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Species Characterization of Plasma Nitrite/Nitrate (NO_x) Concentration

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Experiments were designed to detect and determine differences between nitrite/nitrate concentration ([NO_x]) in plasma across 15 species selected from seven classes of vertebrates. Blood collected in syringes was placed immediately into ethylenediaminetetraacetic acid (EDTA)-containing tubes and was centrifuged. Plasma [NO_x] was determined by measurement of chemiluminescence. Across classes of vertebrates, baseline plasma [NO_x] ranged from 0.6 to 171.3 nmol/ml. Mean ± SD plasma [NO_x] was highest in a fresh-water, jawless fish (lamprey, 95.5 ± 9.1 nmol/ml) and lowest in a saltwater cartilaginous fish (skates, 1.1 ± 0.4 nmol/ml). Both amphibians tested had a wide range in plasma [NO_x], which was explained partly by temporal changes during the year. Within the mammalian class, plasma [NO_x] ranged from 3.8 to 43.2 nmol/ml. Results of this study indicate that NO_x is detectable in plasma of all classes of vertebrates and that baseline concentration varies among species.

Endogenous nitric oxide is formed from oxygen and L-arginine by nitric oxide synthase (NOS) (1). Nitric oxide is converted subsequently to nitrite and, in the presence of hemoglobin, to nitrate (2, 3), a stable metabolite that can be detected in mammalian plasma. It has been assumed that, in a healthy state, baseline plasma nitrite/nitrate concentration ([NO_x]) reflects not only nitric oxide produced principally from endothelial NOS (type III), but also nitric oxide produced from neuronal (type I) and inducible (type II) NOS isozymes. Background of endogenous nitrates from microorganisms and diet can also cause fluctuations in circulating nitrate anion (4).

The vascular endothelium, when stimulated by physical forces or autocoids, produces nitric oxide as well as other factors that promote either relaxation or contraction of vascular smooth muscle (5, 6) and changes in microvessels (7-9). Endothelium-dependent relaxation occurs in all classes of vertebrates that have been tested; however, the mediators of these responses, nitric oxide, prostanoids, and/or endothelium-derived hyperpolarizing factors, may differ among species. For example, metabolites of arachidonic acid predominate in bony fishes (10-13), whereas nitric oxide appears to be produced in amphibians, reptiles, and mammals (11, 14, 15). Further, nitric oxide is reported to decrease permeability of postcapillary venules in cats and rats (8), yet maintain basal capillary permeability in frogs (16). These differences in predominance of endothelium-derived factors may indicate various levels of NOS activity among different species and, thus, differences in nitric oxide production within the cardiovascular system.

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To date and to the authors' knowledge, there are no reports of values for plasma [NO_x] measured across species by use of the same method, which makes comparisons of baseline values difficult. The purpose of the study reported here was to determine baseline [NO_x] in the plasma of healthy animals and humans. The selected species represented the six lower classes of vertebrates (Agnatha, Chondrichthyes, Osteichthyes, Amphibia, Reptilia, and Aves) as well as common species from the seventh vertebrate class, Mammalia. Data have been reported previously in abstract form (17, 18).

Materials and Methods

Animal and human subjects. All animals were cared for and used humanely. Animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Missouri–Columbia and the Mayo Clinic and Foundation, Rochester, Minn. Approval for the project was obtained from the Institutional Review Board at the Mayo Clinic and Foundation. Written informed consent for study participants was obtained from all human subjects.

Blood samples were collected from 15 species (n = 290). Adult animal and human subjects were selected to represent the seven classes of vertebrates and included seven common species of mammals (Table 1). Among the 14 animal species were six wild-caught and eight captive-bred. The wild-caught species were lamprey (caught in Lake Michigan, obtained from U.S. Fish & Wildlife Service, Marquette, Mich.), skate (45-cm length, caught in the southwest end of Martha's Vineyard Sound, obtained from Aquatic Resources Division Marine Biological Laboratories, Woods Hole, Mass.), frog (caught in Lake Champlain, Vt., obtained from JM Hazen, Alburg, Vt.), toad (caught in Boone County, Mo. obtained from University of Missouri, Columbia, Mo.), alligator (caught in Rockefeller Wildlife Refuge, La., obtained from Department of Wildlife & Fisheries, Grand Chenier, La.), and opossum (caught in Boone County, Mo., obtained from the University of Missouri). The captive-bred species were trout (Department of Interior, U.S.G.S., Midwest

Table 1. Class, species, number (N), and diet status

Class name	Species/breed, strain, or race	N	Diet status
Agnatha	Lamprey (*Petromyzon marinus*)	10	NF 90 d
Chondrichthyes	Skate (*Raja erinacea*)	10	NF 24 h
Osteichthyes	Rainbow trout (*Oncorhynchus mykiss*)/Montana	14	NF 24 h
Amphibia	Leopard frog (*Rana pipiens*)	102	NF 3–20 d
	Toad (*Bufo woodhousei fowleri*)	5	NF 12 h
Reptilia	Alligator (*Alligator mississippiensis*)	4	NF 48 h
Aves	Chicken (*Gallus gallus*)/Cornish cross	5	ad libitum
Mammalia	Opossum (*Didelphis virginiana*)	7	NF 24 h
	Rat (*Rattus norwegicus*)/Sprague-Dawley	9	NF 12 h
	Hamster (*Mesocricetus auratus*)/Golden	3	NF 24 h
	Ferret (*Mustela putorius furo*)/European	10	NF 24 h
	Cat (*Felis catus*)/closed colony of Abyssinian cross	4	ad libitum
	Dog (*Canis familiaris*)/purpose-bred mixed-breed coon hound	61	NF 24 h
	Pig (*Sus scrofa*)/Yorkshire cross	35	NF 24 h
	Human (*Homo sapiens*)/Caucasian (10), Asian (1)	11	ad libitum

NF = not fed.

