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# Abnormal Feeding Behavior of Western Toads (*Bufo boreas*) Kept in a Hyperosmotic Environment. I. A Quantitative Behavioral Analysis as Related to Cerebral Amino Acid Concentrations

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ROSE, B. B., C. F. BAXTER, J. W. DOLE, K. H. TACHIKI AND R. A. BALDWIN. *Abnormal feeding behavior of western toads* (Bufo boreas) *kept in a hyperosmotic environment. I. A quantitative behavioral analysis as related to cerebral amino acid concentrations.* PHARMACOL BIOCHEM BEHAV **24**(5) 1315–1321, 1986.—Discriminant analysis of eleven behavioral variables associated with feeding permitted the assignment of hyperosmotically-acclimating (HOA) toads (*Bufo boreas*) to six different behavioral states. These behavioral states could be correlated with specific alterations in the level of select amino acids in three regions of the toad's central nervous system. By considering only those amino acids that showed equivalent levels in corresponding brain regions of normally-behaving, freshwater-acclimating (FWA) and HOA toads, it was possible to focus upon just nine amino acids as possible modulators of feeding behavior. Four of these amino acids were markedly elevated in two or more abnormal behavioral states: gamma-aminobutyric acid (GABA) in all three brain areas; glutamate in the cerebral hemispheres; aspartate in the cerebral hemispheres and olfactory bulbs; and phenylalanine in the olfactory bulbs and optic lobes. Other possible behavior modulators identified were: lysine and threonine in the cerebral hemispheres; carnosine in the olfactory bulbs; and valine and alanine in the optic lobes.

Brain amino acids Feeding behavior Toad Hyperosmotic Neurotransmitters

PREY-CATCHING by toads and frogs is one of the most thoroughly studied behaviors among vertebrates. The prey-capture sequence and the muscular actions involved in the tongue-flick have been described in detail [1, 15, 29]. Careful, quantitative analyses have been made of the characteristics of the visual stimuli that trigger the action ([14,18], many others). Much of the neural circuitry involved in the recognition of and response to visual feeding stimuli has been localized [13,14], and a role for olfaction in initiating the response has been demonstrated [11, 31, 32, 33]. The ease with which the prey-capture sequence can be elicited, the highly predictable nature of the action as a result of its innate basis, and the abundance of available background information make this behavior a good subject in a search for neurochemical correlates.

Recently, it has been demonstrated that the prey-capture response of western toads (*Bufo boreas*) is altered during acclimation to a hyperosmotic environment [10]. Tongue strikes of toads acclimated to freshwater (FWA toads) hit the intended prey with an accuracy of 80 to 100%. The accuracy of toads during acclimation to a hyperosmotic environment of 400 mOs NaCl (HOA toads) decreased to less than 20%. Other aspects of feeding behavior, e.g., rate of feeding and time delay before feeding began, also were altered in HOA toads during acclimation [28]. Exposure to hyperosmotic environments provides a means of altering specific behavioral responses in these animals.

Acclimation of western toads to a hyperosmotic environment is also known to alter the levels of amino acids in brain tissues [4, 6, 30]. This includes the elevation of the putative neurotransmitters: glycine, gamma-aminobutyric acid (GABA), glutamate and aspartate, suggesting the possibility that alteration in the behavioral responses might be related to changes in levels of the transmitter amino acids. After several days in a hyperosmotic environment, the levels of most amino acids in brain tissue tend to revert toward the normal levels of FWA toads; behavior also reverts toward normal.

The western toad is an osmoconformer and occasionally

encounters hyperosmotic conditions in its natural habitat. Although the blood plasma of toads, placed in a hyperosmotic environment, attains an osmolality equivalent to or above that of the environment within a few hours, this acclimation may be stressful as judged by a decreased activity level. However, after about 48 hours of acclimation the activity level of most HOA toads appears normal.

The primary objective of this report is to quantitatively characterize feeding behavior during the acclimation process. For this purpose we classified the behavior of HOA toads by behavioral states using discriminant analysis on a series of behavioral variables associated with feeding (Table 1). Each behavioral state was then further characterized in terms of the content of amino acids in tissues of three different brain areas.

## METHOD

### *Animal Collection and Maintenance*

Adult toads (62–102 mm, snout to vent) were collected in the San Fernando Valley or near Palmdale, Los Angeles County, CA. Each animal was toe-clipped for identification.

