

# A phylogenetic survey of biliary lipids in vertebrates<sup>1,§</sup>

Antonio Moschetta,<sup>2,\*†</sup> Fang Xu,<sup>2,§</sup> Lee R. Hagey,<sup>2,\*\*</sup> Gerard P. van Berge-Henegouwen,\*  
Karel J. van Erpecum,\* Jos F. Brouwers,<sup>††</sup> Jonathan C. Cohen,<sup>§</sup> Molly Bierman,<sup>§</sup>  
Helen H. Hobbs,<sup>§,§§</sup> Joseph H. Steinbach,<sup>\*\*</sup> and Alan F. Hofmann<sup>3,\*\*</sup>

Department of Gastroenterology,\* University Medical Center Utrecht, Utrecht, The Netherlands; Clinica Medica "A. Murri,"<sup>†</sup> Department of Internal and Public Medicine, University Hospital of Bari, Bari, Italy; Department of Internal Medicine and Molecular Genetics<sup>§</sup> and Howard Hughes Medical Institute,<sup>§§</sup> University of Texas Southwestern Medical Center at Dallas, Dallas, TX; Department of Medicine,<sup>\*\*</sup> University of California, San Diego, CA; and Department of Biochemistry and Cell Biology,<sup>††</sup> Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

**Abstract** Biliary lipids (bile salts, phospholipids, cholesterol, plant sterols) were determined in 89 vertebrate species (cartilaginous and bony fish, reptiles, birds, and mammals), and individual phospholipid classes were measured in 35 species. All samples contained conjugated bile salts (C<sub>27</sub> bile alcohol sulfates and/or N-acyl amidates of C<sub>27</sub> and/or C<sub>24</sub> bile acids). Phospholipids were generally absent in the bile of cartilaginous fish and reptiles and were present in low amounts relative to bile salts in bony fish and most birds. In mammals, the phospholipid-bile salt ratio varied widely. The bile from species with low biliary phospholipid-bile salt ratios often contained a high proportion of sphingomyelin, confirmed by HPLC-MS. In species with a high phospholipid-bile salt ratio, the predominant biliary phospholipid was phosphatidylcholine (PC). The phospholipid-bile salt ratio correlated weakly with the calculated weighted hydrophobic index value. Cholesterol was present in the bile of virtually all species, with plant sterols uniformly being present in only trace amounts. The cholesterol-bile salt ratio tended to be higher in mammals than in nonmammals, but bile of all species was unsaturated. ¶¶ Thus, most nonmammalian vertebrates have relatively low levels of biliary phospholipid and cholesterol, suggesting that cholesterol is eliminated predominantly as bile salts. Mammals have a higher phospholipid and cholesterol to bile salt ratio, with the dominant phospholipid being PC.—Moschetta, A., F. Xu, L. R. Hagey, G. P. van Berge-Henegouwen, K. J. van Erpecum, J. F. Brouwers, J. C. Cohen, M. Bierman, H. H. Hobbs, J. H. Steinbach, and A. F. Hofmann. **A phylogenetic survey of biliary lipids in vertebrates.** *J. Lipid Res.* 2005. 46: 2221–2232.

**Supplementary key words** bile salts • cholesterol • gas chromatography • mass spectrometry • phosphatidylcholine • sphingomyelin

The major organic constituents of mammalian bile are bile salts (conjugated bile acids and/or bile alcohol sul-

fates), phospholipids, cholesterol, and bile pigments. The presence of bile salts (or bile acids) in bile was recognized in the early 19th century, long before the chemical structure of these compounds was known. Cholesterol was isolated from gallstones and crystallized in 1769 (1), but not until 1932 was the chemical structure of cholesterol iodide determined by Bernal using X-ray diffraction (2). His work enabled Rosenheim and King (3) to deduce the correct molecular structure of both cholesterol and bile acids (3). Lecithin was isolated from egg yolk by Gobley in 1845 (4), and the chemical structure of its dominant phospholipid, phosphatidylcholine (PC), was established by 1900 (1). A half-century later, Polonovski and Bourrillon identified PC as the only phospholipid present in human bile based on chemical analyses (5), and Phillips confirmed this finding by adsorption chromatography in 1960 (6).

