Sodium Uptake by Puddling in a Moth

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Male Lepidoptera commonly visit stands of water to drink, a behavior known as puddling. Males of the notodontid moth *Gluphisia septentrionis* routinely puddle for hours, imbibing hundreds of gut-loads and voiding the fluid as repetitive anal jets. Cationic analyses showed puddling to lead to systemic sodium gain, a potential benefit to *Gluphisia*, whose larval food plant is low in sodium. Male *Gluphisia* are specialized for puddling, possessing a wide oral slit and a highly expanded enteric surface. The acquired sodium is transferred to the female at mating, for eventual incorporation into the eggs. Sodium acquisition may be the primary function of puddling in Lepidoptera.

When animals first emerged from the oceans to colonize land, they faced a new reality. Sodium, an essential ion (*1*, *2*) plentiful in the seas, was in short supply in the new milieu. For terrestrial herbivores the problem was particularly acute because land plants typically contain little sodium (*3*). Herbivorous vertebrates meet their sodium needs on land by resorting to salt licks or feeding on foliage of higher sodium content (*4*). We here document how one insect, the notodontid moth *Gluphisia septentrionis*, procures sodium by puddling (*5*).

Puddling is widely practiced by butterflies and moths. The behavior, restricted almost exclusively to males, involves drinking from stands of water or moist soil. It has long been assumed that puddling serves for procurement of sodium (*6*–*8*), but evidence for uptake of the cation has never been presented. We here demonstrate that puddling does indeed lead to sodium uptake.

Gluphisia males exemplify puddling in its extreme form. The moths imbibe prodigious quantities of fluid, which they expel as quickly paced anal jets while drinking (*9*–*11*) (Fig. 1A). Observations of male *Gluphisia* (n = 7) drinking at natural puddles (*12*) revealed an anal jet ejection rate of 18.4 ± 2.2 per minute (range 14.2 to 21.4 anal jets per minute), with individual jets averaging 8.2 ± 2.2 μl (range 6.2 to 11.3 μl), an equivalent of 12% of moth body mass. The behavior (n = 8 moths) persisted for long periods (80.3 ± 63.1 min; range 8.5 to 201.9 min), resulting in the passage of large total fluid volumes (13.9 ± 13.9 ml; range 1.1 to 38.4 ml) (*13*). The maximum ejected volume was passed as 4325 jets in 3.4 hours and amounted to more than 600 times the moth's body mass (the human equivalent would be 45,500 liters, passed at 3.8 liter/s).

A comparison of the cationic concentration (*14*) of imbibed and ejected fluids for *Gluphisia* males drinking from two laboratory solutions and from natural puddles revealed that there was invariably sodium uptake (Fig. 2) (*15*, *16*). With the laboratory solutions there was also potassium loss and minimal losses of magnesium and calcium (Fig. 2, A and B). However, with the puddle fluid, where the samples did not necessarily include a moth's first ejections, gains or losses of potassium, magnesium, and calcium were insignificant (Fig. 2C). Separate laboratory determinations (*17*) showed that a disproportionately large quantity of potassium is voided in the initial rather than later ejections (*18*). Early ejections, although they composed only 8 ± 3% of the total fluid passed, accounted for 40 ± 18% of the total potassium loss, but for only 12 ± 2% of the total sodium gain. However, for the entire puddling period, the net sodium gain per moth was generally evenly matched, on a molar basis, by the potassium loss (*19*).

Analysis of *Gluphisia* body parts provided direct evidence that puddling leads to sodium uptake (*20*). The sodium content, but not the potassium content, of males that had puddled exceeded that of controls for all body parts (Fig. 3). In general, potassium concentrations were substantially higher than sodium concentrations, as is typical for herbivorous insects (*3*). Whole body analyses of a separate sample of puddlers (n = 28) revealed a sodium content of 19.0 ± 5.8 μg per moth (*21*). For a sample of nonpuddling controls (n = 10), the corresponding value was 2.3 ± 2.3 μg per moth. The difference in these two values, representing presumably the amount of sodium gained through puddling, is clearly in line with the value calculated for sodium uptake (16.8 ± 9.9 μg per moth) from the imbibition data (Fig. 2B).

Both the duration of puddling and the total volume ejected were found to be inverse functions of the sodium concentration in the imbibed fluid (Fig. 4). Moths drinking a 0.01 mM Na solution puddled longer and passed larger volumes than those imbibing 0.1 and 1.0 mM Na solutions. For the latter two solutions the data did not differ, indicating potential saturation of the ionic uptake mechanism starting at a concentration between 0.01 and 0.1 mM Na.