Table 2. Blood sample collection conditions for each species

Class name	Species	Site	Month	During BSC	After BSC
Agnatha	Lamprey	Heart	Jun	Sedated*	Terminally anesthetized†
Chondrichthyes	Skate	Caudal vein	Jun	Conscious	Terminally anesthetized‡
Osteichthyes	Trout	Heart	Jan, Jun	Conscious	Euthanized§
Amphibia	Frog	Heart	All	Cerebral pith	Euthanized§
	Toad	Heart	May	Cerebral pith	Euthanized§
Reptilia	Alligator	Heart	Jan, Feb	Sedated*	Terminally anesthetized†
Aves	Chicken	Jugular vein	Jan	Conscious	Non-terminal
Mammalia	Opossum	Caudal VC	Apr	Sedated***	Terminally anesthetized†
	Rat	Aorta	Jul	Sedated****	Non-terminal
	Hamster	Heart	May	Anti-mortem, unc	Euthanized#
	Ferret	Heart	Jun	Anti-mortem, unc	Euthanized#
	Cat	Cephalic vein	Apr	Sedated**	Non-terminal
	Dog	Jugular vein	Jan, Feb, Mar, Aug, Sept, Oct, Nov, Dec	Sedated††	Non-terminal
	Pig	Jugular vein	Jan, Feb, Mar, Jun, Jul, Sept, Nov	Sedated‡‡	Non-terminal
	Human	Brachial vein	Aug	Conscious	NA

*Sodium pentobarbital, 50 mg/kg, i.p.; †sodium pentobarbital, 100 mg/kg; ***ketamine, 50 mg/kg, i.p.; ‡tricaine methansulfonate; §spinal transection; ****sodium pentobarbital, 70 mg/kg i.p.; #carbon dioxide; **sodium pentobarbital, 2 to 4 mg/kg, i.v.; ††sodium methohexital, 12 mg/kg, i.v.; ‡‡ telazol (5 mg/kg)/xylazine (2 mg/kg)/glycolpyrrolate (0.006 mg/kg), i.m.
BSC = Blood sample collection; unc = unconscious; VC = vena cava; NA = not applicable.

Science Center, Columbia, Mo., egg source, Ennis National Fish Hatchery, Ennis, Mont.), chicken (Stover Hatchery, Stover, Mo.), rat (Harlan, Indianapolis, Ind.), hamster (Charles River, Wilmington, Mass.), ferret (Marshall Farms, North Rose, N.Y.), cat (Liberty Research Inc., Waverly, N.Y.), dog (Antech Inc., Barnhart, Mo.), and pig (Larson Pigs, Sargent, Minn.).

Microbiological status was unknown; however, all animals appeared healthy and free of disease on the basis of results of physical examination. None had experienced surgery or any other experimental procedure before the samples for this study were obtained. All species except humans were used subsequently for experiments after the blood sample was obtained. When possible, samples were obtained from males and females. The humans (six women and five men) were healthy nonsmokers, and none was receiving medication.

With the exception of skates, opossums, and toads, all animals were housed under controlled laboratory conditions for a minimum of two weeks (dogs and pigs) and maximum of eight months (trout and rats). Light cycles were: 12 h light/12 h dark for skates, trout, frogs, alligators, chickens, rats, hamsters, ferrets, cats, dogs, and pigs; and 9 h light/14 h dark for lampreys. Temperature was maintained at 21°C for alligators, rats, hamsters, ferrets, cats, dogs, and pigs; 8 to 9°C for lampreys; 12 to 14°C for skates; 17°C for trout; 15°C for frogs; and 28 to 30°C for chickens. Lampreys and trout were maintained in fresh-water tanks, and skates were kept in saltwater tanks. Frogs were housed in containers that provided access to wet (fresh water) and dry (gravel or brick surface) areas.

Dietary status and blood collection. Food was withheld from all but three species for at least 12 h before blood sample collection (Table 1). Prior to the nonfeeding period, the captive-bred species received diets that were standard for each and were nitrate free (www.labdiet.com, Purina, St. Louis, Mo.; trout—Zeigler Bros. Inc., Gardners, Pa). With regard to the wild-caught species, the lampreys were not fed for 90 days. The skates ate vertebrates and invertebrates and were caught in high-quality water with a fast current that was assumed to be free of algae accumulation, thus relatively nitrate free. The frogs ate vegetable beef baby food (Gerber Products Co., Fremont, Mich.). The toads were not fed after capture. The alligators ate raw chicken, and the opossums ate standard feline diet (No. 5003, Purina). Three species—chickens, cats, and humans—were fed ad libitum before blood was sampled. Nitrate is toxic to chickens; thus, their diet was nitrate free. Diet for the cats (feline diet, No. 5003, Purina) was also nitrate free. The dietary status for the humans was not restricted before collecting the blood sample and was based on data from Tangphao and co-workers (19), indicating that plasma [NO_x] did not vary with dietary intake in healthy individuals.