In 1968, Admirand and Small (7) proposed that three of the organic constituents of bile—conjugated bile acids, phospholipids, and cholesterol—be termed “biliary lipids,” despite the fact that conjugated bile acids do not have the properties of lipids. This proposal was widely accepted because the relative proportion of these three biliary lipids determines the cholesterol saturation of bile (7). Bile pigments in most vertebrates consist of bilirubin, usually esterified (conjugated) with one or two molecules of glucuronic acid. Conjugated bilirubin is not classified as a biliary lipid for two reasons. First, conjugated bilirubin

Abbreviations: ABCG5, ATP binding cassette transporter G5; HI, hydrophobicity index; PC, phosphatidylcholine; RRT, relative retention time; WHI, weighted hydrophobicity index.

<sup>1</sup>Part of this work was presented at the annual meeting of the American Gastroenterological Association (San Diego, CA, May 2000) and was published as an abstract (*Gastroenterology*. 2000. 118: A139).

<sup>2</sup>A. Moschetta, F. Xu, and L. R. Hagey contributed equally to this work.

<sup>3</sup>To whom correspondence should be addressed.

e-mail: ahofmann@ucsd.edu

§ The online version of this article (available at <http://www.jlr.org>) contains two additional figures and four additional tables.

Manuscript received 6 May 2005 and in revised form 18 July 2005.

Published, *JLR Papers in Press*, August 1, 2005.

DOI 10.1194/jlr.M500178.JLR200

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TABLE 1. RRTs of C<sub>24</sub> conjugated bile acids by HPLC and HI values for taurine conjugated bile acids

Trivial Name	Conjugation	A/B Ring Juncture and A Ring Substituents	B Ring Substituents	C and D Ring Substituents	RRT	HI
I. C <sub>24</sub> bile acids with unsubstituted C <sub>5</sub> side chain						
Cholic	Taurine	5β,3αOH	7αOH	12αOH	1.00	-0.09
	Glycine	5β,3αOH	7αOH	12αOH	1.46	
Allocholic "Ursocholic"	Taurine	5α,3αOH	7αOH	12αOH	1.08	-0.02
	Taurine	5β,3αOH	7βOH	12αOH	0.38	-0.96
β-Muricholic	Taurine	5β,3αOH	6βOH, 7βOH		0.48	-0.75
	Taurine	5β,3αOH	6βOH, 7αOH		0.48	-0.75
α-Muricholic	Taurine	5β,3αOH	6βOH		0.49	-0.74
	Glycine	5β,3αOH	6βOH		0.53	
Hyocholeic	Taurine	5β,3αOH	6αOH, 7αOH		0.65	-0.48
	Glycine	5β,3αOH	6αOH, 7αOH		0.92	
Hyodeoxycholic	Taurine	5β,3αOH	6αOH		0.74	-0.36
	Glycine	5β,3αOH	6αOH		1.07	
Deoxycholic	Taurine	5β,3αOH		12αOH	2.07	0.57
	Glycine	5β,3αOH		12αOH	2.99	
(Allo)deoxycholic	Taurine	5α,3αOH		12αOH	2.24	0.63
	Glycine	5α,3αOH		12αOH	3.23	
Chenodeoxycholic	Taurine	5β,3αOH	7αOH		1.78	0.43
	Glycine	5β,3αOH	7αOH		2.66	
Ursodeoxycholic	Taurine	5β,3αOH	7βOH		0.65	-0.48
	Glycine	5β,3αOH	7βOH		0.90	
"Vulpecholic" NTNP	Taurine	5β,1αOH,3αOH	7αOH		0.36	-1.01
	Taurine	5β,1βOH,3αOH	7αOH		0.34	-1.07
	Glycine	5β,1βOH,3αOH	7αOH		0.41	
"Avicholic" NTNP	Taurine	5β,3αOH	7αOH	16αOH	1.04	-0.05
	Taurine	5β,3αOH	7αOH	15αOH	0.94	-0.15
NTNP	Taurine	3αOH, 5βOH	7αOH		0.41	-0.90
NTNP	Taurine	5β,3αOH	7αOH	14αOH	1.10	0.00
NTNP	Taurine	5β, 3αOH, 4βOH	7αOH		0.88	-0.21
NTNP	Taurine	5β,3αOH	6αOH, 7αOH	12αOH	0.81	-0.28
Lithocholic	Taurine	5β,3αOH			3.62	1.07
	Glycine	5β,3αOH			5.71	
	Sulfate, taurine	5β,3α-sulfo			0.80	-0.29
	Sulfate, glycine	5β,3α-sulfo			1.22	