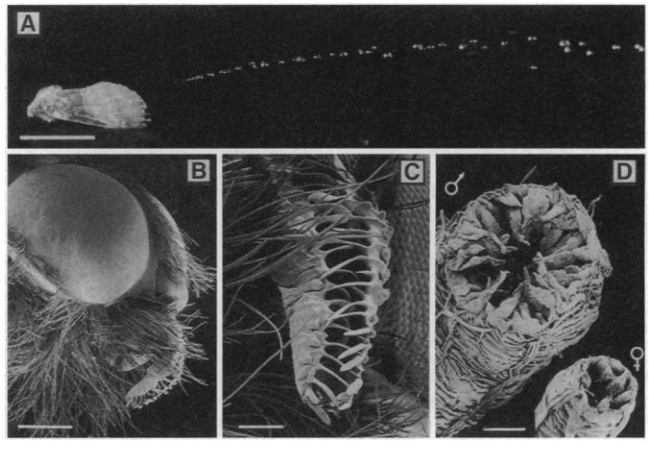

Fig. 1. Puddling behavior and concomitant morphology in *Gluphisia*. (**A**) Male ejecting an anal squirt while puddling. (**B**) Male head, showing proboscis. (**C**) Male proboscis, enlarged, showing oral cleft and the sieve-like arrangement of projections. (**D**) Male and female ileum (cross section). Scale bars: (A) = 1 cm, (B) = 0.5 mm, and (C) and (D) = 0.1 mm.

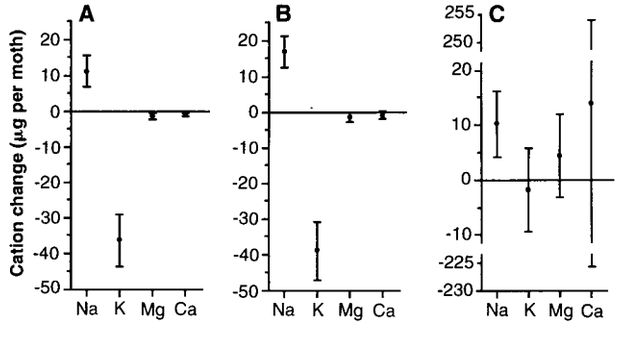

Fig. 2. Net changes in cation content of puddling male *Gluphisia*, as calculated from the concentration differences in the imbibed and ejected fluids. Test solutions: (**A**) 1 mM Na, (**B**) 1 mM for the four cations indicated, and (**C**) field puddles. Mean values with 95% confidence intervals are plotted. Note the scale break in (C). Sample sizes: n = 8 moths for (A), n = 23 for (B), and n = 8 for (C) (*14*–*16*).

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Gluphisia is structurally specialized for puddling. The male, in lieu of the typical elongate lepidopteran proboscis, has a short recurved beak, opening frontally by way of an oral cleft (Fig. 1, B and C). This gaping orifice, ideally suited for ready fluid intake (*22*), is guarded by an interdigitating arrangement of projections, forming a sieve that could shield against uptake of potentially occlusive particulate matter. A reduced proboscis is not uncommon for notodontid moths and appears to be characteristic of species with short-lived, atrophic adults (*23*). Of such species only *G. septentrionis* and two of its congeners (*24*), as well as one other species (*9*), are known to puddle, suggesting that facilitation of massive fluid intake may not be the primary evolutionary justification of proboscis reduction in Notodontidae.

Male *Gluphisia* have intestinal specializations that set them apart from the nonpuddling female. The ileum (anterior hindgut) of the male is 1.65 times as long as and 2.00 times as wide as that of the female (*25*) (Fig. 5), and it bears a dense inner packing of villi, largely absent from the female (Fig. 1D). Because of these differences, the ileal surface area of the male is 19 times that of the female (*26*). The ileum is in all likelihood the site of sodium absorption in *Gluphisia*. The insect hindgut is thought to serve for ionic absorption (*27*). The two congeners of *G. septentrionis* that also puddle by massive fluid throughflow have enlarged male ilea (*24*). Conversely, the ilea of two notodontids not reported to puddle (*Pheosia rimosa* and *Dasylophia thyatiroides*) are sexually monomorphic (*24*).

