled with an identical volume of children's sterilized play-sand (Campbell). Enough distilled water (1.5 liters) to saturate the sand was added to one tray in each of the two rows. Identical volumes of NaCl solutions in distilled water (1 percent, weight to volume), casein hydrolyzate (5 percent, weight to volume, "salt-free," Nutritional Biochemicals), and sucrose (5 percent, weight to volume), respectively, were added to three other trays in each row. The fifth tray in each row contained dry sand. Within each row, the positions of the trays were assigned at random with the stipulation that the two replicates of each treatment could not be adjacent to one another. A dead male tiger swallowtail butterfly was pinned on each tray to act as a decoy. From a raised bank several meters away, we watched the butterflies visiting the trays until puddling ceased. Distilled water was sprayed onto the trays to compensate for evaporation. On subsequent days the same experimental design was used to test other substances, each array of treatments being repeated for two consecutive days. The number of sampling visits (*5*) by butterflies to each tray was scored, and puddling activity was measured for each treatment (Table 1).

Although butterflies made frequent sampling visits to almost all trays, we found that extended puddling visits were restricted almost entirely to those trays containing sodium ions, regardless of which anion was also present (Table 1). The higher the sodium concentration (within the range we used), the more attractive was the sand surface as a puddling substrate. The lowest concentration at which we observed appreciable puddling was $10^{-4}M$ (experiment 8). However, since analysis of the sand at the end of experiment 8 revealed increases of as much as tenfold in sodium ion concentrations during the experiment (*6*), in spite of periodic additions of water to the trays, we can conclude only that the threshold concentration for puddling behavior lies below $10^{-3}M$.

On 15 June we watched tiger swallowtails puddling on exposed mud at an undisturbed site along Six Mile Creek

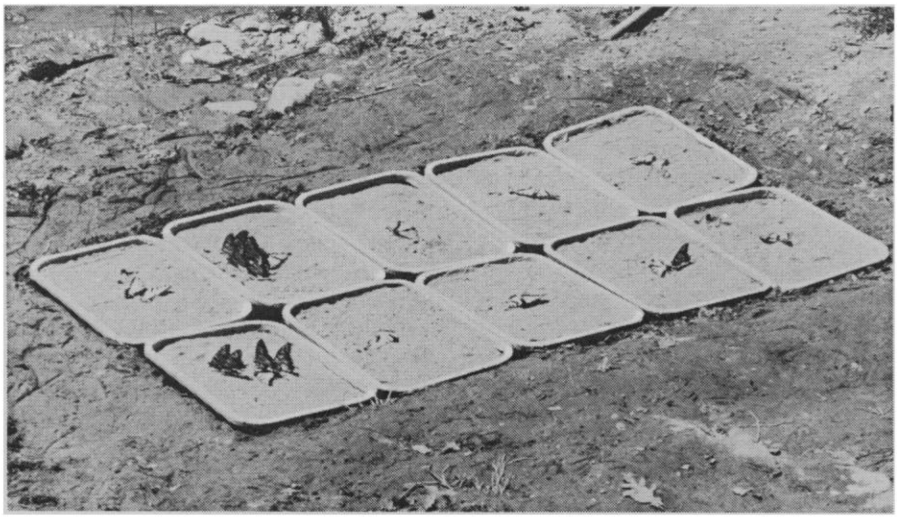

Fig. 1. Male tiger swallowtail butterflies puddling on sand trays during experiment 4 (10 June). The sand was soaked with $0.1M$ aqueous solutions of (far row, left to right) $CaCl_2$, NaCl, Na_3PO_4, $MgCl_2$, and KCl, and (near row, left to right) Na_3PO_4, $MgCl_2$, KCl, NaCl, and $CaCl_2$. A dead butterfly is pinned on each tray to serve as a decoy.

at Banks Road, about 3 km southeast of the Burns Road site. Puddling positions were marked with stakes (the butterflies usually returned within 5 minutes after they were disturbed). Surface mud from these puddling positions contained a sodium ion concentration of 38.0 ± 1.7 mg per liter of mud moisture (*7*), which is above the threshold concentration established in experiment 8.

Aqueous solutions of commercial casein hydrolyzate stimulated puddling behavior (Table 1, experiments 1 and 2). However, analysis of the 2.5 percent solution of this casein hydrolyzate (as used in experiment 2) revealed a sodium concentration of 252.6 ± 72 mg/liter, far above the sodium threshold level. We do not know if amino acids uncontaminated by salt can act as stimulants for puddling behavior by tiger swallowtails, but we have evidence to suggest that they do not. Analysis of puddling mud at the Banks Road site revealed a total amino acid concentration of about $10^{-5}M$ (*7, 8*). On 15 June, two sand trays were set out at this site, one saturated with an aqueous solution of 18 pure amino acids, and the other with an aqueous solution of the "salt-free" casein hydrolyzate, each solution equivalent to a total amino acid concentration of approximately $10^{-4}M$ (*9*). Sodium concentrations in these two solutions were 0.34 ± 0.05 mg/liter and 2.18 mg/liter, respectively (*6*). During a 2-hour period, 11 sampling visits were made to the pure amino acids by at least three butterflies and 21 visits to the casein hydrolyzate tray, but in neither case was puddling observed, although puddling occurred on mud adjacent to the trays. We conclude that if amino acids can act as stimuli for puddling, the threshold concentration is likely to be significantly higher than the amino acid content of Banks Road mud and that they were not the stimulus involved in puddling at this place.

