# Shock induces a long-lasting elevation of blood glucose in *Aplysia*

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*Abstract.* Glucose, and not trehalose, was found to be the main blood sugar in *Aplysia californica*. Changes in blood glucose in response to stress produced by electric shock were measured in blood obtained both from animals dissected within ten minutes of shocking and from catheterized animals at various intervals, up to two and a half hours after the shock. Electric shock increased blood glucose levels. The rise in blood sugar continued as long as two and a half hours after shock.
*Key words.* Glucose; trehalose; *Aplysia californica*; carbohydrate metabolism; blood sugar; stress-induced hyperglycemia.

Responses to stress in mammals have been well documented. Selye[1] divided the stress response into three stages: (1) the alarm reaction, (2) the stage of resistance, and (3) the stage of exhaustion. Physiological responses triggered early in the response to stress were described recently by Tepperman and Tepperman[2]. The immediate reaction is concerned with responses that maintain blood supply to life-sustaining organs. Neural, cardiovascular and lung tissue are given priority in this response. Subsequently, hormonal responses occur to adapt the body to the new stress conditions. Norepinephrine and epinephrine released from the adrenal medulla stimulate glucagon release and inhibit insulin release. Cortisol and other glucocorticoids released from the adrenal cortex stimulate glucagon release. The resultant rise in blood glucose is a manifestation of these changes. Longer-term adaptations to stress include the synthesis of new proteins and the stimulation of cell proliferation.

The invertebrate response to stress is not as well elucidated. Walters et al.[3] reported that electric shock caused central sensitization and the inhibition of the feeding response in *Aplysia*. Cooper et al.[4] identified substances in the blood of traumatized *Aplysia* that were chemically similar to classical neuropeptides. Krontiris-Litowitz et al.[5] observed that the blood of electrically or mechanically stimulated *Aplysia* caused longer lasting contractions on isolated body wall sections than blood of unstimulated animals. In addition, Krontiris-Litowitz (personal communication) also observed the elevation of amino acids, including alanine, glutamine, and taurine after the administration of shock or parapodectomy. The research reported here was begun to determine whether the trauma response in *Aplysia* included a rise in blood glucose as it does in the mammalian response to stress.

In a previous study we established baseline data on carbohydrate stores in *Aplysia*[6]. High concentrations of glycogen were found in both the hepatopancreas and penis; however, the major mobile store of glycogen is in the hepatopancreas. The concentration and the total amount of glycogen in the hepatopancreas rose in well-fed animals, compared to animals that were starved. In contrast, the amount of glycogen in the penis stayed comparatively constant between the two conditions. Blood glucose, which at 2–4 mg/dl is much lower than in the mammal, was not significantly different between starved and fed animals, indicating indirectly that a means of regulating blood glucose levels in the presence of vastly different availability of carbohydrates must be present.

The present study began with a further characterization of the major transport sugar found in *Aplysia* blood since a disagreement about its chemical identity was present in the literature. Goddard and Martin[7], in 'Physiology of Mollusca', stated that glucose was the transport sugar present in *Aplysia*, but Kandel[8], in 'Behavioural Biology of *Aplysia*', stated that trehalose functioned in that capacity. The present study shows that glucose, but not trehalose, is present in *Aplysia* blood.

The effect of shock on the mobilization of glucose was then investigated. We hypothesized that vigorous electrical stimulation would traumatize the animal. In response to this stressor, the animal would mobilize glucose from internal stores and thus elevate the amount of free glucose in its circulatory system. Upon demonstrating that shock stimulated a rise in blood glucose, the time course of the glucose response was investigated.

## *Materials and methods*

*Animals and nutritional status. Aplysia californica*, 200–400 g, from Marinus Inc. (Long Beach, CA) were used in the experiments, except as otherwise stated. Animals were maintained at Wayne State University at 18 °C with an LD 12:12 light-dark cycle and were fed Romaine lettuce daily for nine or more days prior to shock treatment. Animals were given 10 or 20 g lettuce per day, which most animals usually ingested completely. As indicated in Results, some experiments were also done at the University of Miami's Rosenstiel School of Marine and Atmospheric Science (RSMAS, Key Biscayne, FL) which provided maricultured *Aplysia* (40–60 g). These animals were maintained at 22 °C and were fed daily with maricultured *Gracilaria* seaweed.