Sodium uptake may be a necessary pursuit for *Gluphisia*, given that its primary larval foodplant, quaking aspen (*Populus tremuloides*), contains foliar sodium concentrations [2.9 ± 1.2 parts per million (ppm) dry weight, $n = 8$ trees] (*28*) substantially lower than average for trees (*29*). Animal diets typically contain higher sodium concentrations (an average human meal in an industrial society contains 5000 ppm of sodium) (*30*). Potassium, on the other hand, which is plentiful in quaking aspen leaves (8120 ± 990 ppm dry weight, $n = 8$ trees) (*28*), as well as within the adult moth, is unlikely to be a limiting resource for *Gluphisia*. The close correspondence between sodium gain and potassium loss in the puddling moth suggests that the mechanism of sodium uptake involves a balanced exchange with potassium.

Elsewhere we provide an answer to the question of why puddling is restricted to male *Gluphisia*. The female, potentially as deficient as the male in sodium because her larval diet is the same as the male's, receives supplemental sodium from the male at mating, with the sperm package (*24*). Such copulatory transfer of sodium has been demonstrated also for a butterfly (*Thymelicus lineola*) (*8*). The female *Gluphisia*, however, does not retain the acquired sodium herself, but bestows it in large measure on the eggs, thereby endowing the offspring with a first dose of the valuable ion (*24*). Other puddling Lepidoptera may similarly "salt their eggs."

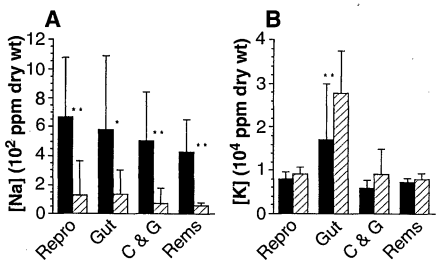

Fig. 3. Concentration of sodium (**A**) and potassium (**B**) of body parts for male *Gluphisia* that puddled (black bars) and their paired, nonpuddling controls (striped bars). Body parts: Repro = reproductive parts; Gut = digestive system; C & G = coremata and genitalia; Rems = remaining parts. Eight pairs of all body parts were analyzed. Paired *t* tests (*, $0.01 \leq P < 0.05$; **, $P < 0.01$).

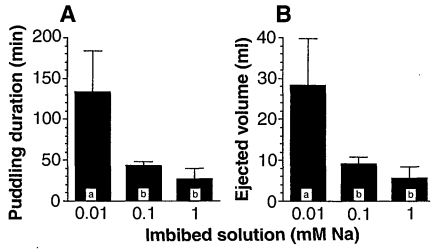

Fig. 4. Puddling duration (**A**) and total fluid ejected (**B**) as a function of the sodium concentration of the imbibed solution (10 moths per solution). Columns not sharing letters are significantly different (experiment-wide $\alpha = 0.05$, Tukey test) (*31*).

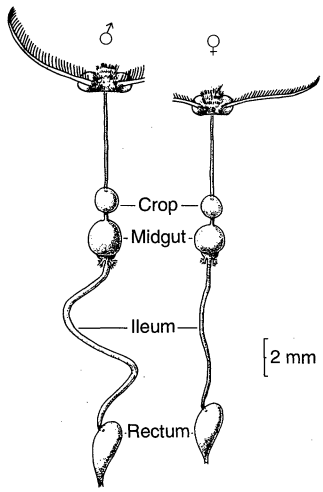

Fig. 5. Digestive system of *Gluphisia*, showing ileal sexual dimorphism (malpighian tubules omitted).