Blood was collected into syringes, using sampling sites and sedation or anesthesia that was appropriate for each species (Table 2). Blood samples from frogs were obtained at the same

time of day and between the tenth and twentieth day of each month throughout the year. Blood samples from the skates, opossums, and toads were obtained within one to two days of capture. Table 2 provides the blood sample collection sites, condition of the animals during and after blood sample collection, and the month(s) that blood samples were obtained for each species.

Measurement of [NO_x] in plasma. Whole blood samples were placed immediately into ethylenediaminetetraacetic acid (EDTA)-containing tubes that were kept cool in wet ice, and were centrifuged within one hour at 3,200 ×*g* for 15 min (4 to 5°C). The plasma was removed and injected into siliconized vacuum tubes for storage at –70°C. In all instances, [NO_x] was measured within one month of acquiring the plasma samples.

Plasma samples were heated to 85°C, and [NO_x] was measured, using chemiluminescence (Sievers nitric oxide analyzer; Model 270B, Boulder, Colo.; detection limit, 1 nmol/ml of sample). Plasma (5 to 300 µl/sample) was injected into a receptacle that contained 5 ml of 0.1*M* vanadium III chloride (Aldrich Chemical Co., Milwaukee, Wis.) in 3.0*M* HCl (J.T. Baker Co., Phillipsburg, N.J.). The vanadium III/HCl solution was made fresh weekly. Area under the peak obtained from the nitric oxide analyzer was integrated and recorded on a Shimadzu Chromatopac Integrator (Model CR601; Shimadzu Corp., Kyoto, Japan).

Vanadium III reduces nitrite (NO_2) to nitric oxide at room temperature (20°C) and reduces NO_x to nitric oxide at 85°C (20, 21). Initial tests were performed where both [NO_2] (at 20°C) and [NO_x] (at 85°C) were measured in plasma obtained from frogs, pigs, and humans to determine the contribution of [NO_2] to the total [NO_x] value.

Water samples. Water was collected from the environments of the lampreys, skates, trout, and frogs, simultaneously with blood sample collection. The water samples were injected into siliconized vacuum tubes, kept cool in wet ice, and stored at –70°C for measurement of [NO_x] as described previously.

Nitric oxide hemoglobin (HbNO). Blood samples collected from trout and frogs were frozen and stored at –70°C. After thawing, the red blood cells were lysed in either oxygenated or deoxygenated H_2O. Absorbance spectra were generated at 560 and 575 nm on a UV/visible recording spectrophotometer (UV-160, Shimadzu) and were analyzed for the presence of oxyhemoglobin (HbO_2) and HbNO (22). The ratio of peak absorbance at 575 nm relative to trough absorbance at 560 nm was calculated to determine the presence of HbO_2 and HbNO in each sample. Total hemoglobin concentration was determined using Drabkin's reagent. Sensitivity of the hemoglobin assay (cyanmethemoglobin) was 0.04 g/dl.

Data analysis and statistics. Plasma [NO_x] data for each species were tested for normality (Shapiro-Wilk), and are presented as mean ± SD unless indicated otherwise. Data were analyzed, using one-way analysis of variance or, in the case of unequal variance (Bartlett test), a Welch analysis of variance was used. The Tukey-Kramer post-hoc test was used to compare between species and months, and coefficient of variation served as an index of variability. Wilcoxon rank sums non-parametric test was used to compare toad and frog data sets. Least squares linear regression was calculated to assess the relationship between number of nonfeeding days and plasma [NO_x], and values of [NO_x] measured in plasma versus water (JMP, SAS Institute Inc., Cary, N.C.). Statistical significance was set at $P < 0.05$ prior to the experiments.

Results

Initial measurements of plasma indicated that nitrite (NO_2) concentration was minimal and ranged from 0.1 to 0.2 nmol/ml. Therefore, the contribution of NO_2 to total [NO_x] in plasma was considered not significant, leaving nitrate, the stable metabolite of nitric oxide in biological fluids, as the major variable.

