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Freezing Survival and Metabolism of Box Turtles, *Terrapene carolina*

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Adult eastern box turtles *Terrapene carolina carolina* from Ohio readily recovered after 44 h of freezing exposure at -2°C . During thawing at 25°C , motor responses resumed in a sequence of increasing complexity with a reflex twitch in response to poking seen after 1.2 ± 0.5 h, coordinated retraction of the limbs after 2.0 ± 0.18 h, and voluntary locomotion after 2.7 ± 0.4 h. Turtles dissected immediately after freezing exposure had ice in body cavities and surrounding skeletal muscles in limbs. Analysis of putative cryoprotectants in serum and seven organs showed that all organs accumulated glucose during freezing. Net glucose accumulations of $6\text{--}20\text{ }\mu\text{mol/g}$ wet weight represented 4-22-fold increases; the highest glucose levels were in liver, heart, and serum. Liver glycogenolysis was identified as the source of the glucose; this was supported by both metabolite (a 62.5-fold increase in the precursor glucose-6-phosphate) and enzyme (the percentage of glycogen phosphorylase in the active α form rose from 21.3-57.5%) responses to freezing. Freezing exposure also caused an increase in lactate levels in serum and four organs, but glycerol ($<2\text{ }\mu\text{mol/g}$), sorbitol ($<0.3\text{ }\mu\text{mol/g}$), and free amino acid levels in organs were unchanged. Both the low levels of putative cryoprotectants and measurements of serum osmolality, 244 ± 4.3 and $315 \pm 15.4\text{ mOsmol/l}$ for control and freezing exposed turtles, indicated that box turtles can endure freezing without an accumulation of large pools of low molecular weight cryoprotectant.

THE eastern box turtle *Terrapene carolina carolina* is one of a number of species of terrestrially hibernating reptiles and amphibians that has developed the ability to endure the freezing of a high percentage of its body water as an aid to winter survival (for review Storey and Storey, 1992). Costanzo and Claussen (1990) demonstrated that these turtles recovered, without injury, after freezing exposures of 48-73 h with core body temperatures as low as -3.6

C and up to 58% of total body water frozen. Box turtles hibernate in burrows under loose soil and organic debris, and radiotelemetry measurements of the body temperatures of hibernating turtles in Ohio over three years showed some periods of subzero body temperatures each winter, the lowest recorded temperature being -1.4°C (Claussen et al., 1991). Freeze tolerance may be a necessary aid to survival whenever temperature falls below the freezing point of body fluids (about -0.5°C) either within the hibernacula or while turtles are active in the late fall or early spring. Freeze tolerance in turtles was first described for hatchlings of the Midland painted turtle, *Chrysemys picta marginata* (Storey et al., 1988) and is equally developed in the Western subspecies *C. p. bellii* (Churchill and Storey, 1992a). Hatchlings of the closely related pond slider *Trachemys* (= *Pseudemys*) *scripta elegans* also tolerate short-term freezing (Churchill and Storey, 1992b).

One of the key adaptations supporting freeze tolerance is the accumulation of low molecular weight cryoprotectants. These minimize the extent of cell water loss during extracellular ice formation (resulting from the colligative effects of high cryoprotectant concentrations) and stabilize macromolecular structures (Storey and Storey, 1988). Sugars and polyhydric alcohols are the common cryoprotectants in both vertebrates and invertebrates (Storey and Storey, 1988). For example, wood frogs (*Rana sylvatica*) accumulate 0.2–0.5 molar glucose in body fluids during freezing exposures. However, studies with painted turtle hatchlings have shown them to be unusual among freeze-tolerant animals because only low levels of putative cryoprotectants (glucose, lactate, taurine) accumulated during freezing (Storey et al., 1988; Churchill and Storey, 1991).

The present study assesses some of the characteristics of freezing in box turtles including an analysis of the time dependence of the resumption of various motor functions during thawing and the measurement of various metabolic responses to freezing.