*Shock treatment.* Shock treatment used either of two different time courses. In the first and second shock ex-

periments and in all catheterized animal experiments, animals received shock for a total of eight minutes, fractionated into four 2-min periods with three intervening 2-min recovery periods. In the third shock experiment, RSMAS animals were used. Perhaps due to their smaller size, these animals were unable to withstand the 8-min shock used on larger animals. Hence, for these animals, shock consisted of one 90-s shock period. Prior to shock treatment, the animal was placed in a plexiglass 'shock-box' (23.5 cm × 18 cm × 11 cm) filled with artificial seawater (ASW, made according to directions from Instant Ocean, Mentor, OH). Electrodes, carrying an AC voltage of ten volts, were lightly touched on and around the outer body wall. ASW was changed during the recovery periods in order to remove the ink and mucus secreted by the animal; care was taken to insure that the electrodes remained clean. After treatment, non-catheterized, shocked experimental animals were sacrificed and blood was collected. Catheterized, shocked experimental animals were returned to the aquarium and food provided; blood was collected via the catheter at specific time periods.

*Blood and tissue collection.* Blood was collected either through a pedal incision at the time of dissection or from live animals via catheters installed as described below. Whole blood was drawn with a syringe, separated into vials and stored at −80 °C until assay for glucose as described below. For tissue glycogen assays, animals were opened via a pedal incision, tissues were dissected out, immediately frozen on dry ice and stored at −80 °C until assay for glycogen as described below.

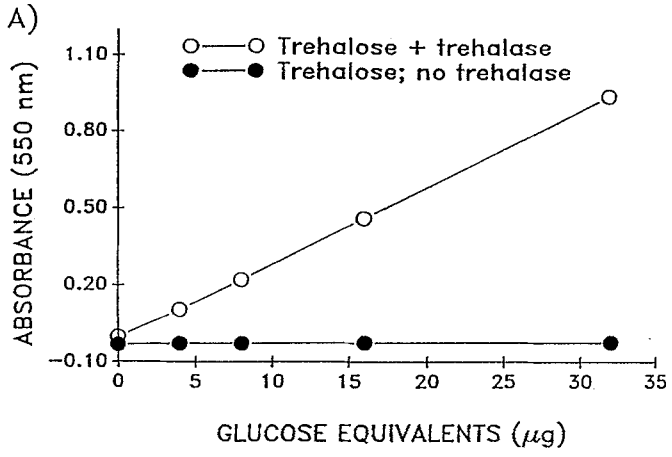
Catheters, for collection of blood from live animals, were made from specially modified bubble diffusers connected to 90 and 190 polyethylene (PE) tubing with a three-way stopcock and needle at the end. PE tubing was obtained from Clay Adams (Parsipany, NJ); unmodified bubble diffusers were obtained from B and F Medical Products, Inc. (Toledo, OH); stopcocks were obtained from American Pharmaseal Co. (Valencia, CA). Prior to surgery, the animal was placed in a plexiglass holding container (23.5 cm × 18 cm × 11 cm) filled with ASW. Isotonic magnesium chloride (0.375 M) was injected to anesthetize the animal in an amount equal to half the animal's volume. The anesthetized animal was placed on its right side and the target area hooked and elevated above the water surface. A small incision at the left lower dorsal region was made and the catheter tip inserted into the animal. The opening was closed by stitching the inner muscle layer and the outer muscle layer separately with surgical silk. Animals recovered from surgery within one day. Catheters were cleansed by autoclaving the filter tips and washing out the tubing with absolute ethyl alcohol followed by ASW. When blood was collected, 0.7 ml was first removed to clear blood in the 'dead space' of the catheter; after clearance, the 1-ml sample to be assayed was removed.

*Assays.* The glucose oxidase-peroxidase-chromophore reaction was the method by which glycogen, glucose, and trehalose levels were measured. The principle of the reaction involves the conversion of glucose, oxygen and water to gluconic acid and peroxide by glucose oxidase, followed by the reduction of peroxide to water and the oxidation of the colorless chromophore to a colored solution by peroxidase; the colored solution can then be read spectrophotometrically. All chemicals were from Sigma Co. (St. Louis, MO); all assays were read with a Bausch and Lomb Spectronic 21.