We have shown that male tiger swallowtails can incorporate amino acids which they ingest during puddling, whether or not they can detect the presence of these compounds. On 12 June at the Burns Road site we put out a sand tray containing 250 ml of a 1 percent casein hydrolyzate solution to which had been added [2-^{3}H]glycine (4 μc/ml) and [1-^{3}H]leucine (4 μc/ml) (New England Nuclear). Thirteen male tiger swallowtail butterflies settled for more than 5 minutes on this tray and were caught for subsequent analysis. These butterflies were held in the laboratory for 24 hours after capture and then killed. Two of the butterflies which had fed on the labeled solution for more than 15 minutes were found (*10*) to contain 15.4 and 59.1 count/min per microgram of protein (*11*), respectively. Autoradiographic examination of other insects revealed that the radioactivity was concentrated in muscles, under the cuticle, and in the gut wall, although grains were found in most tissues (*12*). We conclude that the butterflies will incorporate amino acids, which they may ingest during puddling, into their body proteins although we have not yet determined whether de novo protein synthesis is involved in this incorporation.

Ability to make use of amino acids may be related to their widespread occurrence in nectar (*3*). Although the puddling mud at the Banks Road site had a very low amino acid content, many sites that attract puddling are likely to have a much higher content of amino acids (*2*). It seems unlikely to us, however, that ingestion of amino acids represents the major function of puddling behavior. The sodium content of many land plant tissues is so low (*13*) that the availability of this ion could be a limiting factor in the diet of many herbivores (*14*). Some herbivorous insects appear to have a potassium-regulated instead of the commoner sodium-dependent pump in their excretory systems (*15*), and to excrete fluid that is essentially free of sodium (*16*). It seems, however, that no ion can substitute completely for sodium in the neuromuscular system, although the sodium concentration surrounding nerve cells in herbivorous insects may be maintained at a higher level than that in the hemolymph by glial cells (*16*).

Table 1. Numbers of sampling visits (S) and time (butterfly minutes) spent puddling (P) by male *Papilio glaucus* adults on trays containing and saturated with various substrates. Data for replicate treatments in each experiment are presented separately. In these experiments puddling was observed between 20° and 29°C and 45 to 79 percent relative humidity. The distilled water contained 0.028 to 0.057 mg of Na^+ per liter of solution.

Experiment	Period (hr)	Date (1973)	Visits and times on sand plus substrate:										Minimum number of insects*
			S	P	S	P	S	P	S	P	S	P	
			Dry sand alone		*Dist. H_2O*		*Casein hydrolyzate*†		*5% Sucrose*		*NaCl* (*0.17*M)		
1	6.5	7 June		0		1.5		164.0	0	8.0		176.5	6
				0		0		0.5		2.0		215.0	
2	9	8 June	26	0	47	0.5	27	205.5	60	0.5	74	320.5	10
			48	0	27	0	169	303.5	25	0	81	403.0	
			KCl (*0.1*M)		*$MgCl_2$* (*0.1*M)		*$CaCl_2$* (*0.1*M)		*Na_3PO_4* (*0.1*M)		*NaCl* (*0.1*M)		
3	5	9 June	8	0	0	0	18	0	17	16.0	17	141.5	3
			15	7.0	13	0	9	0	4	0	15	69.5	
4	8	10 June	33	0	36	0	48	1.5	43	79.5	65	362.0	12
			21	1.0	17	0.5	18	0	108	449.5	74	474.0	
			NH_4Cl (*0.1*M)		*KNO_3* (*0.1*M)		*K_3PO_4* (*0.1*M)		*Na_3PO_4* (*0.1*M)		*$NaNO_3$* (*0.1*M)		
5	7	11 June	9	0	6	0	6	0	3	0.5	86	279.5	6
			1	0	0	0	6	0	3	0	10	45.5	
6	4.5	12 June	17	2.0	24	0	19	0	36	145.0	51	125.0	8
			18	6.0	16	0	3	0	40	133.0	48	220.0	
			Dist. H_2O		*NaCl* (10^{-5}M)		*NaCl* (10^{-4}M)		*NaCl* (10^{-3}M)		*NaCl* (10^{-2}M)		
7	7	14 June	4	0	2	0	11	0	10	2.5	5	56.0	2
			3	0	15	0	8	0	3	30.0	5	62.0	
8	4	15 June	2	0	7	1.5	16	27.5	32	172.5	22	195.5	10
			2	0	12	0	13	0	33	242.0	37	261.5	

* Calculated by counting the maximum number of insects seen puddling on the trays at the same time. † In experiment 1, 5 percent was used and in experiment 2, 2.5 percent was used.