MATERIALS AND METHODS

Six adult box turtles, *T. c. carolina* (two males, four females) of mean body mass 408 ± 21.7 g were captured in Athens County, Ohio, in the summer of 1990. Turtles were held at room temperature in the Nazareth College laboratory and fed regularly. In mid-March 1991, turtles were cold acclimated to 4°C for 3–4 weeks; animals were kept in the dark without feeding but with water available. Subsequently, super-

cooling point, freezing survival, and the sequence of recovery of reflexes after thawing were tested. For the first test of freezing survival, five turtles were placed in plastic containers that were immersed in an RTE-210 refrigerated bath held constant at -2°C for 48 h. Body surface temperatures were recorded for three animals using an Omega RD-106 multi-channel recorder with two thermocouples per animal, one taped near the rear of the carapace and the other on the thigh of the hind leg in contact with the skin. After freezing exposure, turtles were removed from the bath and thawed at 25°C . Animals were monitored visually for the resumption of three responses: the reflex retraction of an individual limb when directly poked, the coordinated retraction of all limbs and head into the shell in response to the same poking of one limb, and voluntary locomotion. These observations were begun 30 min into the thaw and were repeated every 15 min. Following recovery, turtles were placed in a cage at room temperature with normal springtime photoperiod. Turtles were believed to have recovered fully when they locomoted and fed normally.

In early May, the turtles were air freighted to the Carleton lab for analysis of biochemical parameters related to freezing. Animals were again placed in an incubator at $4 \pm 0.3^{\circ}\text{C}$ for two weeks before use. Three control turtles (two female, one male) were killed by decapitation. A blood sample was taken from the severed neck blood vessels. Brain, liver, kidney, heart, gut (intestine + stomach), and red and white skeletal muscles were quickly dissected out and frozen in liquid nitrogen; using a team of three people, this was accomplished within 5 min for all organs and less than 2 min for heart, brain, and liver. Frozen tissues were stored at -80°C until use. Blood samples were chilled on ice, allowed to clot, then centrifuged in an Eppendorf microcentrifuge (12,000 g for 5 min); the serum was then removed and frozen at -80°C .

The other three turtles (two females, one male) were given freezing exposure at an air temperature of $-2 \pm 0.1^{\circ}\text{C}$ for 48 h. Two thermistors were attached to each turtle, one taped to the plastron and one inserted into the cloaca. Only one animal, however, retained the cloacal thermistor until the end of the experiment; with the others, this thermistor was found in contact with the tail skin at the end of the experiment. Each turtle was then put in a plastic bucket and placed in the constant temperature incubator. After 48 h of exposure, turtles were removed one at a time and killed by decapitation as above. A small amount of blood was

collected by severing the aorta and processed as above. Organs were then dissected out and frozen as above.

Measurements of serum osmolality were made using a Wescor 5100C vapor pressure osmometer. For metabolite measurements, perchloric acid extracts of organ samples (1:5 w/v) and serum (1:5 v/v) were prepared as previously described (Storey and Storey, 1984). Glucose, glucose-6-P, lactate, alanine, glycerol, and sorbitol were measured by coupled enzyme assays (Lowry and Passonneau, 1972; Bergmeyer, 1974). For amino acid analysis, organ samples were homogenized 1:10 in 0.5% w/v sulphosalicylic acid, centrifuged at 25,000 g for 10 min, and the supernatant removed and neutralized with KOH. Amino acids in the supernatant were quantified using a Waters HPLC with precolumn derivatization using orthophthalaldehyde.

To measure organ glycogen phosphorylase activities, tissue samples were homogenized 1:4 w/v in ice-cold 20 mM imidazole buffer, pH 7.2 containing 15 mM 2-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, and 50 mM NaF with 0.1 mM phenylmethylsulfonyl fluoride added immediately prior to homogenization. The homogenate was left to settle for 30 min on ice, and then activity was assayed spectrophotometrically at 21°C. Assay conditions for glycogen phosphorylase were 50 mM imidazole buffer pH 7.2, 50 mM potassium phosphate, 10 mM MgCl_2 , 0.4 mM NADP, 4 mg/ml glycogen, 10 μM glucose-1,6-bisphosphate, 0.5 U/ml phosphoglucomutase, and 0.5 U/ml glucose-6-phosphate dehydrogenase in the absence vs presence of 1 mM AMP for the determination of phosphorylase *a* vs total (*a* + *b*) phosphorylase, respectively. Data are reported as means \pm SEM; statistical comparisons used unpaired *t*-tests or, for the comparison of multiple means, ANOVA plus Scheffé tests.