HI, hydrophobicity index; NTNP, no trivial name proposed; RRT, relative retention time. RRT values are given for both glycine and taurine conjugates, but for the calculation of HI values, glycine conjugates were assumed to be taurine conjugates, as the RRT for glycine conjugates is strongly influenced by the apparent pH of the mobile phase.

bin is water soluble and not extractable into organic solvents. Second, it is present in bile at a concentration well below that of cholesterol (8), and its concentration in bile appears not to affect biliary cholesterol saturation.

The predominance of PC in bile (5, 6) and the observa-

tion that biliary PC has a well-defined fatty acid structure (9, 10) strongly suggested that the secretion of PC into canalicular bile was carrier mediated. A transporter mediating PC secretion into bile was identified when it was discovered that mice deficient in the *Abcb4* (ATP binding cas-

TABLE 2. RRTs and hydrophobic index values for C<sub>27</sub> conjugated bile acids and bile alcohol sulfates by HPLC

Trivial Name	Conjugation	A/B Ring Juncture and A Ring Substituents	B Ring Substituents	C and D Ring Substituents	Side Chain	RRT	HI
II. C <sub>24</sub> bile acids with substituents on the C <sub>5</sub> side chain							
αOH-cholic	Taurine	5β,3αOH	7αOH	12αOH	23(R)OH	0.57	-0.60
αOH-deoxycholic	Taurine	5β,3αOH		12αOH	23(R)OH	1.07	-0.03
Phocaecholic (αOH-chenodeoxycholic)	Taurine	5β,3αOH	7αOH		23(R)OH	0.90	-0.19
III. C <sub>27</sub> bile acids with or without substituents on the C <sub>8</sub> side chain							
Varanic	Taurine	5β,3αOH	7αOH		[25ε]	5.94	1.52
	Taurine	5β,3αOH	7αOH	12αOH	[25R]	2.48	0.73
	Taurine	5β,3αOH	7αOH	12αOH	[25S]	2.86	0.86
	Taurine	5β,3αOH	7αOH	12αOH	24(R)OH	1.14	0.03
	Taurine	5β,3αOH	7αOH	12αOH	22(R)OH	1.22	0.09
	Taurine	5β,3αOH	7αOH	16αOH	[25(R)]	2.75	0.83
	Taurine	5β,3αOH	7αOH	16αOH	[25(S)]	2.88	0.87
IV. C <sub>27</sub> bile alcohols							
Scymnol	Sulfate	5β,3αOH	7αOH	12αOH	24OH	0.87	-0.22
5α-cyprinol	Sulfate	5β,3αOH	7αOH	12αOH	26OH	1.91	0.50

sette transporter B4) gene had bile devoid of PC (11). Because inflammation of the biliary tract (cholangitis) developed when hydrophobic bile acids were fed to *Abcb4* knockout mice (12), the hypothesis was advanced that biliary PC not only solubilizes cholesterol in bile but also protects cholangiocytes from the toxic effects of bile acids. The protective effect of PC would result from its cooperative association with bile acids to form mixed micelles, a process that decreases the monomeric activity of bile acid anions and should decrease the cytotoxicity of bile (13–15).