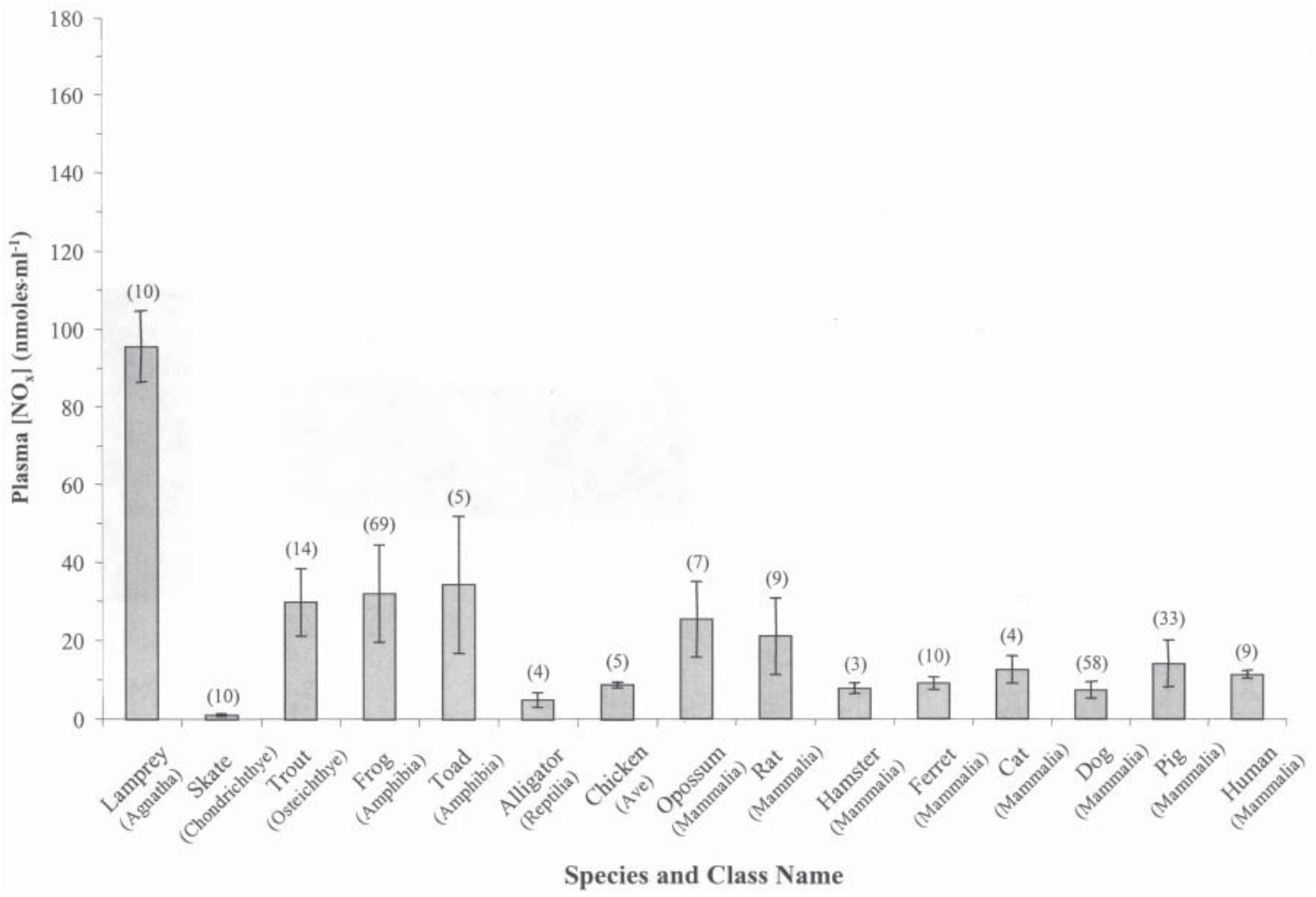
Baseline values of [NO_x] were detected in the plasma of all 15 species (range, 0.6 to 171.3 nmol/ml). Figure 1 presents average baseline plasma [NO_x] by species. Specific comparisons between species are provided in the caption for Fig. 1. The data sets were all distributed normally with the exception of that for frogs, which was not included in the analysis, but is presented in Fig. 1 for comparison and completeness.

The lowest average value for circulating [NO_x] in plasma was measured in skates (1.1 ± 0.4 nmol/ml). The highest average value was found in lampreys, 95.5 ± 9.1 nmol/ml, which differed statistically from average values for all other species. Average values for trout and toads were not different from each other, but were higher than those for all species accept lampreys and opossums. Average values for alligators were not different from those for chickens, but average values for both differed from those for lampreys, trout, toads, opossums, and rats. Average values for alligators and chickens also were statistically similar to those for skates and the remainder of the mammals. Among the seven common mammals tested (n = 140), values of plasma [NO_x] ranged from 3.8 (dog) to 43.2 (opossum) nmol/ml. The greatest variability in the data sets occurred in frogs and pigs (Table 3).

Box plots for the two amphibians tested for baseline plasma [NO_x] are presented in Fig. 2. Data from May for toads and frogs are presented along with data for frogs collected in all 12 months. The entire data set for frogs was skewed ($P = 0.0001$) toward the lower values and had the widest range (14.4 to 171.3 nmol/ml) of the 15 species tested. Mean ± SD plasma [NO_x] in toads was 34.3 ± 17.5 (median, 31.9) nmol/ml and was similar to that for frogs in May ($P = 0.58$; mean, 29.7 ± 7.9, median, 29.2 nmol/ml) as well as for frogs in all months ($P = 0.94$; mean, 32.0 ± 12.5, median, 29.8 nmol/ml with outliers excluded, n = 69). Values for toads ranged between 17.3 and 53.0 nmol/ml. Plasma [NO_x] in two groups of frogs (n = 12) that manifested signs of infection were excluded from the baseline data set. The [NO_x] for the infected frogs (395.5 ± 68.0 nmol/ml) was 10-fold higher relative to baseline values (Fig. 2).

