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Classification Modeling of Physiological Stages in Captive Balkan Whip Snakes Using Blood Biochemistry Parameters

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Abstract.—We studied captive Balkan Whip Snakes (*Hierophis gemonensis*) to determine blood biochemical parameters that are useful indicators of physiological status during different periods of the biological cycle, including pre- and posthibernation, hibernation, sexual activity, and normal activity. In addition to classic statistical analyses, six machine-learning methods using 10 times 10-fold cross-validation evaluation were used to determine the best classification model for the blood biochemistry data. Results of the machine-learning models indicated that using three of the blood biochemistry variables simultaneously—urea, glucose, and lactate dehydrogenase—is enough to discriminate accurately between different physiological conditions during the biological cycle. This approach clarifies the role and importance of physiological processes, which show diversity of functional characteristics of various biochemical parameters in ecological relation to snakes held under laboratory conditions mimicking natural environmental changes.

The Balkan Whip Snake (*Hierophis gemonensis*) is found in Croatia from sea level to 800 m but is most abundant at low altitudes. It is a slender snake with a well-defined head, smooth scales, and prominent eyes with round pupils. Adults can reach over 130 cm in length but are usually shorter. The snake is olive gray, gray-brown, or yellowish-brown, with dark spots on its foreparts, which are often divided by irregular light streaks. Its belly is yellowish or whitish, with dark blotches on the sides of the neck. It usually has 19, sometimes 17, dorsal scales, 160–187 belly scales, and 80–116 pairs of subcaudal scales. Small white spots are often present on the edges of some dorsal scales. Balkan Whip Snakes inhabit dry rocky places, bushy terrain, vineyards, open woods, and low macchia (a Mediterranean shrubland), as well as edges of roads (Lymberakis and Ajtic, 2005). Although it is considered harmless to humans, the Balkan Whip Snake may bite when handled. It feeds mainly on large insects, reptiles, small mammals, and nestling birds. Females lay 4–10 oval eggs, 25–40 $\times$ 15–20 mm in size. Balkan Whip Snakes become dormant during the winter and show seasonal adaptations similar to those found in hibernating mammals, with a few hibernation characteristics that are common among ectotherms (Strunjak-Perovic et al., 2010).

Blood biochemistry reflects physical and chemical changes in organisms, indicating general metabolic and physiological status. However, studies of reptilian blood have been performed for few species to date (Olufemi and Adeyinka, 1994). In general, the composition of blood plasma varies widely in reptiles, and there are no baseline measurements of blood biochemistry for most species, including the Balkan Whip Snake, that can be used as reference values to describe physiological condition (Saint Girons, 1970). Pienaar (1962) and Duguy (1970) identified age, sex, reproductive status, nutrition, and season as factors influencing hematological values in reptiles. Dutton and Taylor (2003) used morphometric measurements, hematology, and blood chemistry to evaluate the health status of adult viperid snakes, and found pre- and posthibernation differences only in uric acid.

Classic statistical techniques have frequently been used to analyze blood parameters. In our experience, however, approaches based on machine learning methods provide better classification models and greater insight into the data. Machine learning is a scientific discipline that is concerned with the design and development of algorithms that allow computers to evolve behaviors based on empirical data. A major focus of machine-learning research is to develop the ability in machines to learn to recognize complex patterns and make intelligent decisions based on data. Machine-learning methods do not necessarily introduce prior assumptions about sample distributions or relationships between variables (Mitchell, 1997; Duda et al., 2000). Moreover, machine-learning methods have an inherent ability to uncover complex patterns in the data. Their use in analyzing data sets from diverse biological sources is steadily increasing (McQueen et al., 1995; Mastrorillo et al., 1997; Dzeroski and Drumm, 2003). For example, decision-tree algorithm C4.5 (Quinlan, 1992), implemented within the WEKA data mining suite (Witten and Frank, 1999), was used to generate classification models, and PARF (Topic and Smuc, 2004), a parallel implementation of the well-known Random Forest algorithm (Breiman, 2001, Topic et al., 2005), was used to develop predictive and descriptive models, for differentiation of various fish groups.