For at least 30 days prior to and during an experiment, toads were housed in glass aquaria (30×59 cm) in groups of six to 12. Aquaria were kept in an environment of 20°C and a 12/12 light/dark cycle. Each aquarium was tilted at an angle of 10 degrees from the horizontal, with tap water covering the lower half at all times. Tanks were cleaned and the water replaced daily. Toads were fed mealworms (larval *Tenebrio mollitor*) ad lib at least three times weekly.

### *Collection of Behavioral Data*

Behavioral observations of toads were made in a small room partitioned into a lighted and dark side by an opaque curtain. Small holes in the curtain permitted an observer on the dark side to watch the behavior of toads placed, one by one, into a 15×30 cm aquarium (observation chamber) on the lighted side. All observations were made between noon and 5 p.m.

To observe a toad's feeding behavior, 12 active mealworms were placed into the observation chamber at the end nearest the observer. At time zero a toad was removed from its home tank and placed, alone, into the observation chamber at the end farthest from the mealworms and the observer, and facing away from both. A transparent lid was then placed on the observation chamber; holes in the lid permitted free exchange of air.

After the introduction of the toad, the observer recorded: (1) lag time (TLG), time from time zero until the toad first attempted to capture a mealworm; (2) hits (HT), the number of feeding attempts which resulted in the capture of at least one mealworm; (3) feeding time (FT), time elapsed from the first feeding attempt until the toad had made six successful hits or was observed for 15 minutes, whichever came first; and (4) misses (MS), the number of feeding attempts in which the toad failed to capture a mealworm. Misses were subdivided into close (CM) and far misses (FM), depending on whether the tongue missed its target by an estimated distance of less than or more than 5 mm; far misses were usually readily identifiable because the tongue often failed to extend from the mouth, or, if it did, frequently left a wet spot where it struck the aquarium floor or wall. All times were expressed in minutes.

Other behavioral variables were calculated for those toads that fed, including: (1) trial time (TT)=TLG + FT; (2) total number of feeding attempts (AT)=HT + MS; (3) strike accuracy as a percentage of total feeding attempts (SA)=(HT × 100)/(HT + MS); (4) prey-capture rate (PCR)=HT/FT; (5) feeding attempt rate (FAR)=(HT + MS)/FT; (6) far misses as a percent of all misses (PFM)=(FM × 100)/MS. To permit comparison of a toad's response at the beginning of a test with its response for the test as a whole, we also determined HT(B), MS(B), CM(B), FM(B), AT(B) and SA(B), variables comparable to those with similar symbols but based only upon the first six feeding attempts in a given test.

Often a toad, ignoring the mealworms, would either pace along the aquarium wall, snout pressed to the glass, or extend its body upward on the wall while standing on its hind feet, as if attempting to climb out of the test chamber. The occurrence of such "escape behavior" was also recorded.

### *Experimental Protocol*

*Pretests.* To establish a baseline, and to determine the normal response of each toad, feeding behavior of each animal while acclimated to freshwater was monitored every other day for six days. After the third pretest, seven toads that had not fed actively in the observation chamber or with a mean strike accuracy markedly below the mean of all the toads were eliminated. The remaining animals were then ranked according to their mean strike accuracies and divided between a control and an experimental group so that the means of the two groups were essentially the same.

*Tests.* On the day following the third pretest, the tap water in the tanks housing the experimental group was replaced with a 200 mOs NaCl solution. Twenty-four hours later, and every day thereafter, a 400 mOs NaCl solution was placed into each tank after it was cleaned. The use of the 200 mOs NaCl prior to the 400 mOs solutions permitted the experimental group of toads to acclimate gradually to the new hyperosmotic environment. The FWA control group was maintained in freshwater throughout the experiment.

Behavioral observations were made of all toads, control (FWA) and experimental (HOA), every other day. The day the experimental toads were first subjected to 400 mOs NaCl was designated as day zero. Pretests, therefore, occurred on days −6, −4, −2, and the experimental toads were first subjected to 200 mOs NaCl on day −1. In addition to the pretests, behavior was monitored on day zero (immediately before the experimental toads were exposed to 400 mOs NaCl in their environment, but after 24 hr in 200 mOs NaCl) and on every even-numbered day thereafter for a maximum of 28 days. Toads were not fed between tests or pretests.