Glycogen and glucose were assayed as described previously[6]. For determination of trehalose, we developed a method based on the hydrolysis of trehalose to glucose by trehalase. Trehalose is a non-reducing dimer of glucose; trehalase hydrolyzes trehalose to its two constituent glucose molecules. Therefore, after trehalase treatment, the amount of trehalose previously present can be detected with the standard glucose assay. Trehalose standards consisted of 0, 20, 40, 80, 160 or 320 µl trehalose at a concentration of 0.1 g/l ASW, 100 µl trehalase at a concentration of 1 mg/ml and enough ASW to raise the volume to 800 µl. To verify that the glucose assay did not respond to unhydrolyzed trehalose, a trehalose standard without trehalase was also run. In addition, in order to determine if trehalase interferes in the detection of glucose, glucose standards with and without trehalase were run. Samples to be assayed for trehalose consisted of 400 µl *Aplysia* blood, 100 µl trehalase and 300 µl ASW. Since the samples would also be expected to contain glucose, to which this assay also responds, duplicate samples were prepared in which trehalase was substituted with 100 µl of ASW. The amount of trehalose in the blood sample would therefore be the difference between the samples with and without trehalase added. For the standards and the samples, mixture of the initial solution components was followed by a pre-incubation period of 20 min at 37 or 55 °C (we found that either temperature could be used). After preincubation, 1000 µl chromophore/buffer reagent, 100 µl peroxidase and 100 µl glucose oxidase were added and standards and samples allowed to incubate for 60 min. After incubation, the absorbance of the standards and samples was read spectrophotometrically at 550 nm.

## *Results*

*Baseline levels of blood sugars.* Because the identity of the *Aplysia* circulating sugar was a matter of controversy (see Introduction), initial assays were conducted to determine whether glucose or trehalose was present in the *Aplysia* circulatory system. A trehalose assay was established with four standards: trehalose with and without trehalase and glucose with and without trehalase (fig. 1). The trehalose standards proved that trehalase is necessary for the detection of trehalose; without trehalase, trehalose is not cleaved to glucose and hence cannot be detected by the glucose assay (fig. 1 A). The glucose standards proved



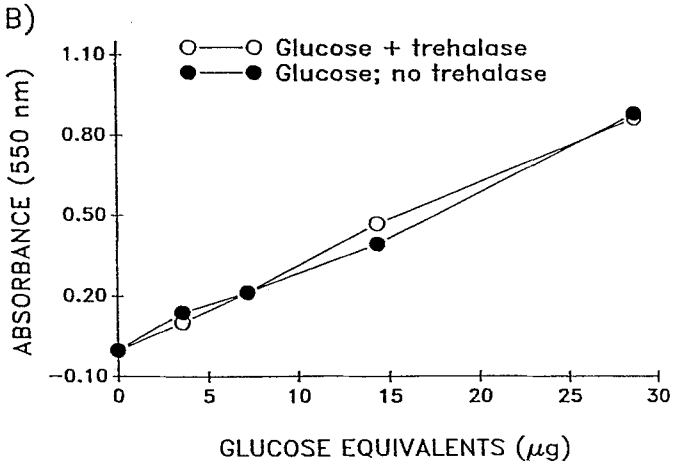




Figure 1. *A* Standard curves for trehalose with and without trehalase. The points represent the average of replicate samples of 0, 4, 8, 16 and 32 μg trehalose. The units on the graph are of the amount of glucose that would have given the equivalent number of glucose subunits (calculated at two per each trehalose molecule). The lines represent at least squares fit to the experimental data. *B* Standard curves for glucose with and without trehalase. The points represent the average of replicate samples of 0, 3.6, 7.2, 14.4 and 28.8 μg glucose.

that trehalase did not interfere in the detection of glucose; glucose absorbance values with and without trehalase present were nearly identical (fig. 1 B). After proving the validity of the trehalose assay, this assay was done on *Aplysia* blood with and without trehalase; the results of the two groups were nearly identical (fig. 2). In addition, an experiment was done in which the *Aplysia* blood was boiled for 10 min prior to assay for trehalose and glucose, to be certain that any endogenous trehalose that might have been present had not been hydrolyzed by blood-borne trehalase. Also in this experiment, no change in measured glucose levels were brought about by the addition of trehalase in the assay. These experiments indicate that trehalose in the blood is either non-existent or in low, undetectable amounts. Furthermore, the non-zero values for glucose when trehalase was absent indicate that *Aplysia* blood definitely contains glucose.