The present study tested the hypothesis that blood biochemical parameters could be used as tools for evaluating the physiological condition of Balkan Whip Snakes and the influence of environmental factors on snake metabolism during biological cycles (pre- and posthibernation, hibernation, reproduction, and normal activity). Machine learning techniques were used to develop classification models and determine individual importance, mutual interactions, and reliability of particular blood biochemistry parameters. The results also contribute to the general understanding of this relatively unknown species.

Materials and Methods

Animals.—Fourteen specimens of *H. gemonensis* were collected on the island of Vis, Croatia. The specimens were marked, measured, and weighed, and their sex was determined (females $N = 7$, males $N = 7$). They were held individually in plastic terrariums (45 $\times$ 25 $\times$ 17 cm). Hiding places were provided in the form of clay flower pots placed upside down. Paper towels were used as substrate. Snakes were fed every two weeks with one subadult laboratory mouse; water was given ad libitum. During the active period, daytime temperature was 28–30°C, and nighttime temperature was 25–

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TABLE 1. Blood biochemistry parameters ($\bar{x} \pm SD$) determined for captive Balkan Whip Snakes *(Hierophis gemonensis)* during different periods.

Parameter	Posthibernation	Sexual activity	Normal activity (July)	Normal activity (September)	Prehibernation	Hibernation
TRIG mM	0.037 ± 0.092	0.061 ± 0.132	0.067 ± 0.157	0.311 ± 0.326	0.210 ± 0.093	0.082 ± 0.073
CHOL mM	12.8 ± 2.27	11.7 ± 3.63	13.9 ± 5.22	13.1 ± 0.970	13.1 ± 1.11	13.4 ± 1.50
GLU mM	0.928 ± 0.346	1.99 ± 1.17	2.34 ± 0.795	4.05 ± 1.86	3.71 ± 1.44	0.627 ± 0.214
TP g L^{-1}	51.7 ± 12.6	39.7 ± 5.17	40.1 ± 8.84	48.9 ± 8.88	52.4 ± 6.26	51.8 ± 3.43
BUN mM	0.623 ± 0.043	0.776 ± 0.265	1.31 ± 0.309	0.658 ± 0.337	0.275 ± 0.105	0.340 ± 0.051
AST IU	0.384 ± 0.960	7.54 ± 9.12	11.2 ± 19.3	2.91 ± 9.65	7.00 ± 17.0	243 ± 289
CK IU	845 ± 679	482 ± 345	606 ± 668	255 ± 315	330 ± 356	2,036 ± 890
LDH IU	337 ± 280	481 ± 484	513 ± 465	220 ± 227	273 ± 230	1,472 ± 791
ALKP IU	127 ± 79.1	125 ± 233	66.0 ± 17.0	108 ± 62.4	120 ± 49.5	115 ± 50.6
PHOS mM	0.751 ± 0.163	0.963 ± 0.292	0.73 ± 0.222	0.692 ± 0.150	0.710 ± 0.126	0.857 ± 0.107
Ca mM	3.30 ± 0.312	2.85 ± 0.207	3.05 ± 0.282	3.33 ± 0.199	3.30 ± 0.153	3.42 ± 0.125

27°C. The room in which snakes were held had a very large glass window, allowing natural light to penetrate and expose snakes to seasonal differences in photoregime. The snakes hibernated from the end of December until the beginning of March. For three weeks pre- and posthibernation, the snakes were held in the same terrarium with the same light conditions as in the active phase but with a different temperature regime oscillating from 21°C during the day to 13°C during the night. Snakes were not fed during this period, mimicking ceasing of feeding behavior prior to and immediately after hibernation. Hibernating animals were kept in small (30 × 20 × 8 cm) plastic terrariums in 6–8°C at constant darkness. Small terrariums were filled with paper towels and water bowls to prevent dehydration.