RESULTS

During the first freezing exposure experiment, turtles cooled in the -2°C bath showed a slow decrease in body surface temperature to a mean supercooling point of $-1.6^\circ\text{C} \pm 0.06^\circ\text{C}$ SEM ($n = 3$). The mean time to the onset of freezing was 4.0 ± 0.6 h ($n = 3$); therefore, the mean time of actual freezing was 44 h (out of 48 h total exposure at -2°C). After freezing, five turtles were used for an analysis of the recovery of motor functions during thawing in air at room temperature (25°C). The mean times (\pm SEM, $n = 5$) for the resumption of these three responses were 1.2 ± 0.22 h for the reflex response to poking an individual limb, 2.0 ± 0.18

h for a coordinated retraction of the limbs and head in response to poking one limb, and 2.7 ± 0.18 h for voluntary locomotion, respectively (each significantly different from the others by the repeated measures ANOVA/Scheffé test, $P < 0.025$). All animals recovered fully from this freezing exposure and appeared completely normal within 1 day and remained so until used again for the second freezing exposure.

For metabolic studies, these same animals (4–5 weeks later) were again given freezing exposure for 48 h in a incubator with an air temperature of -2°C . In this trial, we found no pronounced supercooling before freezing began; body temperatures (T_b) fell over 5–7 h to -0.5 – -0.8°C and then stabilized. All turtles pulled their body extremities into their hinged shells as T_b fell below 0°C , but the tip of the tail of one and a foot of another protruded. A quick examination of these after 7 h confirmed that freezing had begun within this time. After 48 h of exposure to -2°C (representing at least 40–42 h of freezing), the mean body surface temperature for each animal registered by the thermistors was $-0.81 \pm 0.07^\circ\text{C}$ ($n = 3$); being higher than the air temperature of the incubator, this indicated that ice formation was still continuing in the animals. During dissection, we observed ice in all three turtles; ice surrounded the brain within the skull and was found around pectoral, pelvic, and neck muscles and throughout the body cavity, particularly in the dorsal area. No heartbeat was observed in any of the animals, and the lungs were a mass of icy tissue.

Mean (\pm SEM, $n = 3$) serum osmolality was 244 ± 4.3 mOsmol/l for control turtles and significantly higher 315 ± 15.4 mOsmol/l for frozen turtles (unpaired *t*-test, $P < 0.01$); based on these values, the calculated freezing points of box turtle serum should be -0.45°C and -0.59°C , respectively. The levels of some common cryoprotectants were assayed in serum and seven organs; Table 1 shows the effect of freezing on glucose, glucose-6-phosphate, lactate, and alanine contents. Freezing resulted in a sharp increase in the amounts of glucose in all organs. Liver glucose rose by 22.3-fold to a mean 20.7 $\mu\text{mol/g}$ wet weight; mean glucose levels in heart (19.1 $\mu\text{mol/g}$) and blood (18.3 $\mu\text{mol/ml}$) were similar. Glucose also rose by 4–10-fold in other organs to final concentrations of 6.6–8.6 $\mu\text{mol/g}$. Glucose-6-phosphate rose by 62-fold in liver, clearly indicating an activation of glycogenolysis in this organ. Glucose-6-P also increased significantly in heart and gut during freezing. Freezing exposure resulted in an accumulation of lactate in serum and four tissues (liver, heart,

brain, gut), with amounts ranging up to 7.66 $\mu\text{mol/g}$ in brain. Skeletal muscle lactate levels were high and variable in both control and frozen individuals. Lactate levels did not change in kidney during freezing, but this organ was the only one to show an increase in alanine, an alternative product of glycolytic metabolism. Levels of two common cryoprotectants, glycerol and sorbitol, were measured in serum and liver of box turtles, but neither changed significantly as a result of freezing exposure. Overall mean values for the two polyols were 1.29 ± 0.18 and 1.82 ± 0.46 $\mu\text{mol/g}$ for glycerol and 0.11 ± 0.06 and 0.29 ± 0.22 $\mu\text{mol/g}$ for sorbitol in serum and liver, respectively.

The levels of amino acids in liver and red muscle of control and freezing-exposed box turtles are shown in Table 2. Freezing exposure had no effect on the organ concentrations of any individual amino acid. The peak of taurine + alanine formed a major part of the total amino acid pool in both organs, as also occurs in painted turtle and pond slider hatchlings (Storey et al., 1988; Churchill and Storey, 1991, 1992b). When compared with values for alanine measured enzymatically (Table 1), it is apparent that alanine accounts for virtually all of this peak in skeletal muscle but for 40% or less of the total peak in liver. Other principal contributors to the amino acid pool were aspartate, glutamate, threonine, tyrosine, and methionine. An analysis of brain amino acids ($n = 2$ control and frozen) similarly showed no apparent differences in the levels of any amino acid in control vs frozen tissue (data not shown).