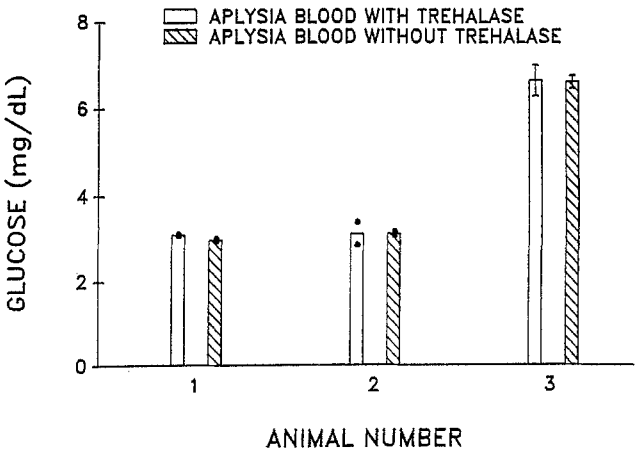



Figure 2. Blood glucose levels in *Aplyisa* blood with and without trehalase. Animals one and two were fed, nonshocked animals, whereas animal three was a fed, shocked animal. Points on the bars for animals one and two are the values for replicate determinations. Error bars for animal three represent the standard errors for triplicate determinations.

*Effects of shock*

*Behavioral response. Aplysia* had a stereotyped behavioral response to shock that included withdrawal from the noxious stimulus, copious secretion of purple ink and mucous, and generalized contraction of body wall producing a 'balled up' posture. Animals that remained in holding tanks or were put in the shockbox but were not shocked showed none of these behaviors.

*Rise in blood glucose.* To investigate the effect of shock on *Aplysia* carbohydrate levels, blood glucose and tissue glycogen from nonshocked and shocked animals were measured (fig. 3). Animals were sacrificed 5–10 min after termination of the last shock. Tissue glycogen concentrations in the hepatopancreas, the penis, the heart, and the foot were not significantly different between the two groups. However, the blood glucose of the shocked group was significantly higher than the nonshocked group. This experiment was repeated with similar results;

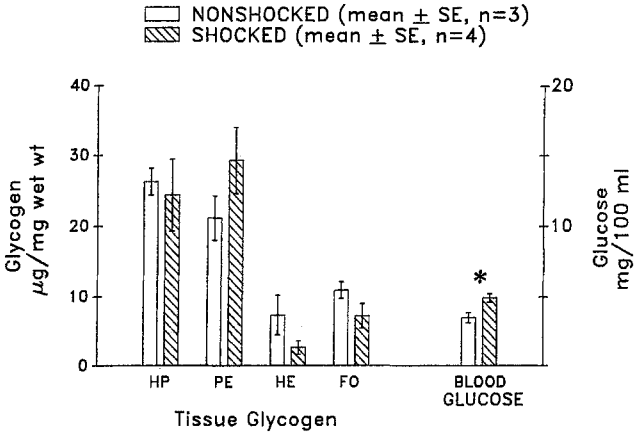



Figure 3. Tissue glycogen and blood glucose levels in nonshocked and shocked animals. Shock consisted of four 2-min shock periods with three intervening 2-min recovery periods. Abbreviations represent tissues as follows: HP, hepatopancreas; PE, penis; HE, heart; FO, foot. * = significantly different from nonshocked animals ($p < 0.05$, t-test).

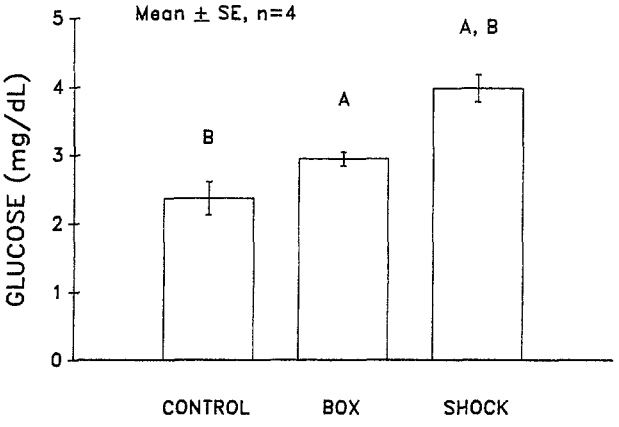



Figure 4. Effect of shock and/or translocation of animal from aquarium to box in which shock is delivered on blood glucose. Control animals were dissected immediately upon removal from aquarium holding tank. 'Box' animals were placed in the shock apparatus for the same period of time as shocked animals but not shocked. 'Shock' animals were exposed to four 2-min shock periods with three intervening 2-min recovery periods and were dissected 5–10 min after termination of shock. A = significantly different from control animals ($p < 0.05$, t-test). B = significantly different from 'box' animals ($p < 0.05$, t-test).