Blood Collection and Processing.—Snakes were fasted for two weeks before taking blood samples. Blood was collected from the caudal vein of each snake during January (hibernation), March (posthibernation), May (sexual activity), July and September (normal activity), and November (prehibernation). The snakes were not anesthetized during blood collection. The volume of blood withdrawn varied individually but ranged from 0.7 to 1.0 mL. The blood was promptly centrifuged (15,800 × G for 95 sec). The blood plasma of the Balkan Whip Snake is a greenish-yellow to light orange fluid and makes up to 60–80% of blood volume. Plasma samples were analyzed by the colorimetric method used previously for fish blood biochemistry (Edsall, 1999; Coz–Rakovac et al., 2005, 2008a,b, 2009; Topic Popovic et al., 2006), using a VetTest 8008 biochemical analyzer (Idexx Laboratories, Inc., Westbrook, ME). The coefficients of variation for the VetTest 8008 range from 1.2% to 5.1% (Tschudi, 1995). In the present study, the measured biochemical parameters were total protein (TP), glucose (GLU), cholesterol (CHOL), urea (BUN), plasma triglycerides (TRIG), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK), alkaline phosphatase (ALKP), calcium (Ca), and phosphorus (P). They were selected as suitable markers for environmental influences, stress effects, metabolism, condition, and health, AST and ALKP being the most important aminotransferases relating to amino acid metabolism, GLU, TRIG, and CHOL indicating energy status, BUN reflecting renal function, LDH and CK as markers of tissue breakdown, TP indicating infection, with Ca and P as markers of electrolyte disturbances.

Statistical Analyses and Modeling.—All data were first assessed by conventional statistical methods, using SigmaStat software package version 2001, 1986–94 (Jandel Corp., San Rafael CA). Basic descriptive statistics included measures of central tendency and dispersion (mean and standard deviation, $\bar{x} \pm SD$). The nonparametric Kruskal-Wallis test was used to determine statistical significance of differences (at $P \leq 0.05$) between mean values from different sampling times. Two state-of-the-art machine learning methods were used to produce classification models from the plasma biochemical parameters: the C4.5 decision tree algorithm in the WEKA package (Witten and Frank, 1999); and PARF (Topic and Smuc, 2004), a local implementation of the Random Forests algorithm (Breiman, 2001). The results were compared to the results of the classic statistical approach. The process of finding the best classification model provided an assessment of how important the individual blood biochemistry parameters are for the classification of, or for the discrimination between, different times in the annual cycle.

RESULTS

The snake samples included females and males; however, classic statistical analyses showed no significant differences in variables between sexes. Therefore, all 14 snakes were treated as a single group. The measured range of each blood biochemistry parameter was compared to the normal range for reptiles published in the VetTest 8008 database and in other previous studies. Values for TP, GLU, PHOS, and Ca were generally within the normal range. AST, CK, and LDH values were within the normal range but were higher during hibernation. ALKP values were within the normal range but were lower during normal activity. TRIG levels were only within the normal range during normal activity and otherwise were low. CHOL values were generally higher, and prehibernation BUN values were lower than those reported for other reptile species (Dutton and Taylor, 2003).

All of the measured metabolites and enzymes exhibited variation during the biological cycle (Table 1). TRIG was

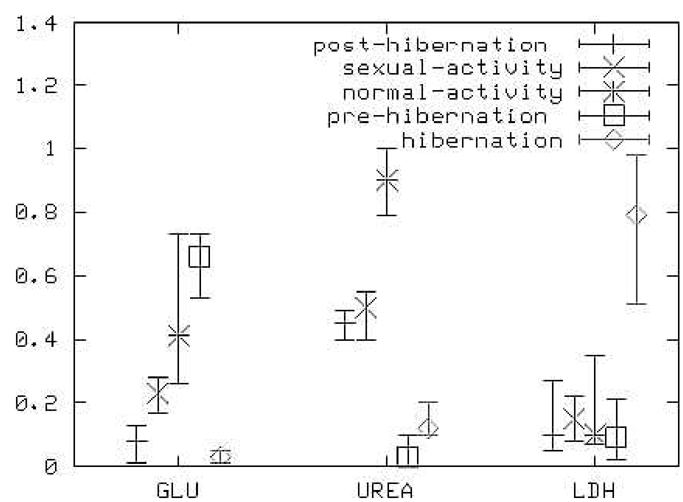

FIG. 1. PARF prototypes shown using box-plots (three most important variables: BUN [UREA], GLU, and LDH). Prototypes inferred from PARF classification results depict distinct mean value and range levels in captive Balkan Whip Snakes during different measurement periods. Data were normalized to the interval 0–1 to allow direct/parallel comparison over variables.