### *Identification of Behavioral States*

For the purpose of identifying behavioral states, we considered the observation period for each animal, i.e., each pretest or test, to be a separate case. In 278 (56.8%) of the 489 behavioral tests of 82 HOA toads, the mealworms failed to elicit a feeding response; these tests, in which behavioral variables were either identical for all animals or were unobtainable, were considered to form a self-defined, "nonresponder" state. Discriminant analysis using the variables HT, AT, MS(B), FM, FM(B), PFM, SA, SA(B), FT, TT, and FAR was used to categorize the remaining 211 (43.2%) HOA tests. Normal feeding behavior in HOA toads was identified by comparing these variables with the same variables in 492 cases (pretests of all toads plus tests of control animals) obtained from 122 FWA toads.

Prior to the discriminant analysis, all statistical assumptions of the procedure [35] were evaluated and none was considered to be violated. Unequal group size was not considered a problem since the smallest group notably exceeded in size the number of discriminating variables [35]. Multivariate outliers in each preliminary group were identified ($p<0.01$) and removed from the analysis phase to avoid bias; all cases, however, were included in the classification process.

Statistical analyses were performed on a CDC 750 computer using two commercially available programs, SPSS, the Statistical Package for the Social Sciences [17,23] and BMDP, BioMedical Data Program Statistical Software [9].

*Biochemical Analysis*

Experimental HOA toads were sacrificed by decapitation immediately following a test on a designated day after determining their feeding behavioral pattern. Randomly selected FWA control animals were sacrificed at regular time intervals during the course of the experiment at the same time as the HOA toads.

Just before decapitation, approximately 1.5 ml of blood was obtained from each toad by cardiac puncture, using a 2 ml syringe containing 5 units of $NH_4^+$ heparin. The blood was transferred immediately to plastic centrifuge tubes, cooled to 4°C and spun for 12 minutes in an Eppendorf microfuge to precipitate all cellular components. The supernatant plasma was collected and a 250 $\mu$l aliquot used for the determination of plasma osmolality by freezing point depression (Advanced Osmometer Model 3D).

Decapitated heads were frozen immediately in a mixture of dry ice and acetone and stored at −70°C in sealed plastic bags. The brains were removed from the skull at −70°C and then transferred to a dissection block maintained at −18°C in a walk-in cold room at 4°C. The brains (at −18°C) were then trimmed to remove any connective and vascular tissue, as well as frozen cerebro-spinal fluid, and dissected into three parts—olfactory bulbs, optic lobes and remaining cerebral hemispheres. Brain stem, including the infundibulum and parts of the hypothalamus, were discarded. Included with the cerebral hemispheres were portions of the thalamus and hypothalamus (including the optic chiasma). The frozen dissected parts were weighed and immediately homogenized in 4 volumes of ice cold 0.25 M sulfosalicylic acid (w/v). The sulfosalicylic acid solution also contained 150 $\mu$M norleucine which was used as the internal standard in the subsequent analysis. After homogenization the precipitated proteins were sedimented by centrifugation and the clear supernatant extracts placed into separate tubes. The extract of each tube was adjusted to an approximate pH of 2.0, using a few microliters of concentrated lithium hydroxide. Extracts were stored at −70°C until analyzed using an automated amino acid analyzer equipped with a 15 cm, 4 mm microbore column of Durrum DC 5A resin as described in detail previously [36]. Ten to 20 $\mu$l of extract, representing from 2.5 to 5.0 mg of tissue, were used for analysis of amino acid levels in brain areas.

*Guidelines for the Identification of Possible Amino Acid Modulators of Feeding Behavior*

Two guidelines were used to identify amino acids in toad brain that are likely candidates for influencing feeding behavior: (1) Any amino acid selected had to be at comparable levels in tissues of corresponding brain areas in the two groups of normally behaving toads: FWA controls and HOA normals. Comparisons were made using Student's $t$-test; if the probability that the amino acid levels in the two groups belonged to the same population was greater than 20% ($p>0.20$), they were considered to be the same and were included for further consideration. Any amino acid in a given brain area that failed this test was eliminated as a possible candidate to modify feeding behavior. (2) The amino acid level in one or more of the abnormal behavioral states of HOA toads had to differ significantly ($p<0.05$) from that present in comparable brain areas of the HOA normally-behaving animals; Student's $t$-test was used for all statistical comparisons. Amino acids included in Table 4 were selected using these guidelines.