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5. Specimens of the moth, henceforth referred to as *Gluphisia*, were deposited in the Cornell Insect Collection (lot number 1214).
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10. Anal fluid expulsion has been reported for other puddling lepidopterans, but in lesser quantities than in *Gluphisia* [W. J. Reinthal, *ibid.* **17**, 35 (1963) and references therein; G. O. Krizek and P. A. Opler, *J. Res. Lepid.* **27**, 143 (1989)].
11. Unless otherwise specified, moths were raised from eggs in the laboratory on fresh foliage of their foodplant, quaking aspen (*Populus tremuloides*). Puddling solutions in laboratory tests were prepared from the chloride salt of the appropriate cation or cations and quartz-distilled water. Data are presented as mean ± SD, unless otherwise noted.
12. Moths were located drinking at night from puddles along dirt roads (Scotia Barrens, Centre County, PA). The ejectate was collected in a plastic receptacle placed in the trajectory of the squirts and quickly (to prevent evaporative changes in concentration) transferred to sealed polyethylene vials. Ejection frequency = total number of ejections per period of observed puddling (minutes); volume of individual ejections = total ejected volume (microliters) per number of ejections.
13. These durations and volumes are minimal measures and more variable than in actuality because the moths had already been puddling for unknown periods when discovered. Ejectates were virtually particle-free (silt content = 0.02 ± 0.02% by mass).
14. Cation concentrations were determined by inductively coupled plasma emission spectrophotometry [*ICAP 61 Operator's Manual* (Thermo Jarrell Ash Corporation, Franklin, MA, 1988)].
15. For the two laboratory solutions (Fig. 2, A and B), male *Gluphisia* [both laboratory-reared and field-collected (light traps, Ithaca, NY)] were placed individually in plastic cylinders (8.2 cm in diameter) and were induced to puddle by wetting their mouthparts with test solution, which then was presented as a continuously replenished supply. Ejected fluid was collected as it accumulated. For each moth we determined the cationic concentration of the imbibed and ejected fluids in order to calculate the cationic change (micrograms per moth): Δ[cation] × volume ejected, where $\Delta[\text{cation}] = [\text{cation}]_{\text{imbibed}} - [\text{cation}]_{\text{ejected}}$.
16. For the field data (Fig. 2C), ejectate was collected as described (*12*), and a sample of actual puddle fluid was taken from directly in front of the moth. The cation change was calculated as in (*15*). Sodium concentration in the puddles was somewhat lower (0.07 ± 0.06 mM; $n = 8$) than in the laboratory solutions.
17. The data are from 6 moths, and the puddling solution was as in Fig. 2B. Ejectate was collected from each moth, for an initial period (3.7 ± 1.8 min, representing 13 ± 3% of the total puddling period) after the onset of puddling, and then for the remainder of the puddling period (second sample).
18. The difference in potassium loss between initial and subsequent ejections was calculated as follows: $[\text{K}]_{\text{initial ejections}} - [\text{K}]_{\text{subsequent ejections}} = 45.3 \pm 43.7$ ppm ($n = 6$); Wilcoxon signed rank $Z = -2.201$,

probability (P) = 0.03.
19. Data were gathered for 28 moths puddling on three Na solutions: 0.01 mM (n = 8), 0.1 mM (n = 10), and 1 mM (n = 10). The slope ± 1 SE of linear regression (Na uptake versus K loss, expressed as micromoles per moth) was 0.97 ± 0.15, coefficient of determination (r^2) = 0.62, $P < 0.001$.
20. Males were paired to minimize differences in age and relatedness. Within a pair, one moth was randomly assigned to puddle (solution as in Fig. 2B), and the other was kept without fluid as a control (if former moth failed to puddle, the pair was discarded). After puddling (16 ± 1 hours after cessation), pair members were dissected (in the dry) into four components: reproductive system (testes, simplex, accessory glands); gut (complete digestive system, including malpighian tubules); coremata (the pheromonal brushes) plus genitalia; and remains (all other parts combined). Dissected parts were oven-dried (55°C) and weighed, then digested [quartz tubes; boiling mixture of 0.25 ml of nitric acid (70%) and 0.10 ml of perchloric acid (70%)], taken to dryness, solubilized [0.15 ml of hydrochloric acid (37%)], and brought to 3.00 ml with quartz-distilled water, then analyzed (*14*).
21. The puddling solution was as in Fig. 2B, and the analytical technique was as in (*14, 20*) except that the digestion step (*20*) was done twice.
22. The energetic cost of fluid transport through a tube decreases linearly with shortening and exponentially (fourth power) with bore increase [M. LaBarbera and S. Vogel, *Am. Sci.* **70**, 54 (1982)].
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25. Ileal length and midpoint width were as follows: males (13.9 ± 1.8 mm and 0.30 ± 0.06 mm; n = 11); females (8.4 ± 1.3 mm and 0.15 ± 0.02 mm; n = 10). Independent t tests for both between-sex comparisons: $P < 0.001$.
26. The surface area of the ileal lining was estimated from the gross linear measurements (*25*) and from measurements from electronmicrographs (Fig. 1D), which provided a basis for calculation of villi dimension and numbers (the villus was assumed to be a right circular cone).
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28. Leaf samples were taken fresh from aspen (Ithaca, NY). After they were oven dried (55°C) and ground, the samples (0.4 g each) were dry ashed (450°C, 6 hours) in quartz tubes. On cooling, 0.25 ml of hydrogen peroxide (30%) was added, and samples were ashed (2 hours). The ash was then solubilized as in (*20*), except that 0.5 ml of hydrochloric acid and 9.5 ml of water were added. Analyses as in (*14*).
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30. Calculation based on (*1*).
31. For both measures [variances showed heteroscedasticity (Bartlett's test; $P < 0.001$) that remained after logarithmic transformation], significant differences were detected between treatments [Kruskal-Wallis analysis of variance: duration, H = 23.1, degrees of freedom (df) = 2, $P < 0.001$; volume, H = 22.7, df = 2, $P < 0.001$].
32. We thank D. Deutschman, M. Eisner, P. Fraissinet, J. Franclemont (to whom this paper is fondly dedicated), J. F. Hare, H. Hurrell, K. Moy, M. Oliva, M. Rutzke, and M. VanEvery for assistance. Supported by NIH grant AI 02908 and a National Institute of Mental Health training grant, the Edna Baily Sussman Fund, Sigma Xi, the Theodore Roosevelt Memorial Fund, and Cornell Graduate School stipends.