Typically, liver glycogen is the substrate for cryoprotectant synthesis by freeze-tolerant vertebrates, and the effect of freezing on the activity of glycogen phosphorylase is a good indicator of the activation of cryoprotectant production. In box turtle liver, an activation of phosphorylase in response to freezing was readily apparent; the percentage of phosphorylase in the active *a* form rose significantly by 2.7-fold during freezing exposure from $21.3 \pm 2.7\%$ in controls to $57.5 \pm 5.3\%$ in frozen animals (Table 3). Phosphorylase in other organs, however, was unaffected by freezing exposure, with neither the maximal activity of the enzyme nor the percent *a* altered during freezing. The maximal activity of phosphorylase was highest in skeletal muscles, intermediate in liver, heart and brain, and lowest in kidney and gut.

DISCUSSION

Box turtles show a good ability to endure freezing. Costanzo and Claussen (1990) showed

TABLE 1. LEVELS OF SOME METABOLITES IN SERUM AND ORGANS OF CONTROL VS FROZEN BOX TURTLES.

	Glucose		Glucose-6-P		Lactate		Alanine	
	Control	Frozen	Control	Frozen	Control	Frozen	Control	Frozen
Serum*	1.72 \pm 0.26	18.3 \pm 2.81 ^b	—	—	3.03 \pm 1.05	7.19 \pm 0.49 ^c	0.52 \pm 0.02	0.38 \pm 0.07
Liver	0.93 \pm 0.30	20.7 \pm 4.48 ^b	0.02 \pm 0.01	1.25 \pm 0.39 ^b	1.42 \pm 0.16	3.80 \pm 0.45 ^b	0.87 \pm 0.08	1.04 \pm 0.06
Skeletal muscle								
White	2.15 \pm 0.79	8.64 \pm 0.81 ^b	2.03 \pm 0.44	2.31 \pm 0.20	9.78 \pm 1.66	5.79 \pm 2.04	1.66 \pm 0.33	2.27 \pm 0.89
Red	1.88 \pm 0.79	8.24 \pm 0.20 ^b	1.11 \pm 0.51	2.06 \pm 0.22	7.19 \pm 4.52	3.62 \pm 0.42	2.24 \pm 0.37	2.99 \pm 0.90
Heart	1.58 \pm 0.52	19.1 \pm 3.49 ^b	0.39 \pm 0.06	0.88 \pm 0.09 ^b	0.79 \pm 0.40	2.79 \pm 0.76 ^c	1.42 \pm 0.21	1.13 \pm 0.21
Kidney	0.98 \pm 0.24	7.48 \pm 2.67 ^c	0.18 \pm 0.06	0.26 \pm 0.13	3.99 \pm 1.29	3.92 \pm 1.10	1.57 \pm 0.16	2.21 \pm 0.24 ^c
Brain	0.69 \pm 0.21	6.62 \pm 0.55 ^b	0.11 \pm 0.05	0.23 \pm 0.04	5.49 \pm 0.09	7.66 \pm 0.79 ^b	0.54 \pm 0.09	0.63 \pm 0.07
Gut	0.83 \pm 0.28	7.10 \pm 1.17 ^b	0.14 \pm 0.02	0.33 \pm 0.08 ^c	2.96 \pm 0.42	7.38 \pm 0.79 ^b	0.59 \pm 0.08	0.38 \pm 0.12

Data are means \pm SEM, $n = 3$. Gut combines stomach and intestines.

* Values for serum are in $\mu\text{mol/ml}$.

^b Significantly greater than the corresponding control value by the one-tailed, unpaired *t*-test, $P < 0.01$; ^c $P < 0.05$.

TABLE 2. LEVELS OF SOME AMINO ACIDS IN LIVER AND RED SKELETAL MUSCLE OF BOX TURTLES.