Data presented were obtained in four separate replicated experiments, spanning a three year period. Three replicates were conducted during late December through February and one was performed during March and April. All data were combined for analysis.

TABLE 1

FREQUENCY OF HOA STATES AMONG 489 BEHAVIORAL TESTS AND SUBJECTIVE DESCRIPTION OF EACH BASED ON LEVEL OF FEEDING ACTIVITY AND STRIKE ACCURACY

| | Frequency (and %) | Feeding Activity Level | Strike Accuracy |
|---|---|---|---|
| FWA Control | 518 | active | good |
| HOA Normal | 22 (4.5%) | active | good |
| HOA Poor-hitter | 31 (6.3%) | active | fair |
| HOA Very-poor-hitter | 47 (9.7%) | active | poor |
| HOA Poor-responder | 39 (8.0%) | sluggish | fair |
| HOA Very-poor-responder | 72 (14.7%) | very sluggish | poor |
| HOA Non-responder | 278 (56.8%) | absent | unknown |

FWA controls have been included for comparison.

## RESULTS

*Behavior*

Six behavioral states are identified among the HOA toads: a self-defined non-responder state, and five others produced by the discriminant analysis prcedure applied to the remaining 211 tests. Each state is defined quantitatively in Table 2; data for the FWA controls are also included, primarily to illustrate the great similarity of this group with the normally-behaving HOA toads. (A briefer, more subjective description of each group, together with the frequency of occurrence among HOA toads, is presented in Table 1.)

The following are verbal descriptions of each state (and FWA controls) based on the data in Table 2. In these descriptions we also note the occurrence of excessive amounts (>2 minutes/test) of escape behavior; these data were not used in deriving the behavioral states, and are not included in Table 2.

*Freshwater control.* FWA toads typically respond rapidly to prey; on the average they begin feeding 0.57 min after being placed in the chamber, score the allotted six hits in 2.13 min, feed at a rate of 4.3 attempts per min, and complete