Most of the outliers depicted in Fig. 2 (Frog All Months) were clustered in August, October, and December, suggesting a temporal component to plasma [NO_x] in frogs. Data for the frog are presented by month in Fig. 3. Differences between months are included in the list insert for Fig. 3 and accounted, in part, for the wide range in basal plasma [NO_x] (Fig. 2) and high coefficient of variation in frogs (Table 3). Among those species tested in more than one month, plasma [NO_x] in trout, alligator, and pig did not differ ($P > 0.05$) by month. Dog plasma [NO_x] values, however, were different ($P = 0.03$) between January and August and, unlike frog values, had equal variance between months. A trend toward a temporal component was apparent in dog values (data not shown); however, we did not have access to samples in all 12 months to complete the analysis.

With the exception of chickens, cats, and humans, blood samples were obtained from animals from which food was withheld for a minimum of 12 h (Table 1). To examine further the



Species	L	S	Tr	Td	A	Ch	O	R	Ha	Fe	Ct	D	P	Hu
Lamprey (L)		*	*	*	*	*	*	*	*	*	*	*	*	*
Skate (S)	*		*	*			*	*					*	*
Trout (Tr)	*	*			*	*		*	*	*	*	*	*	*
Toad (Td)	*	*			*	*		*	*	*	*	*	*	*
Alligator (A)	*		*	*			*	*						
Chicken (Ch)	*		*	*			*	*						
Opossum (O)	*	*			*	*			*	*	*	*	*	*
Rat (R)	*	*	*	*	*	*				*		*		*
Hamster (Ha)	*		*	*			*							
Ferret (Fe)	*		*	*			*	*						
Cat (Ct)	*		*	*			*							
Dog (D)	*		*	*			*	*					*	
Pig (P)	*	*	*	*			*					*		
Human (Hu)	*	*	*	*			*	*						

Figure 1. Mean ± SD plasma nitrite/nitrate concentration ([NO_x]) measured in 15 species. Number of each species is in parentheses. Frog data were not distributed normally and were not included in the analysis; however, they are presented for comparison. Outliers were identified in the dog (3), pig (2), and human (2) data sets and removed from the analysis. Analysis of variance indicated differences ($P < 0.0001$) between species, which are presented in the list within the graph. *Indicates difference from the species listed in the left-hand column. *See* Table 1 for scientific nomenclature.

potential contribution of feeding on plasma [NO_x] levels in a species with high endogenous plasma [NO_x], we tested for a relationship between number of nonfeeding days and plasma [NO_x] in frogs. Number of nonfeeding days ranged from three to 20 and, consistent with a minimal effect of feeding on plasma [NO_x] in frogs, there was no relationship ($P = 0.61$) between nonfeeding days and plasma [NO_x] (data not shown).

Blood sample collection sites differed depending on the species. We measured [NO_x] in paired plasma samples obtained from central (heart puncture) versus peripheral (skin vein) sites in frogs (n = 11). Plasma [NO_x] in samples obtained from the central sampling site (27.8 ± 7.6 nmol/ml) was not different ($P = 0.53$) from that in samples obtained from the peripheral site (29.8 ± 6.9 nmol/ml).

Water is a potential source of NO_x in animals that exist in aqueous or aqueous and terrestrial environments. Consequently, the water in which these animals were housed, in addition to

Table 3. Coefficient of variation (CV) for average plasma [NO_x] measured in 15 species

Class	Species	CV
Agnatha	Lamprey	0.10
Chondrichthyes	Skate	0.33
Osteichthyes	Trout	0.29
Amphibia	Frog	0.71
	Toad	0.51
Reptilia	Alligator	0.38
Aves	Chicken	0.09
Mammalia	Opossum	0.38
	Rat	0.47
	Hamster	0.19
	Ferret	0.19
	Cat	0.28
	Dog	0.38
	Pig	0.72
	Human	0.24

temporal variation and feeding, represented another potential source of NO_x. Samples were obtained from the fresh-water tanks that held the lampreys, the sea-water tanks used for the skates, the pond water from the trout farm, and the aquarium water of the frogs. Plasma [NO_x] plotted as a function of water [NO_x] is presented in Fig. 4. A relationship ($P = 0.22$) did not exist between water and plasma [NO_x] among these four species.

Red blood cells in whole blood also were tested as a potential sink for nitric oxide in two species that were accessible and had high plasma [NO_x]. For trout (n = 4) and frogs (n = 4), the pooled mean ratio for peak (575 nm) relative to trough (560 nm) absorbance was 1.64 ± 0.03 in oxygenated and deoxygenated samples. These data indicated 100% HbO_2 species (expected ratio, 1.6) and minimal HbNO (expected ratio, 1.0) in the samples of whole blood (22). Additionally, minimal values for blood nitrite were detected.