Although accumulation of NaCl from saline soils may act as a defense of plants against herbivores in areas where excretory water is not readily available (*17*), we suggest that the opposite adaptation is more common: relative exclusion of sodium from the tissues of most land plants may help defend them against grazing by making it difficult for the grazers to obtain as much of this ion as they need.

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5. When a butterfly landed on a tray and remained on the surface for less than 15 seconds, a sampling visit was scored. Puddling was scored when a butterfly remained on the surface for 15 seconds or longer. "Butterfly minutes" represent the product of the number of butterflies times the number of minutes each "puddled." Through binoculars it was possible to see proboscises extended, in some cases before the butterflies landed.
6. Samples of sand (20 to 30 g) were scraped from the surface of each tray and weighed; after being dried at 110°C, the samples were weighed again (to calculate moisture content) and then extracted five times with twice-distilled water. The aqueous extracts were made up to 100 ml each, and samples were analyzed for sodium in a Perkin-Elmer model 403 atomic absorption spectrophotometer, alongside distilled water controls. The sodium content of distilled water washings (100 ml) of 25 g of the untreated sand was not detectably different from that of the distilled water controls (0.028 to 0.057 mg/liter).
7. Surface mud (about 5 mm deep) was scraped from approximately 5 cm^2 around each of four points where butterflies had been observed to puddle within the previous 2 hours. One portion of the combined samples was oven-dried to determine the moisture content (23.7 percent); a second portion was extracted five times with distilled water; combined supernatants after centrifuging were concentrated by vacuum distillation, and the volume was adjusted to 100 ml with distilled water; a third portion of mud was extracted five times with ethanol; combined supernatants were evaporated to dryness under vacuum and the residue taken up in 100 ml of distilled water. Sodium analyses of the extracts and controls were performed by J. S. Eaton with the atomic absorption spectrophotometer.
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9. The amino acids included in the mixture were lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, and tryptophan. Total amino acid concentrations in the tray solutions were found by the method above to be $1.83 \times 10^{-4}M$ (amino acid mixture) and $4.28 \times 10^{-5}M$ (casein hydrolyzate).
10. Butterflies were homogenized in 10 percent sucrose, $0.1M$ tris-HCl, *p*H 7.0, and extracted four times with 5 percent trichloroacetic acid, and the suspension was counted in toluene-based counting fluid (Packard Tri-Carb scintillation counter). In the final pellet, 90 percent of the radioactive material was solubilized after incubation with protease (Worthington).
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25 October 1973 ■

Modulation of the Copulatory Sequence of the Male Rat by a Schedule of Reinforcement

Abstract. *Copulating male rats were permitted a maximum of seven intromissions in which to ejaculate. This experimental constraint of the male rat's sexual behavior produced an increase in the number of sessions in which the male ejaculated before the seventh intromission. This species-specific behavior pattern is therefore susceptible to environmental conditioning.*

The sexual behavior of the male rat consists of three distinguishable classes of copulatory responses: mounts, intromissions, and ejaculations. During each of these responses, the male rat grasps the receptive female's flanks with his forelegs and begins a series of pelvic thrusts. During mounts, these pelvic thrusts do not lead to vaginal penetration; during intromissions and ejaculations, however, vaginal penetration is achieved. After approximately 5 to 15 intromissions, a copulatory sequence is terminated by the occurrence of the ejaculatory response.

Because the reflexive components of its copulatory behavior are so readily apparent (*1*), learning has not been considered important in most descriptions of the male rat's mating activities. The present experiment was designed to assess whether rats could learn to modulate the stereotypic pattern of its copulatory sequence. Such a demonstration would support the argument that the structure of this behavior is not controlled solely by endogenous factors.

In order to determine whether learning could contribute to the control of sexual behavior, a schedule of reinforcement was used: for one group of male rats (experimental group), the opportunity to ejaculate was dependent on its occurrence within the first seven intromissions of the copulatory series. Each rat was given free access to a receptive female until the occurrence of the ejaculatory response or of seven intromissions, whichever occurred first. If ejaculation is a more potent reinforcer than intromission, and if the male rat can learn to modulate its copulatory behavior in order to produce reinforcement (that is, ejaculation), then he should learn to ejaculate with fewer preceding intromissions.

In addition to the experimental group, two control groups were used. The rats in the maturation-experience (M-E) control group, which were the same age as the males in the experimental group, were given free access to receptive females with the same weekly frequency as the experimental group; however, these males were always permitted to intromit until ejaculation. By comparing the number of intromissions preceding ejaculation (intromission frequency) for the males in this group with those of the experimental group, it should be possible to determine whether changes in the experimental group's intromission frequencies were generally due to maturation and sexual experience or to the specific contingencies of this experiment.

A second control group, called the frustration (F) control group, was used to evaluate the noncontingent effects (for example, frustration) which might follow the interruption of a rat's copulatory sequence. Each