TABLE 2

MEANS±SD OF BEHAVIORAL VARIABLES FOR FWA CONTROLS AND SIX HOA BEHAVIORAL STATES

| Behavioral Variable | FWA Controls | HOA Behavioral State: Non-Resp. | Very-Poor-Resp. | Poor-Resp. | Very-Poor-Hitter | Poor-Hitter | Normal |
|---|---|---|---|---|---|---|---|
| Time lag | 0.6± 0.7 | 15.0±0 | 3.0± 3.5 | 0.9± 0.9 | 1.6± 2.3 | 0.9± 1.1 | 0.8± 0.7 |
| Feeding time | 2.1± 1.2 | 0 | 12.0± 3.5 | 14.0± 1.0 | 12.8± 2.9 | 4.2± 2.0 | 2.4± 0.8 |
| Trial time | 2.7± 1.5 | 15.0±0 | 15.0± 0 | 14.9± 0.5 | 14.4± 2.1 | 5.1± 2.1 | 3.2± 1.1 |
| Attempts | 7.5± 1.8 (6) | 0 | 7.6± 4.9(4) | 10.8± 5.9 (5) | 40.7±20.6(30) | 2.5±10.4 (12) | 8.1± 1.6 (7) |
| Attempts (B) | 6.0± 0 (6) | 0 | 4.8± 1.7(6) | 5.6± 0.9 (6) | 6.0± 0 (6) | 6.0± 0 (6) | 6.0± 0 (6) |
| Feeding attempt rate | 4.3± 2.0 | — | 0.7± 0.5 | 0.8± 0.5 | 3.7± 2.9 | 5.8± 2.3 | 3.7± 1.4 |
| Hits | 6.0± 0 (6) | 0 | 0.9± 1.0(0) | 4.2± 0.9 (4) | 1.5± 2.0 (0) | 6.0± 0 (6) | 6.0± 0 (6) |
| Hits (B) | 4.8± 1.2 (6) | 0 | 0.4± 0.6(0) | 2.4± 1.3 (3) | 0.1± 0.7 (0) | 1.0± 1.0 (0) | 4.4± 1.1 (3) |
| Prey capture rate | 3.6± 1.6 | — | 0.1± 0.1 | 0.3± 0.1 | 0.2± 0.3 | 1.7± 0.6 | 2.9± 1.2 |
| Misses | 1.5± 1.8 (0) | 0 | 6.6± 4.5(1) | 6.6± 5.5 (3) | 39.3±19.5(23) | 16.5±10.4 (6) | 2.1± 1.6 (1) |
| Misses (B) | 1.2± 1.2 (1) | 0 | 4.4± 2.0(6) | 3.3± 1.7 (3) | 6.0± 0.2 (6) | 5.0± 1.0 (6) | 1.6± 1.1 (1) |
| Strike accuracy (%) | 84.0±15.2(100) | — | 17.1±23.2(0) | 49.2±23.8(100) | 3.0± 3.9 (0) | 31.7±12.2 (15) | 76.7±15.1(86) |
| Strike accuracy (B) (%) | 80.2±19.6 (83) | — | 13.5±24.0(0) | 44.4±28.0 (17) | 0.4± 2.5 (0) | 17.2±15.7 (0) | 72.7±18.1(50) |
| Close misses | 1.4± 1.5 (0) | 0 | 1.4± 2.7(0) | 3.0± 2.8 (0) | 2.1± 5.0 (0) | 4.9± 4.4 (0) | 1.6± 1.5 (0) |
| Close misses (B) | 1.1± 1.1 (1) | 0 | 0.8± 1.7(0) | 1.2± 1.5 (0) | 0.3± 1.1 (0) | 1.5± 2.1 (0) | 1.2± 1.2 (0) |
| Far misses | 0.1± 0.8 (0) | 0 | 5.2± 4.6(0) | 3.6± 4.6 (0) | 37.2±20.0(18) | 11.6±11.3 (5) | 0.5± 1.0 (0) |
| Far misses (B) | 0.1± 0.5 (0) | 0 | 3.5± 2.4(6) | 2.1± 1.9 (0) | 5.7± 1.1 (6) | 3.5± 2.2 (4) | 0.4± 0.7 (0) |
| % Far misses | 2.3±12.8 | — | 73.7±40.1 | 47.4±35.6 | 94.2±15.1 | 63.3±31.6 | 21.2±38.0 |

Mode (of discrete variables) in parentheses. Blanks indicate variable cannot be calculated. All HOA states differed significantly ($p<0.01$) when tested with Roy-Bargman Stepdown Manova [30]. N=frequency (Table 1). All time in minutes. Behavioral variables are defined in text.

the test in only 2.7 min. Strike accuracy is high (mean=84%; mode=100%), as is rate of prey-capture (mean=3.6/min); the toads average only 1.5 misses, only 2% of which are off-target by more than 5 mm. Escape behavior lasting more than 2 minutes is seen in only 2% of the tests.

*HOA normal.* The 22 HOA cases classified as normal are behaviorally similar to the FWA controls; only three behavioral variables differ significantly ($p<0.05$) from those of the FWA controls, as tested with Student's $t$-test. Far misses at the start of the tests are more common (mean=0.4), resulting, in turn, in lower strike accuracy (mean=76.7%) and rate of prey-capture (mean=2.9/min). The fact that among normal HOA toads far misses are seven times as frequent for the first six strikes as compared to FWA toads, but only four times as common when measured for the entire test period suggests that these differences probably occur because most HOA toads that behave normally have only just recovered from an abnormal state in which lower strike accuracies are common. HOA toads in this state are never seen to exhibit escape behavior lasting more than 2 minutes.

*HOA poor-hitter.* Toads in this behavioral state possess apparently normal interest in food; average delay before feeding begins is 0.93 min, only slightly longer than among HOA normal toads (mean=0.78 min), and all toads score six hits. Mean strike accuracy, however, is only 31.7%, about one-third that of normally-behaving animals. Misses are eight times as common (mean=16.5) as among normally behaving HOA animals, and prey are missed by distances greater than 5 mm (far misses) more than 63% of the time. More frequent misses result in a feeding time (mean=4.2 min) almost twice that of the normal HOA toads. Strike accuracy tends to improve during tests, from a mean of 17.2% at the beginning to 31.7% for the test as a whole. More than 2 minutes of escape behavior is seen in 10% of the tests; in no case does it continue for as long as 10 minutes.