Discussion

Plasma was obtained from 15 species that represented six non-mammalian classes of vertebrates as well as common species of mammals, including humans. Nitrite/nitrate was detected in the plasma of all species including 10 (lamprey, skate, trout, frog, toad, alligator, opossum, hamster, ferret, and cat) that had not been tested previously for presence of the anion in their circulation. Plasma [NO_x] was highest in a jawless fish (lamprey) and lowest in a cartilaginous fish (skate), indicating a wide range in baseline, circulating [NO_x] between two primitive vertebrates. Frogs, toads, rats, and pigs had the highest variability in plasma [NO_x]. A temporal component to the data set was identified in frogs.

Plasma nitrate concentration: Comparisons among classes of vertebrates. We hypothesized that variability in baseline plasma [NO_x] among classes of vertebrates would reflect differences in the contribution of nitric oxide to vascular tone (5, 6, 23) and permeability (7-9). In sharks, prostaglandin E has been reported as the prevalent endothelium-derived relaxing factor (24), an observation that is consistent with minimal plasma [NO_x] reported here for skates, another cartilaginous fish. Aortic rings obtained from trout responded with endothelium-dependent relaxation on activation of prostanoids, but not nitric oxide (10). In contrast, studies performed in whole heart (25) and brain (26) indicated that nitric oxide may be released from microvessels and be involved in controlling coronary and cerebral blood flow of rainbow trout. On the basis of results of those studies, one would predict detectable [NO_x] in plasma of

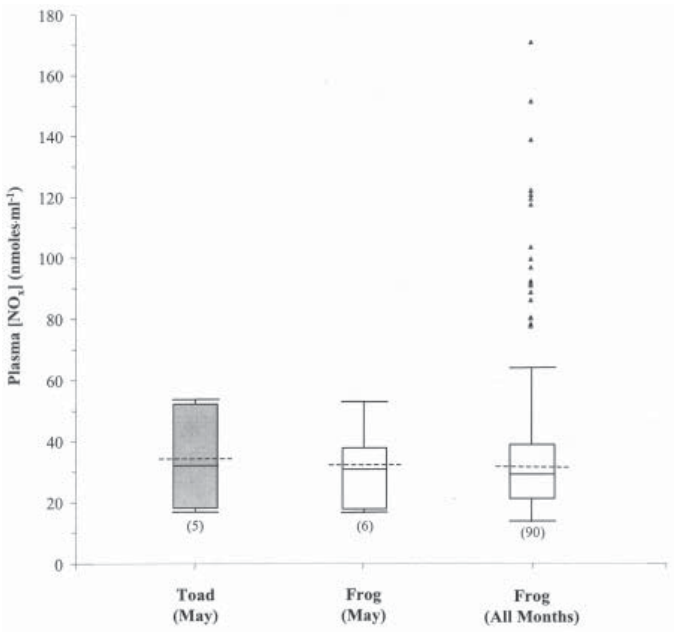


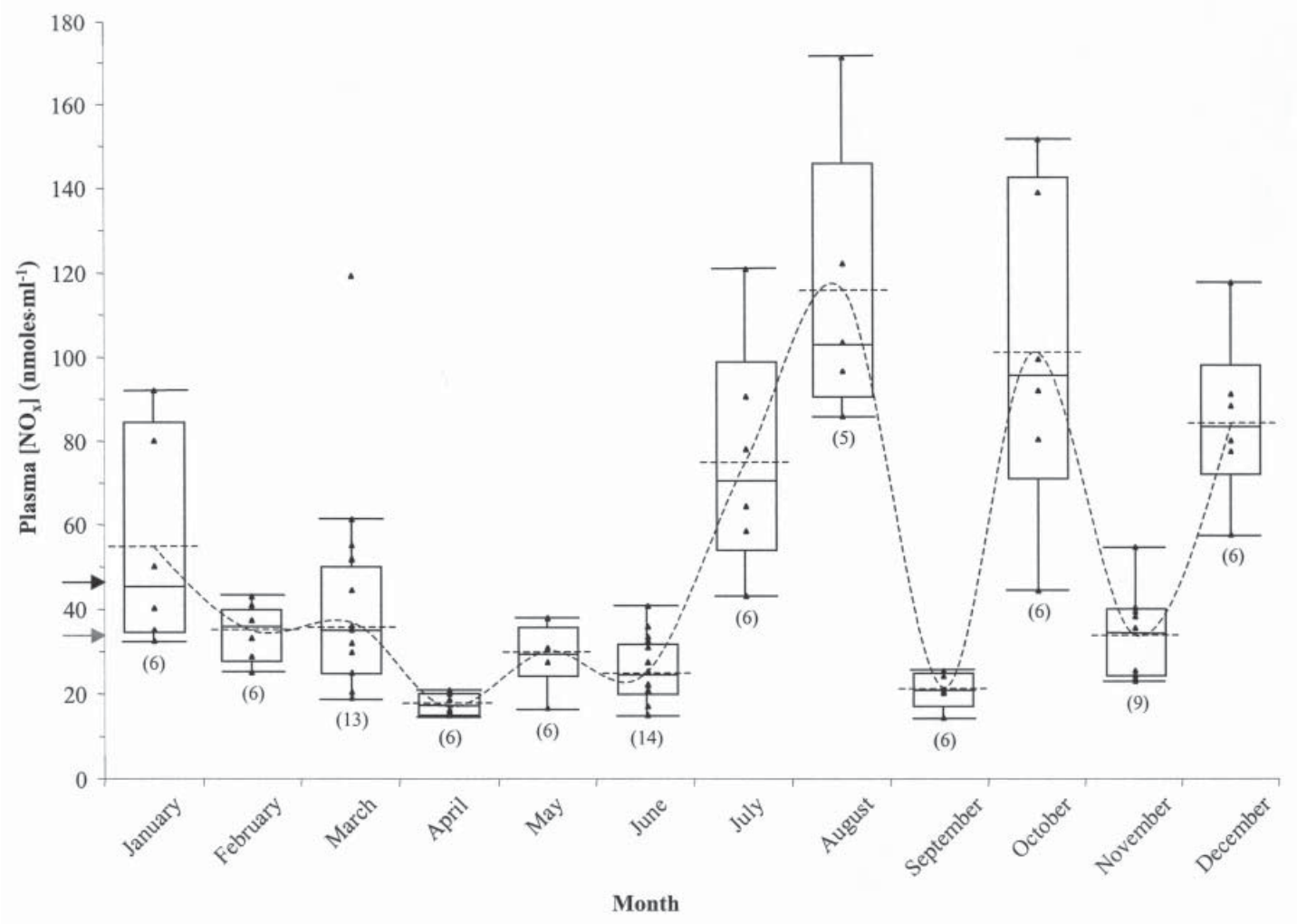
Figure 2. Box plots of plasma [NO_x] data for toads and frogs. Solid horizontal lines located within each box signify medians, and broken lines indicate means. The length of each box along the ordinate indicates the 25th and 75th percentiles of the data sets, and the lines extending from each box indicate the minimum and maximum for the data after outliers were excluded. The 21 outliers for frogs (all months) are pictured as individual data points. The number in each group is in parentheses.