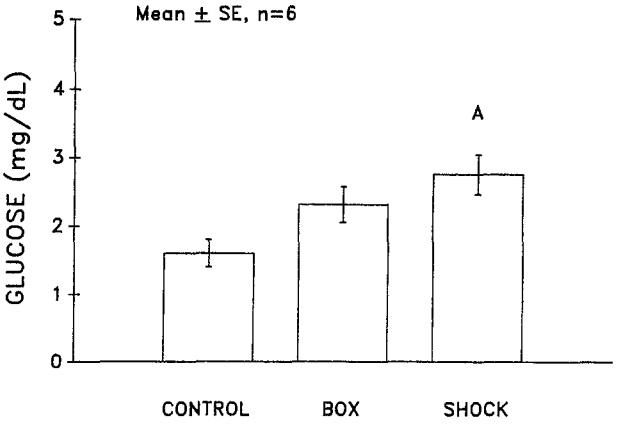



Figure 5. Effect of shock on blood glucose. Control animals were dissected immediately upon removal from their aquarium holding tank. 'Box' animals were placed in the shock apparatus for the same period of time as 'shock' animals but were not shocked. 'Shock' animals were exposed to one 90-s shock period and were dissected 60 min after termination of shock. A = significantly different from control ($p < 0.05$, t-test).

in experiment two of this series, three groups were examined: control animals not placed in the shockbox and not shocked, animals placed in the shockbox and not shocked, and animals placed in the shockbox and shocked (fig. 4). Shocked animals had significantly elevated blood glucose levels when compared to animals that were put in the shock apparatus but were not shocked. Shocked animals also differed significantly from animals taken directly from the tank. In addition, animals placed in the shockbox but not shocked had significantly higher levels than tank animals.

The above experiments sampled blood taken only 5–10 min post-shock. In order to test whether the high levels of glucose last as long as 1 h post-shock, an experiment was done in which animals were sacrificed at 60 min post-shock rather than 5–10 min post-shock. RSMAS *Aplysia* were shocked, returned to holding tanks immediately after shock treatment, and dissected 1 h later. The results showed a significant increase in blood glucose concentration when comparing the shocked group to the tank control, demonstrating that shock effects on blood glucose last at least 1 h (fig. 5).

In order to study the time course of the rise of blood glucose and also to eliminate the possible confounding effect of stress caused by dissection, animals were catheterized so that multiple blood samples could be taken without stressing the animal. Blood glucose levels of catheterized animals rose over a period of time following shock. In contrast, control levels remained constant or decreased over the same time period. As illustrated in figure 6, immediately after cessation of the shock stimulus, blood glucose rose precipitously in three of four animals. In a fifth animal (not illustrated), blood glucose increased by 138% over its initial level (time = 0) within 10 min after transferral into the shock apparatus and prior to shock. Following shock, blood glucose rose further, changing by 430% over the initial level within 6 min after shock and staying elevated at this level for at least 26 min after shock (last time point sampled for this animal).

*Discussion*

Our results clearly indicate that electrical shock leads to an increase in blood glucose levels (figs 3–6). The exact mechanism that couples trauma to heightened free circulating glucose is unknown. We hypothesize that electrical shock stimulates the release of endogenous neurotransmitters or hormones that regulate blood glucose level. The identity of these regulatory factors is unclear but serotonin may be one of the mediators of the stress response in *Aplysia*. Serotonin has been shown to exert effects in *Aplysia* similar to catecholamines in mammals. In mammals, catecholamines stimulate cardiac activity and inhibit gastrointestinal tract motility. Similarly, in *Aplysia*, serotonin activates the heart [9] and inhibits spontaneous contractions of the anterior portions of the gastrointestinal tract [10]. Furthermore, removal of serotonin with a specific serotonergic neurotoxin, 5-dihydroxytryptamine, decreases gill and siphon withdrawal and dishabituation in response to shock [11]. The fact that serotonin mediates several of the responses to shock in *Aplysia* suggests that it may similarly mediate the rise in blood glucose.