It has been assumed that PC is the dominant phospholipid in the bile of vertebrates. A few notable exceptions, however, have been found. PC is virtually absent from the bile of the guinea pig, in which bile formation is driven by bicarbonate secretion and the bile acid concentration is low (16). The bile of the Asiatic carp and the skate, a cartilaginous fish, has been reported not to have any detectable phospholipids (17, 18).

The major mechanism by which cholesterol is secreted into bile was provided by the identification of ATP binding cassette transporter G5 (ABCG5) and ABCG8 (19), which are two apical proteins that heterodimerize to promote biliary cholesterol secretion. In mice, the amount of cholesterol secreted into the bile is proportional to the expression of these transporters (20). Not all vertebrates have cholesterol in their bile; Boyer and colleagues reported that the little skate, *Raja erinacea*, has no biliary cholesterol (18).

During the past decade, the bile salts of a large number of vertebrates have been characterized by Hagey, Hofmann, and Scheingart (21, 22), building on the work of Haslewood (23) and others. In this report, we have extended this work by analyzing the phospholipid and cholesterol contents of a wide spectrum of vertebrates to test the hypothesis that PC is a predominant phospholipid in bile and plays a cytoprotective role in species in which the circulating bile acid pool is hydrophobic. We also sought to deter-

mine when during evolution the ability to secrete cholesterol into bile was acquired.

## MATERIALS AND METHODS

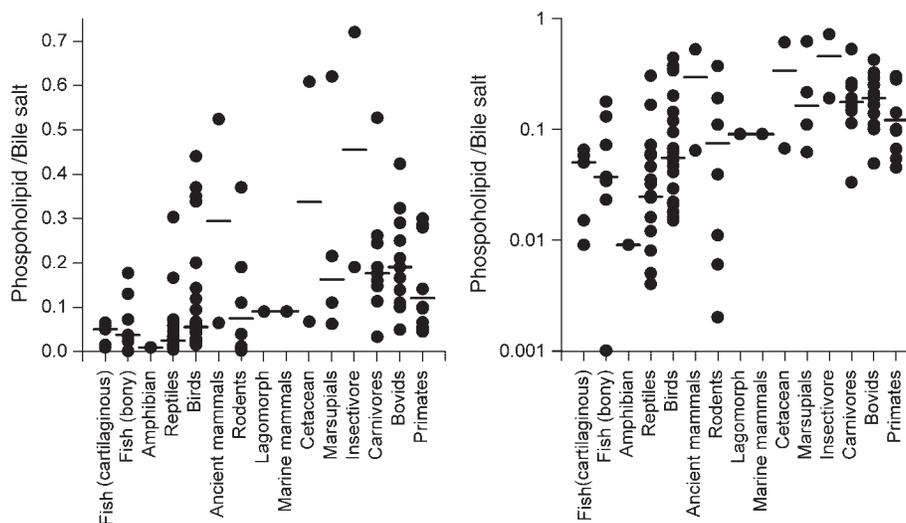
### Animal bile samples

Bile samples (112) from 95 vertebrate species were analyzed in this study, and values from 89 species had sufficiently high bile salt concentrations (>0.5 mM) to be included in the study. Values for biliary lipids in 12 species (horse, rat, mouse, hamster, prairie dog, rabbit, cat, dog, guinea pig, rhesus monkey, squirrel monkey, and human) were obtained from the literature, for a total of 101 species. Taxonomic data, the source of the bile samples, and literature references are provided in supplementary Tables I and II (nonmammalian species) and supplementary Tables III and IV (mammalian species). Most bile samples were obtained at autopsy from the pathology laboratory at the Zoological Society of San Diego. Gallbladder bile or bile duct bile (in species lacking a gallbladder) was obtained by aspiration with a 25 gauge needle.

The bile sample was immediately added to at least four volumes of reagent-grade isopropanol, and the samples were stored at 4°C until lipid analysis was performed. Samples of bile from marine mammals were obtained from Sea World (San Diego, CA, and Coral Gables, FL). Other samples were collected by G.A.D. Haslewood (now deceased; at Guy's Hospital, London, UK) (23). Samples obtained by Haslewood had been heated in ethanol and filtered, and the residue was dried. These residues were transferred to the United States in 1996, with permission of the Bureau of Wildlife and Fisheries. Additional samples were provided by Dr. Valentine Lance (Department of Biology, San Diego State University) and Hubert Johnston (Veterinarian of the County of San Diego).