trout (Fig. 1). In leopard frogs, conduit arteries and capillaries had endothelium-dependent relaxations and changes in hydraulic conductivity (permeability to water), respectively, which involved nitric oxide (15, 16). In addition, chickens have a nitric oxide pathway and no prostanoid pathways (27, 28), again, consistent with the detectable [NO_x] observed here in avian (chicken) plasma (Fig. 1).

Animals of the lower classes of vertebrates exist in aqueous or aqueous and terrestrial environments. Consequently, high plasma [NO_x] could reflect equilibration of plasma with the local habitat. However, plasma [NO_x] measured in lampreys, skates, trout, and frogs did not correlate with [NO_x] measured in the water of their respective surroundings (Fig. 4). Additionally, similar values for plasma [NO_x] between toads, which are terrestrial amphibians, and leopard frogs (terrestrial/aquatic amphibians) (Fig. 2) suggest that an aquatic environment may not be the major influence on [NO_x] in the circulation of these animals. At the other extreme, low plasma [NO_x] in skates may relate to factors associated with a saltwater habitat and transition in the osmoregulatory function of chloride cells located in the gills (29).

The frogs were maintained on a nitrate-free diet throughout the year, and values for plasma [NO_x] were similar between fed and nonfed frogs. Additionally, neither lampreys nor trout feed on plant life. As such, feeding was eliminated as an explanation for the different values for plasma [NO_x] in the lower vertebrates (lamprey, skate, and trout) reported here.

In frogs, a temporal component was identified in plasma [NO_x] (Fig. 3). Blood from toads was sampled during only one month, which explained the narrow range in values for toads, compared



Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
January				*				*		*		
February							*	*		*		*
March							*	*		*		*
April	*						*	*		*		*
May							*	*		*		*
June							*	*		*		*
July		*	*	*	*	*		*	*		*	
August	*	*	*	*	*	*	*		*		*	
September							*	*		*		*
October	*	*	*	*	*	*			*		*	
November							*	*		*		*
December		*	*	*	*	*			*		*	

Figure 3. Box plots of plasma [NO_x] measured in frogs during the middle of each month across the year. Black arrow indicates mean, and gray arrow represents median for the complete data set minus one outlier in the month of March. Number of animals in each month is indicated in parentheses below each bar. Broken line depicts temporal component to these data.*Indicates difference from the month listed in the left-hand column (list insert). *See* Figure 2 for key.

with frogs (Fig. 2). We did not have access to samples in all months of the year for any other species. Neither the stimulus nor the mechanism for the temporal patterns of plasma [NO_x] observed in frogs is known; further study is required.

Plasma nitrate concentration: Comparisons within the class Mammalia. In 1981, baseline values of endogenous nitrate were detected in the blood of rats (30), an observation that could not be explained by either diet or infection. The circulating [NO_x] presented here for a variety of common laboratory mammals, including rats, documents differences among these animals (Fig. 1). In general, the lower average plasma [NO_x] of hamsters, ferrets, cats, dogs, pigs, and humans, compared with opossums, could indicate that these species regulate NOS within a more narrow range. Likewise, in dogs, endogenous nitric oxide production is limited principally to the coronary circulation (31) and can be altered by exercise (change in shear stress) (32). Thus, one would anticipate low plasma [NO_x] in healthy, sedentary dogs, relative to pigs (Fig. 1), where production of endogenous nitric oxide is presumed to occur throughout the cardiovascular system.