TABLE 2. Raw importances and *Z*-scores for individual blood biochemistry parameters as obtained from PARF analysis.

Parameter	Raw importance	Z-Score
BUN	19.57	32.74
GLU	13.92	29.70
LDH	3.94	14.17
TRIG	3.71	12.07
Ca	2.76	10.00
TP	2.24	8.80
CK	1.29	5.85
PHOS	1.21	5.69
ALKP	0.66	3.27

significantly lower during posthibernation and sexual activity than at the end of normal activity and during prehibernation. CHOL values were higher at the beginning of normal activity than during the rest of the year, but the differences were not significant. GLU was low during hibernation, then increased significantly during posthibernation, and remained high through normal activity and prehibernation. TP concentrations were lowest during sexual activity and rose gradually during normal activity, reaching a significantly higher level that lasted from prehibernation through posthibernation. BUN was highest during normal activity and sexual activity and decreased significantly during prehibernation and hibernation period. AST and LDH showed little variation through the active periods but were significantly elevated during hibernation. CK measurements during hibernation were omitted from the analysis, because of a low number of readings, but remaining data showed no significant variations. ALKP was significantly lower at the beginning of normal activity than during the rest of year. PHOS showed no significant temporal variation. Ca was significantly lower during sexual activity than during the other periods.

Data quality was checked prior to further analysis with machine learning algorithms. CHOL and ALT data were removed completely, because of a larger number of missing values. The prototypes generated with PARF provided particularly useful insights into this application of machine-learning methods. The PARF algorithm generated a forest of decision trees from the measured variables (Fig. 1) and then used a detailed distance matrix to determine distinct clusters or prototypes (one for each biological stage). The variable importance method in PARF was then used to assess the individual importance of each parameter with respect to their discrimination power in the classification models. The most important variables were BUN and GLU followed by LDH (Table 2). Models were built incrementally, by adding parameters in order of decreasing importance, and were then evaluated with six machine learning methods, using 10 times 10-fold cross-validation. The three top-ranked parameters provided a highly reliable classification model, with up to 81% overall accuracy (Table 3). The PARF algorithm gave the best performance (Table 4), although only marginally better than the SVM-RBF. The decision-tree model (Fig. 2) confirmed the PARF results (Table 5) but had lower accuracy. With the latter model, it was difficult to discriminate between post-hibernation and sexual activity periods or between normal activity and prehibernation periods.

DISCUSSION

The 14 Balkan Whip Snakes used in the present study were held under environmental and nutritional conditions that simulated the natural seasonal cycle in their native habitat. Therefore, the observed changes in plasma biochemistry are assumed to be similar to those occurring in wild snakes, for which data on seasonal changes in activity and biochemistry are scarce.

The blood biochemistry parameters measured showed different patterns through the annual cycle of the Balkan Whip Snake that can be understood in the context of the snake's life history. For example, Ca levels were low during sexual activity, whereas PHOS was slightly elevated. Previous studies concluded that Ca and PHOS levels in reptiles are under endocrine control and linked to internal physiological changes, rather than to environmental conditions (Alcobendas et al., 1992). Other reptile species showed increased plasma Ca and PHOS levels during reproduction (Alcobendas et al., 1992; Allender et al., 2006), because of higher estrogen concentrations, which induce synthesis of the Ca-binding protein vitelin (Dessauer, 1970). Lower Ca values during sexual activity in Balkan Whip Snakes may have been related to eggshell production or were a result of the methodology, which measured only free Ca ions in plasma but not Ca bound in proteins.

Plasma proteins in reptiles are of vital importance in immune and coagulation functions, transport of ions and hydrophobic molecules, enzymatic activity, and regulation of plasma osmolality (Dessauer, 1970). The Balkan Whip Snakes in the present study exhibited higher TP values from prehibernation through posthibernation, even though they were not fed for much in these periods. Plasma TP is affected by nutrition in reptiles (Dessauer, 1970). Elevated TP may have been related to a lower plasma volume during the period of lower culture temperatures, a phenomenon observed in other reptiles (Dessauer, 1970; Wojtaszek, 1992). Alternatively, a metabolic disorder, such as insufficient fat stores, may have necessitated unusually high catabolism of protein reserves during hibernation.