*HOA very-poor-hitter.* Toads in this behavioral state also have a normal interest in food, as indicated by the greatest number of feeding attempts (mean =40.7) of any state and feeding attempt rate (mean =3.7/min) comparable to that of HOA normal. However, they are nearly incapable of capturing prey; 97% of the feeding attempts are unsuccessful, and 94% of the misses are off-target by at least 5 mm. Prey-capture rate, consequently, is very low (mean =0.15/min). However, during the tests, strike accuracy improved 8-fold, from 0.36% at the beginning to 2.98% overall. In 88% of the tests escape behavior persists for more than 2 minutes, but in only 24% does it continue as long as 10 minutes.

*HOA poor-responder.* Toads in this behavioral state have a below normal interest in food; although delay before beginning to feed (mean =0.93 min) is only slightly longer than among HOA normal, feeding commonly ends prematurely, after an average of only 4.18 hits. Mean strike accuracy is two-third that of normal (mean =49.2%), but there is great variability (range 16% to 100%); percentage of far misses (mean =47.4%) is the lowest of any abnormal state. Because toads usually capture fewer than six prey, hence use the full 15 min test time, prey-capture rate is 10% of normal (mean=0.3/min). Escape behavior lasting more than 2 minutes occurs in 94% of the tests; in 69%, it continues for more than 10 minutes.

TABLE 3

MEAN CONCENTRATION (μmoles/g)±STANDARD DEVIATION AND (N), BY BRAIN AREA AND BEHAVIORAL STATE, OF ALL AMINO ACIDS THAT MET OUR SELECTION GUIDELINES

| Brain Region | Amino Acid | FWA Control | HOA Behavioral State | | | | |
|---|---|---|---|---|---|---|---|
| | | | Normal | Poor-Hitter | Very-Poor-Hitter | Very-Poor-Resp. | Non-Resp. |
| Cerebral Hemispheres | GABA | 1.58±0.43(8) | 1.74±0.26(7) | 1.94±0.41(4) | 2.12±0.39(4) | 2.78±0.48(6)‡ | 2.30±0.85(17)* |
| | GLU | 5.36±0.57(7) | 5.80±1.01(7) | 6.61±0.33(4) | 7.99±0.56(5)‡ | 7.46±1.48(6)* | 7.96±1.90(16)† |
| | ASP | 1.30±0.39(7) | 1.34±0.36(7) | 1.48±0.07(4) | 1.99±0.27(5)† | 1.98±0.38(6)† | 2.04±0.69(16)* |
| | LYS | 0.13±0.06(8) | 0.13±0.03(7) | 0.16±0.03(4) | 0.11±0.06(4) | 0.11±0.06(6) | 0.08±0.03(16)‡ |
| | THR | 0.18±0.07(8) | 0.15±0.04(7) | 0.21±0.07(4) | 0.26±0.11(5) | 0.22±0.12(6) | 0.22±0.10(17)* |
| Olfactory Bulbs | GABA | 2.42±0.38(6) | 2.41±0.62(6) | 2.98±0.73(4) | 3.54±0.54(4) | 3.63±1.20(6)* | 2.91±0.74(12) |
| | PHE | 0.06±0.01(5) | 0.05±0.02(6) | 0.06±0.02(4) | 0.13±0.10(4) | 0.12±0.06(6)* | 0.12±0.06(11)† |
| | ASP | 1.10±0.17(5) | 1.03±0.32(6) | 1.50±0.51(4) | 1.90±0.69(4)* | 1.94±0.56(6)† | 1.94±0.89(11)* |
| | CAR | 0.10±0.03(3) | 0.08±0.03(4) | 0.16±0.05(2)* | 0.09±0.01(2) | 0.13±0.05(5)* | 0.09±0.02 (4) |
| Optic Lobes | GABA | 2.86±0.58(7) | 3.61±0.39(6) | 4.06±0.80(4)* | 4.23±0.80(4)* | 5.28±1.52(6)* | 4.56±1.26(14)† |
| | PHE | 0.04±0.01(6) | 0.03±0.01(7) | 0.04±0.01(4) | 0.05±0.02(4)* | 0.06±0.02(6)† | 0.07±0.03(14)‡ |
| | VAL | 0.07±0.02(7) | 0.09±0.03(7) | 0.12±0.15(4) | 0.16±0.04(4)* | 0.12±0.05(6) | 0.08±0.03(14) |
| | ALA | 0.26±0.04(7) | 0.26±0.11(6) | 0.35±0.08(4) | 0.52±0.11(4)† | 0.47±0.24(6) | 0.44±0.22(14) |