	Liver		Red muscle	
	Control	Frozen	Control	Frozen
	nmol/g wet weight			
Aspartate	205 ± 28	243 ± 45	143 ± 18	141 ± 22
Glutamate	510 ± 20	651 ± 75	1443 ± 69	1427 ± 253
Serine	96 ± 28	192 ± 57	411 ± 109	442 ± 20
Glutamine	64 ± 10	79 ± 15	48 ± 6	72 ± 16
Histidine	9 ± 2	7 ± 1	15 ± 4	31 ± 10
Threonine	375 ± 55	360 ± 71	426 ± 75	292 ± 78
Arginine	68 ± 5	63 ± 4	38 ± 5	44 ± 4
Taurine + Alanine	2805 ± 650	2617 ± 103	2464 ± 812	2719 ± 822
Tyrosine	422 ± 40	556 ± 125	262 ± 57	209 ± 5
Methionine	160 ± 44	109 ± 9	1052 ± 281	921 ± 124
Phenylalanine	81 ± 1	63 ± 11	255 ± 74	197 ± 23
Isoleucine	51 ± 8	60 ± 11	42 ± 15	44 ± 13
Leucine	31 ± 7	25 ± 8	19 ± 3	37 ± 15
Lysine	45 ± 8	35 ± 6	156 ± 45	155 ± 43
Total	5046 ± 657	5441 ± 259	7291 ± 1771	8677 ± 1160

Data are means ± SEM, n = 3 for liver and muscle. Taurine and alanine co-eluted on the column used.

that this species can endure freezing for 73 h to an internal body temperature as low as -3.6 C and an ice content as high as 58% of total body water. This exceeds the minimum test for physiologically relevant freeze tolerance suggested by Storey and Storey (1992). In the present study, box turtles recovered after freezing at an environmental temperature of -2 C for approximately 44 h. However, the body temperature of our turtles did not equilibrate with the air temperature, indicating that they did not reach equilibrium ice content; indeed, Costanzo and Claussen (1990) showed that box turtles accumulate ice very slowly. We cannot state how much ice formed in these turtles without calorimetric data, but based on the time

course of ice formation (Costanzo and Claussen, 1990) and measurements of body surface temperatures at the conclusion of the freezing episodes, we speculate that ice content would be no more than 25% of total body water.

During prolonged freezing, the vital signs and reflex responses of freeze-tolerant animals are gradually lost and become undetectable in the fully frozen animal (Layne et al., 1989). These resume, however, during thawing; in the wood frog, the sequence is heartbeat and blood flow first, followed by breathing, and then hindleg muscle reflexes (Layne and First, 1991). The present results show a sequence in the resumption of muscle response and muscle coordination after freezing. The reflex response of a

TABLE 3. GLYCOGEN PHOSPHORYLASE IN BOX TURTLE ORGANS: MAXIMAL ENZYME ACTIVITIES AND PERCENTAGE OF ENZYME ACTIVITY IN THE ACTIVE *a* FORM.

	Maximal activity Units/g wet weight		Percent <i>a</i>	
	Control	Frozen	Control	Frozen
Liver	4.3 ± 1.4	5.3 ± 1.2	21.3 ± 2.7	57.5 ± 5.3*
Skeletal muscle				
White	9.6 ± 0.7	7.5 ± 0.8	55.9 ± 7.7	68.7 ± 4.0
Red	6.1 ± 1.0	8.1 ± 1.5	58.4 ± 3.3	66.8 ± 2.5
Heart	2.8 ± 0.6	3.9 ± 0.5	27.1 ± 5.3	29.4 ± 2.1
Kidney	1.0 ± 0.1	0.8 ± 0.1	35.5 ± 4.5	32.0 ± 8.2
Brain	3.8 ± 0.3	3.6 ± 0.2	76.0 ± 2.8	73.5 ± 2.2
Gut	1.5 ± 0.1	1.6 ± 0.1	24.6 ± 5.0	34.1 ± 4.3

Data are means ± SEM, n = 3.

* Significantly different from the corresponding control value by the unpaired t-test, *P* < 0.01.

single limb to poking reappeared 0.8 h earlier (on average) than did the more complicated, coordinated reflex response that retracted all limbs and the head in response to the same stimulus. Finally, voluntary muscle movement reappeared after a further 0.7 h of thawing. This indicates that, even though individual muscles can quickly regain their ability to respond to stimuli after thawing, the ability to coordinate muscle movements via the central nervous system remains impaired for a longer time.

Several factors complicate any assessment of the dynamics of postfreeze recovery. Freeze duration, freeze temperature, and thaw rate may influence the recovery process. Prolonged freezing and low freezing temperature likely lengthen the time required for recovery because there is a greater disturbance of normal metabolism and more ice is present in body tissues; conversely, fast thawing accelerates recovery (Layne and First, 1991). The thaw temperature used here (25 °C) greatly exceeds the temperatures likely encountered by box turtles during natural thaws (these would probably be near 0 °C), and motor functions undoubtedly returned faster in our experiments than they would in nature. Experiments on freeze-tolerant frogs, however, indicate that rapid thawing in air at room temperature compromises neither survival nor the order with which functions return (Layne and First, 1991). Therefore, the recovery times reported here for box turtles apply only to the specific set of freeze/thaw conditions used in these experiments.