In general, lipid concentrations in reptiles seem to be quite conservative and do not change markedly even during prolonged starvation (Dessauer, 1970). However, plasma lipids increase during spring in some species, probably in connection with the estrus cycle. In the Balkan Whip Snakes, CHOL values were stable through the year, but TRIG concentrations were higher at the end of normal activity and during prehibernation, possibly reflecting physiological preparation for hibernation period. Similar plasma lipids regime were found in the annual cycle of the Egyptian cobra, *Naja haje haje* (El-Deib, 2005). Elevated plasma TRIG could reflect synthesis in the liver and circulation in the blood to other parts of the body for production of fat reserves used as a secondary energetic source during hibernation.

The model used in the present study identified BUN as one of the most important discriminating factors of physiological changes in the Balkan Whip Snakes. BUN had lowest values during prehibernation and hibernation and highest values during normal activity. Fluctuations in blood urea concentrations have also been observed in other reptiles. For example, viviparous snakes *Thamnophis strialis* (Dessauer, 1970) and *Acrochordus granulatus* (Nitinkumar and Padgaonkar, 1998)

TABLE 3. Mean (± SD) quality measures of models generated using different machine learning methods with the three most important blood biochemistry parameters (BUN, GLU, and LDH). Models were evaluated using 10 times 10-fold cross-validation.

Method	NB	C4.5	RF	SVM (RBF)	LR	MLP
Accuracy (%)	64.5 (14.6)	74.0 (13.3)	81.2 (12.1)	71.7 (10.7)	71.2 (14.0)	68.4 (15.3)
Kappa (%)	0.57 (0.17)	0.68 (0.16)	0.77 (0.15)	0.65 (0.13)	0.65 (0.17)	0.62 (0.18)

TABLE 4. Confusion matrix of the model generated with the PARF algorithm (accuracy = 81.9%).

	Annotation by the model				
Real annotation	Posthibernation	Sexual activity	Normal activity	Prehibernation	Hibernation
Posthibernation	13	1	0	0	0
Sexual activity	1	8	3	0	0
Normal activity	0	2	19	3	0
Prehibernation	0	0	2	9	1
Hibernation	0	0	0	0	10

exhibited increases during pregnancy, and the turtle *Testudo hermanni* had high levels of urea during spring at the end of its inactive period. Elevated BUN during normal activity may reflect greater energy requirements during this period. Elevated urea can indicate starvation (Dessauer, 1970; Dutton and Taylor 2003; Campbell, 2004), and although the captive snakes were fed on a regular basis, the rations may not have been sufficient to satisfy the rising energy demands. Alternatively, a higher metabolic rate may have resulted in greater protein degradation, leading to higher BUN, or renal activity may have been greater during the active period.

GLU was another blood chemistry parameter identified by our model as an important indicator of annual changes in physiology. In reptiles, GLU varies in response to external environmental fluctuations and reflects the physiological state of the animal (Campbell, 2004). However, different species exhibit different patterns in response to the same environmental changes. In the captive Balkan Whip Snakes, GLU was lowest during hibernation and posthibernation and highest at the end of normal activity and during prehibernation. Similar patterns were observed in *Varanus griseus* and *Alligator mississippiensis*. Seasonal changes in metabolism could account for these patterns (Dessauer, 1970; Dutton and Taylor, 2003). Increased energetic demands during autumn, when snakes in nature are searching for a place to hibernate, may have caused the elevated GLU levels, and conversion of GLU to energy reserves stored as TRIG is required. Low temperatures during hibernation and posthibernation may have reduced metabolic activity and cellular transport, accounting for low GLU during those periods.

The plasma enzymes AST, CK, and LDH showed a significant rise during hibernation in the Balkan Whip Snakes. Raised levels of these three enzymes indicate muscle lesions in vertebrates (Shmueli et al., 2000; Cuadrado et al., 2002; Campbell, 2004), suggesting that the hibernating snakes undergo muscle resorption during the inactive period to lower the demand for nutrients and oxygen. The snakes may also use muscle tissue as a source of energy through the winter months. Low ALP at the beginning of the normal activity period could reflect specialized physiology of this species.