The stress syndrome, as described by Selye [12], is a generalized response to noxious stimuli. In the present study, we have shown that glucose rises in response to shock as it does in mammals. The behavioral responses of the animal appear to be those that would accompany responses to any noxious stimulus. For example, Cooper et al. [4] and Krontiris-Litowitz et al. [5] reported similar be-

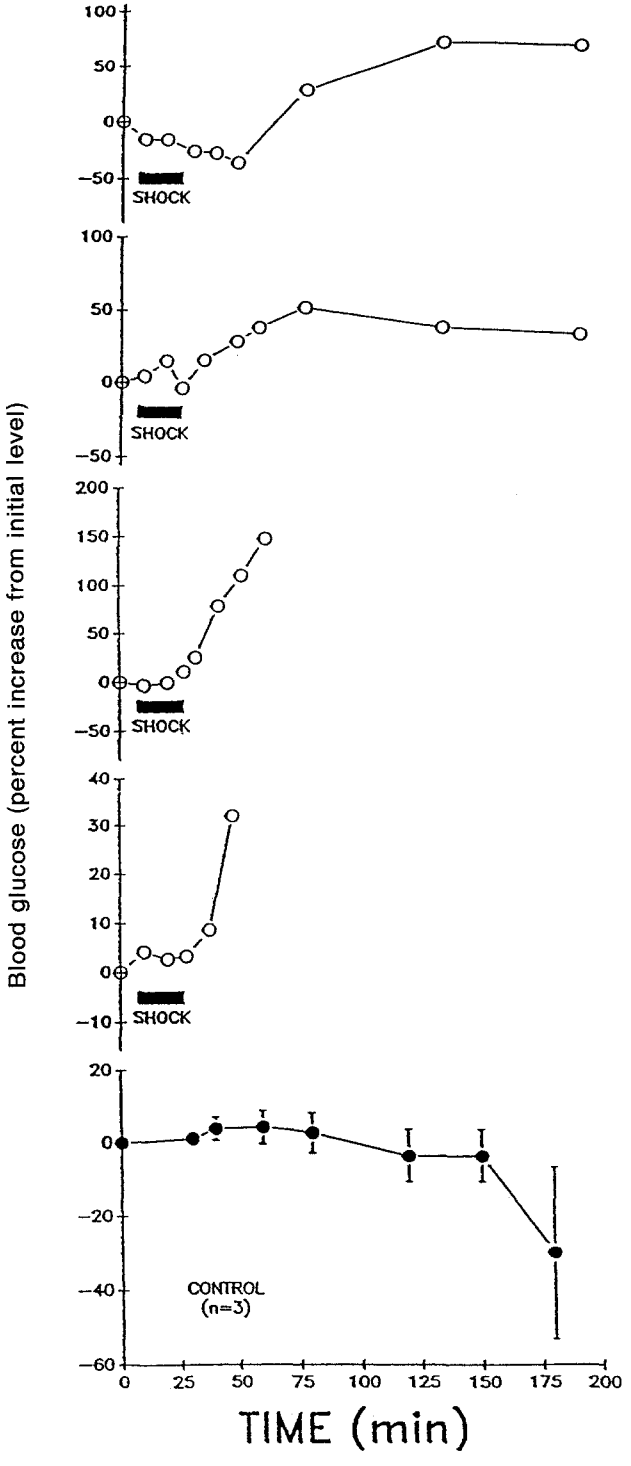



Figure 6. Percent increase in blood glucose of catheterized animals in response to shock. Shock consisted of four 2-min shock periods with three intervening 2-min recovery periods. Shocked animals were placed in the shocking apparatus for only the indicated 'shock' period, after which they were returned to their original aquarium holding tank. Control animals were kept in their original aquarium holding tank throughout the blood collection period.

havioral responses in *Aplysia* using both shock and mechanical trauma (pinching and cutting) as the stressors. If the response to shock is part of a generalized adaptation syndrome[12], then investigations in *Aplysia* would help determine what components of the response are applicable across a broad phylogenetic range.

In previous experiments[6], we observed that newly received animals had higher levels of blood glucose than animals maintained in the laboratory. The blood glucose levels of newly received animals ($4.9 \pm 0.2$ mg/dl) were similar to those of shocked animals in the present study. This may indicate that other stressful stimuli than shock may produce a rise in blood glucose. The shipping process involves confinement of the animal in a small amount of seawater, cooling, mechanical agitation, and oxygen deprivation. The shipping process may mimic some of the stresses normally encountered by *Aplysia* in the field. *Aplysia* is an intertidal gastropod that may undergo periods of exposure to air, dehydration, entrapment in tide pools, oxygen starvation and temperature change. If so, then investigation of the mechanisms mediating stress may be physiologically relevant to the life history of the animal.

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