Concentrations significantly different from that of normal HOA toads indicated by: *0.05; †0.01; ‡0.001. FWA control values are included for comparison; none differed significantly from HOA normal values. Mean blood osmolalities (mOs) for FWA and HOA behavioral groups, in order, are: 261, 418, 422, 440, 414, 430, 426. Mean osmolalities of HOA groups did not differ significantly as tested with ANOVA ($p>0.28$).

*HOA very-poor-responder.* Toads in this state exhibit the least interest in food of any state except the non-responder; they delay longest before starting to feed (mean=3.04 min), average the fewest feeding attempts (mean =7.57), exhibit the lowest feeding attempt rate (mean =0.69/min), and have the lowest prey-capture rate (mean=0.08/min). Mean strike accuracy is only 17.06% (mode=0), but extremely variable (range, 0% to 100%); almost 74% of all strikes missed the target by more than 5 mm. For every variable but feeding time, this state is more extreme than the preceeding one; the shorter feeding time is anomalous, resulting from the long time lag before beginning to feed, leaving less time for feeding before the test is terminated. Escape behavior exceeds 2 minutes in duration in 96% of the tests and exceeds 10 minutes in 79%.

*HOA non-responder.* Toads in this state are highly uniform in behavior. The toads make no attempts to feed, hence have neither hits nor misses. Animals in this state exhibit escape behavior for more than 2 minutes in 96% of all tests and for more than 10 minutes in 93%; escape behavior continuing for the entire test is the norm.

### *Amino Acid Changes Correlated With Behavioral States*

Biochemical data were obtained from 40 HOA toads. Discriminant analysis categorized seven as normal at the time of sacrifice, four as poor-hitters, five as very-poor-hitters, one as a poor-responder, and six as very-poor-responders; 17 were non-responders. The levels of those amino acids in each brain region identified as potentially related to feeding behavior are listed in Table 3. Biochemical data from twelve normally-feeding FWA control toads are included for comparison. Data from the single poor-responder toad permitted no statistical comparison.

In the various brain areas of non-responder toads, six amino acids were identified as possible modulators of feeding behavior (see "guidelines" in the Method section). Five of these amino acids were significantly elevated: gamma-aminobutyric acid (GABA) in the cerebral hemispheres and optic lobes; phenylalanine in the optic lobes and olfactory bulbs; aspartate in the cerebral hemispheres and the olfactory bubls; and glutamate and threonine in the cerebral hemispheres. Lysine levels were significantly depressed in the cerebral hemispheres.

Among toads classified as very-poor-responders, four amino acids in the brain areas satisfied the "guidelines." All were elevated significantly: GABA in all three brain areas; phenylalanine in the olfactory bulbs and optic lobes; aspartate in the cerebral hemispheres and olfactory bulbs; and glutamate in the cerebral hemispheres.

In the brains of toads classified as very-poor-hitters, the "guidelines" identified six possible amino acid modulators of feeding behavior. All were significantly elevated: aspartate in the cerebral hemispheres and optic lobes; glutamate in the cerebral hemispheres; and GABA, phenylalanine, valine, and alanine in the optic lobes.

Among toads in the less behaviorally deviant poor-hitter state, only carnosine and GABA qualified as possible behavioral modulators. The level of each of these compounds was elevated significantly, carnosine in the olfactory bulbs, GABA in the optic lobes.

## DISCUSSION

In this study we identified 18 quantifiable behavioral variables associated with feeding activity, all to some degree interrelated. Eleven were used simultaneously in identifying behavioral "states" using discriminant function analysis, a procedure that permits the objective prediction of group membership based upon the possession of a similar set of

behavioral variables. This technique, widely used in the social sciences, is a potentially valuable tool for identifying neurochemcial parameters important in behavior. Though discriminant analysis involves complex statistical manipulations, commercially available computer programs greatly facilitate its use.