Analysis of blood biochemistry parameters in the present study used a number of machine-learning methods, including Naïve Bayes (NB), Decision Trees (C4.5), Random Forests (PARF), Logistic Regression (LR), Support Vector Machine using Radial Basis Function kernel (SVM-RBF), and Multilayer Perceptron (MLP). The goal was to generate classification models that not only accurately represented different physiological periods in the life history of Balkan Whip Snakes but that were also clear and easily interpretable by experts. The combination of single variable analysis, multivariate analysis, and machine-learning methods provided accurate and clear results. The models showed that in captive Balkan Whip Snakes, the hibernation and posthibernation periods were easily discriminated on the basis of blood biochemistry, whereas sexual activity and prehibernation periods were somewhat more difficult to discriminate. The models used only three parameters, which reduces the cost of future monitoring of this species. Overall, the results of the present study demonstrate that the physiological status of snakes can be evaluated using standardized nonlethal and low-cost methods. Such evaluations could be used to describe metabolic changes through the life history of a species, or to detect physiological disturbances and deficiencies that might lead to a general decline in a population.

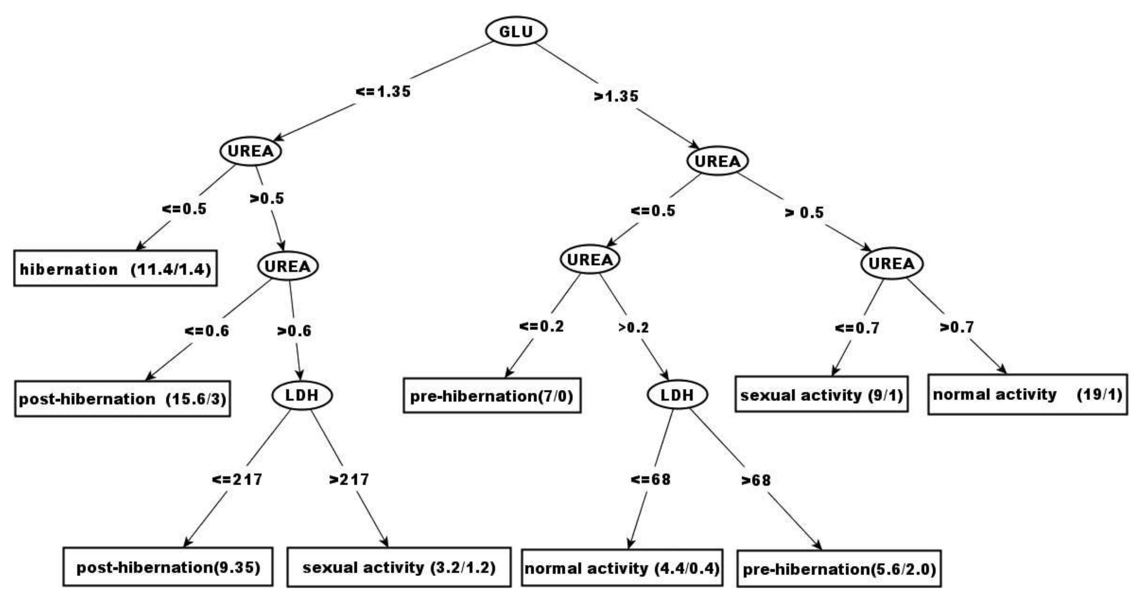

FIG. 2. Simple decision tree, based on the three most important parameters (BUN [UREA], GLU, LDH) from the PARF analysis.

TABLE 5. Confusion matrix for the decision tree model shown in Figure 2 (accuracy = 68.1%).

Real annotation	Annotation predicted by the model				
	Posthibernation	Sexual activity	Normal activity	Prehibernation	Hibernation
Posthibernation	10	3	1	0	0
Sexual activity	5	6	1	0	0
Normal activity	0	1	19	3	1
Prehibernation	0	1	6	4	1
Hibernation	0	0	0	0	10

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