As a metabolic response to freezing, all organs of the box turtles showed significant accumulations of glucose. Liver, heart, and serum (blood collected from the heart) had the highest amounts, 19–21 $\mu\text{mol/g}$ wet weight for tissues and 18 $\mu\text{mol/ml}$ for serum, representing a 10–22-fold increase in glucose content. Other internal organs as well as skeletal muscles showed lower net accumulations of 5.9–6.5 $\mu\text{mol/g}$ glucose. This gradation in glucose content among different organs is also seen in freeze-tolerant frogs and results because of a progressive restriction of blood flow from the periphery inward as the freezing front moves inward, thereby limiting the distribution of glucose from the liver to other organs (Storey, 1990). One caution must be stated, however, and that is that some portion of the increase in organ glucose levels (measured on a per gram wet weight basis) may actually be a result of water loss from organs during freezing. However, if organ dehydration was significant, we would expect to see a general increase in the levels of all measured metabolites (amino acids, lactate, glucose-

6-phosphate, polyols) as well as phosphorylase activities in organs of freezing-exposed, compared with control, turtles. This was not the case.

The evidence in the present study indicates that liver is the site of freezing-induced glucose production in the box turtle, as is also the case in freeze-tolerant frogs (Storey, 1990). This evidence includes (1) liver showed the highest-fold increase in glucose content; (2) liver showed a massive, 62.5-fold, increase in glucose-6-phosphate, the precursor of glucose synthesis, during freezing exposure; and (3) liver was the only organ that showed an activation of glycogen phosphorylase during freezing, the percentage of the active *a* form rising by 2.7-fold from 21% in controls to 57.5% in frozen animals.

The serum osmolality of box turtles increased by a mean 71 mOsmol/l as a result of freezing. Two effects can contribute to this—the concentrating effect on plasma of the net water loss into ice and the addition of cryoprotectants to body fluids. The sum of increases in glucose and lactate in serum of freezing-exposed turtles was 20.7 mmol/l, and this could account for 29% of the net increase in plasma osmolality. The remainder of the osmolality increase may be the result of a water loss into extracellular ice because we found no indication of the addition of other common low molecular weight cryoprotectants (e.g., glycerol, sorbitol) to the serum. Furthermore, levels of putative cryoprotectants were also low in all organs analyzed, and there were no changes in the amino acid content of liver and muscle as a result of freezing. This does not completely rule out the possible presence of unidentified cryoprotectants, but even if such compounds were present, their levels would have to be fairly low. Thus, it appears that box turtles can survive freezing without the need to produce high concentrations of cryoprotectants. This may be a general occurrence for reptiles because neither hatchling painted turtles, pond slider turtles, nor garter snakes produce high levels of cryoprotectants during freezing (Churchill and Storey, 1991, 1992b, 1992c). Because both frogs and turtles can tolerate ice in amounts of 50–65% of total body water (Storey, 1990), it will prove instructive to determine whether the two groups show different strategies of cell volume regulation, tolerances of cellular dehydration, or patterns of intra- vs extraorgan sequestration of ice.

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Evidence for a Monophyletic Gobiinae

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Sixty-nine different oculoscapular canal pore configurations are described for 129 gobioid genera. A modified oculoscapular canal structure consisting of a single median anterior interorbital pore and a single terminal pair of nasal pores located near the posterior nares is recognized as synapomorphic for the gobioid subfamily Gobiinae. Some member genera and species have lost the anterior interorbital pore or the oculoscapular canal itself but are included based on relationships determined by other characters. The apomorphic feature is concordant with other diagnostic nonpolarized characters. Gobiines have one epural and most species have a 3-22110 first dorsal-fin pterygiophore insertion pattern, two prehemal pterygiophores, and 26 or 27 vertebrae. A new classification of gobioid fishes is proposed to accommodate results presented here and to integrate information obtained from the competing two-family and six-family classifications now in use.

THE limits of family-level taxa within the Gobioidae are poorly defined and a matter of much debate. Harrison (1989) recently summarized the major arguments, including the

problem of using different, and often conflicting, character suites on which to base gobioid classifications. I will not review specific arguments of the conflict here, readers are referred