Whereas glucose levels have been implicated as regulators of feeding behavior [2,25], the present findings appear unrelated to this mechanism. In studies with HOA toads in which glucose levels were measured, only transient changes in brain and plasma glucose were observed during the first 24 hours of acclimation to the hyperosmotic environment. However, glycogen stores, in toads acclimating to a hyperosmotic environment, are depleted [5], and this phenomenon may be related to the elevation of non-essential amino acid in the brain tissues.

There is uncertainty concerning the effects of osmotic changes upon respiration and energy metabolism in poikilotherms. Some studies suggest that there is no change over a very wide range of osmolalities [21]. In other studies, increases in the metabolic rate were found when either hyper- or hypoosmotic changes were made in the environment [3,24]. There are reports also that a shift to hyperosmotic conditions decreases energy metabolism and respiration [12,34]. Energy metabolism in *Bufo boreas* under hyperosmotic conditions may be altered, but to date no reliable data are available.

The altered plasma osmolality does not *per se* relate to the changes in behavior we observed. Although some HOA toads did not feed and some fed in an abnormal manner, feeding behavior among other toads fully acclimated to the hyperosmotic environment was similar to that among FWA controls. Escape behavior also cannot be attributed solely to changes in plasma osmolality, since such behavior was occasionally seen among FWA controls, and was noted among normally-feeding HOA toads no more frequently than among those acclimated to freshwater. Furthermore, in the present study comparisons of brain amino acid levels and behavior were made between abnormally-behaving HOA toads and normally-behaving HOA toads. Thus, our data show unequivocally that in toads with similar plasma osmolalities, differences in *some* amino acid concentrations in *some* brain areas (Table 3) are associated with differences in feeding behavior.

Four of the amino acids selected by our procedure—GABA, aspartate, glutamate and phenylalanine—stand out from the others in that each was identified as elevated in several abnormal behavioral states and (except for glutamate) in more than one brain region. All of these amino acids are either neurotransmitters (GABA and glutamate), putative neurotransmitters (aspartate), or metabolic precursors of neurotransmitters (phenylalanine).

The alteration in levels of phenylalanine in optic lobes and olfactory bulbs is associated in the present study with several abnormal behavioral states. Phenylalanine, in addition to its role as a precursor compound for catecholamines, is also an immediate precursor of phenylethylamine, a compound with apparent neuromodulatory properties in tissues of the central nervous system [7,8].

Feeding behavior in toads is stimulated not only by visual cues but also by odors [11, 32, 33]. Carnosine has been implicated as a putative neurotransmitter in olfactory bulbs [16,22]. It is noteworthy therefore that, in the present study, an alteration in feeding behavior is associated with changes in the level of carnosine exclusively in the olfactory bulbs.

Valine and lysine appear to be related to alterations in feeding behavior. Although lysine is not usually associated with neurotransmitters and neuromodulators, changes in blood valine have been shown to affect the activity of the neurotransmitter serotonin [8, 20, 37]. The relationship of these two amino acids to feeding behavior is unclear.

The suggestion that changes in amino acid levels in brain tissue are related to feeding behavior and apostatic mechanisms is not new [20,26]. In fact, GABA has been implicated repeatedly in mammalian feeding behavior [19,27]. However, to the best of our knowledge, our study is the first to document this probable interrelation in amphibians. Furthermore, the diversity of biochemical changes in different brain areas that correlate with a variety of changes in feeding behavior attest to the complexity of these interrelations.

In our analysis, behavioral states were identified solely on the basis of differences in feeding behavior. It is possible that the neurochemical changes observed are associated also with a variety of other behaviors. It was noted, for example, that frequency of escape behavior differed among the behavioral states, occurring in general more frequently in toads with the most abnormal feeding behavior and less frequently in those that fed more normally. The question of whether the changes in feeding and escape behavior are independent, or whether they are in some way related, cannot be answered on the basis of the data presented in this paper.

The behavioral and biochemical observations demonstrate the feasibility of characterizing and classifying amphibian feeding behavior by the use of discriminant analysis applied to various behavioral variables associated with feeding. The association of feeding behavioral states with changes in some brain amino acid levels supports the probable validity of the behavioral states as established by the discriminant analysis.

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