In the study reported here, average plasma [NO_x] measured in healthy human subjects in Minnesota was similar to that

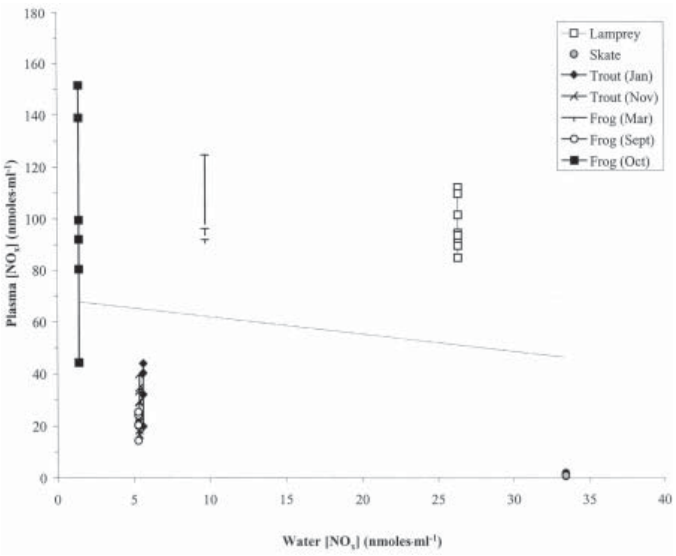


Figure 4. Plasma [NO_x] measured in lower vertebrates and plotted as a function of [NO_x] in water sampled from their immediate environment. Individual data points for each animal are indicated. Two separate measurements of plasma [NO_x] and water [NO_x] were performed for trout, and three were done for frogs. A relationship did not exist between water [NO_x] and plasma [NO_x], $r = 0.17$, $P = 0.22$ (broken line).

(19.7 μ*M*) in people in The Netherlands, as reported by Moshage and co-workers (33). In contrast, data reported by Wennmalm (34) and colleagues indicated higher baseline values in humans (range, 19.0 to 31.0 μ*M* [34, Sweden] and mean ± SEM, 38.0 ± 4.0 μ*M* [4, Sweden]) compared with those reported here. In fact, intake of a seven-day, nitrate-restricted diet lowered plasma [NO_x] only to 29 ± 1 μ*M* (4), a value still higher than that reported here and by Moshage and co-workers (33). These differences could reflect environmental factors other than diet, including temperature acclimation, fitness level, cardiovascular risk factors, such as high cholesterol, and hormonal status. As such, restricting the oral intake of nitrate acutely before blood samples are collected (4) may not be the appropriate control variable for human subjects and patients when using plasma nitrate concentration as an index of endogenous nitric oxide. Additional research into the influence of chronic environmental factors on baseline circulating [NO_x] in plasma is required.

Limitations of the study. Different values for plasma [NO_x] among different species could be accounted for by differences in fed/nonfed states, blood collection sites, different anesthesia methods, and time of year that the samples were obtained. Although we were unable to test these possibilities in all species, we have addressed each in at least one. In frogs, the number of nonfeeding days was varied between three and 20, and a relationship was not found between nonfeeding days and plasma [NO_x]. Also in frogs, blood samples were collected from the heart and skin, and differences in average plasma [NO_x] were not found between these two blood collection sites.

Differences in plasma [NO_x] were detected in species where the method of sedation was the same. For example, lampreys, alligators, rats, and cats all received sodium pentobarbital (Table 2), but had plasma [NO_x] that differed in some instances and was the same in others (Fig. 1). Even more specifically, lampreys and alligators received the same sedation dose, both received the agent intraperitoneally, and blood was collected from the heart of both species, yet plasma [NO_x] differed by almost 20-fold between the two species. Additionally, plasma [NO_x] in venous blood obtained from conscious humans versus conscious skates also was significantly different.

One other explanation for differences between species is different times of the year that we obtained samples from each species. We documented variation of plasma [NO_x] with time of year in one species, the frog (Fig. 3), and between two months in dogs. However, differences between months were not found for trout, alligators, or pigs. More work is required in the area of temporal changes in baseline plasma [NO_x] to better understand the prevalence of these variations as well as the mechanism and biological significance of the observation.

In conclusion, nitrite/nitrate was detected in all plasma samples collected from 15 species that were selected from the six non-mammalian classes of vertebrates as well as common species of the mammalian class and humans. Compared with selected lung breathing species of the reptilian, avian, and mammalian classes, circulating [NO_x] was higher in fresh water lampreys, which represented the more ancient agnathan class. In contrast, endogenous [NO_x] was minimal in skates, a cartilaginous fish (class Chondrichthyes). Circulating [NO_x] had a temporal component in frogs. Detection of NO_x in plasma obtained from a comparative survey of vertebrates as well as the wide variation in baseline plasma [NO_x] among individual species may reflect different mechanisms that have evolved for vasoregulation as well as nitrate clearance and distribution.

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