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Evidence for Developmentally Programmed Transdifferentiation in Mouse Esophageal Muscle

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Transdifferentiation is a relatively rare phenomenon in which cells of one differentiated type and function switch to a second discrete identity. In vertebrate embryos, smooth muscle and skeletal muscle are distinct tissues that arise from separate compartments of the mesoderm. The musculature of the mouse esophagus was found to undergo a conversion from smooth muscle in the fetus to skeletal muscle during early postnatal development. The switch from smooth to skeletal muscle features the transitory appearance of individual cells expressing a mixed phenotype, which suggests that this conversion is a result of programmed transdifferentiation.

Skeletal and smooth muscles of vertebrates differ with respect to structure, innervation, function, and developmental origin (*1*). For example, skeletal muscle is composed of fused multinucleate myotubes containing striated fibers, whereas smooth muscle is composed entirely of mononucleate, nonstriated cells. The musculature of the mammalian stomach and intestine is composed exclusively of smooth muscle, but the esophagus differs because it also contains skeletal muscle. Although some muscle genes are expressed in both smooth and skeletal muscle tissues, others are strictly specific.

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By monitoring the expression of such highly specific smooth or skeletal muscle markers [skeletal fast myosin heavy chain (MHC) and smooth myosin light chain (MLCK)], we found that during early development, the mouse esophageal musculature is composed entirely of differentiated smooth muscle (Fig. 1, A and B) (*2, 3*). Later, esophageal expression of smooth muscle–specific genes declines and expression of skeletal muscle–specific genes increases (Fig. 1, C through F). This transition from smooth to skeletal muscle type occurs in a rostrocaudal progression that begins in late fetal development and continues through the first 2 weeks of postnatal development (Fig. 1, G and H). Smooth muscle myosin heavy chain, another smooth muscle–specific marker, was also expressed in the esophageal musculature at prenatal time points, which confirmed the smooth muscle phenotype of these cells (*3, 4*). The expression pattern of another skeletal muscle–specific protein, α-actinin, was the same as that of skeletal MHC protein for all developmental time points (*5*).

The muscularis layer of diaphragm-level esophagus expressed both skeletal and smooth muscle proteins at postnatal day 3 (P3), which revealed a time and place of transition at the tissue level (Fig. 1C). Greater magnification revealed coexpression of smooth MLCK and skeletal MHC in the cytoplasm of individual cells (Fig. 1D). This unanticipated coexpression of smooth and skeletal differentiation markers within individual cells was observed from embryonic day 16 (E16) in rostral sections to P8 in caudal sections (*5*). Coexpression on the single-cell level was verified by partial dissociation of P3 esophagus tissue (Fig. 1, I through L). The progression from smooth to skeletal tissue type, together with the coexpression of smooth and skeletal markers in individual cells, suggests that functional, differentiated smooth muscle cells in the esophagus switch directly to differentiated skeletal myocytes. The presence of syncytial myofibers in dissociated preparations provided additional cytological evidence for the skeletal character of the final muscle phenotype (*5*).

Expression of the skeletal muscle regulatory factors (MRFs) MyoD, myogenin, Myf-5, and MRF4 marks commitment to the skeletal muscle phenotype, but extensive studies of their expression patterns have never found these markers in smooth muscle or its known progenitors. At least one of the MRFs is expressed before transcription of any muscle-specific structural gene in all skeletal muscle (*6*). At E15 (Fig. 2, A and B) and at birth (*5*), diaphragm-level esophageal musculature was consistent with this pattern and did not stain with monoclonal antibodies (mAbs) to MyoD or myogenin,