### Biliary lipid measurements

**Biliary lipid groups.** The levels of bile acids and bile alcohol sulfates were determined using the 3 $\alpha$ -hydroxysteroid dehydrogenase method (24). Phospholipids were determined by two methods. In the first method, phospholipids were determined by measuring inorganic phosphate in the chloroform phase of a Folch ex-



**Fig. 1.** Phospholipid-bile salt ratios in different vertebrate classes shown in linear scale (left) and logarithmic scale (right). Median values are indicated by horizontal lines for each class. The ratio is significantly higher in mammals than in nonmammals ( $P < 0.001$ ).

tract (25). In the second method, total phospholipids (inorganic phosphate and nonphospholipid phosphorus-containing molecules) were determined using the malachite green method for inorganic phosphate after a perchloric acid combustion step (26). In species in which the level of biliary phospholipids is high, the contribution of inorganic phosphate to total phosphate is small (27), but in species in which biliary phospholipids are low, measuring biliary phospholipids by determining total phosphate may overestimate the amount of phospholipid present (28).

Cholesterol was determined enzymatically in the same bile samples used for phospholipid class analysis (27 samples). Cholesterol levels were determined by GC-MS in the remaining samples (65 samples). For the GC-MS analysis, an aliquot of bile was added to 1 ml of ethanol containing 5 $\alpha$ -cholestane (50  $\mu$ g) and epicoprostanol (2.5  $\mu$ g). The sterols were hydrolyzed by heating (100°C in

ethanolic KOH (100 mM) for 2 h, and the lipids were extracted into petroleum hydrocarbon, dried under nitrogen, and derivatized with hexamethyldisilazane-trimethylchlorosilane. GC-MS analysis was performed using an Agilent 6890N gas chromatograph coupled to an Agilent 5973 mass selective detector. The trimethyl silyl ethers were separated on an HP-5MS 5%-phenyl methyl polysiloxane capillary column (30 m  $\times$  0.25 mm inner diameter  $\times$  0.25  $\mu$ m film), with helium as the carrier gas (flow of 1 ml/min). The temperature program was 150°C for 2 min, increased by 20°C/min up to 280°C, and held at that level for 13 min. The injector was operated in the splitless mode and kept at 280°C. The mass spectrometer was operated in the single ion monitoring mode. The extracted ions were 458.4 (cholesterol), 343.3 (desmosterol), 458.4 (lathosterol), 456.4 (zymosterol), 382.4 (campesterol), 393.4 (lanosterol), and 396.4 ( $\beta$ -sitosterol). No sterols

TABLE 3. Biliary lipid composition and WHI values of fish, an amphibian, and reptiles

Order	Species Name	5H	C <sub>27</sub> Alcohol	C <sub>27</sub> Acids	C <sub>24</sub> Acids	Conjugation	Major Bile Salts	WHI	Phospholipid-Bile Salt Ratio	Cholesterol-Bile Salt Ratio <sup>a</sup>	Cholesterol-Phospholipid Ratio	Campesterol-Cholesterol Ratio <sup>a</sup>	$\beta$ -Sitosterol-Cholesterol Ratio <sup>a</sup>
Fish (cartilaginous)	Thornback ray	$\beta$	•			Sulfate	Mixed C <sub>27</sub> alcohols	NC	0.009				
	Cow-nose ray	$\beta$	•			Sulfate	Scymnol	-0.05	0.058				
	Black tip shark	$\beta$	•			Sulfate	Scymnol	-0.05	0.05	3	0.57		
	Epaulette shark	?	•			Sulfate	C <sub>27</sub> alcohols	NC	0.065	4.3	0.065		
	Swell shark	$\beta$	•			Sulfate	Scymnol	-0.05	0.015	1.5	0.097		
Fish (bony)	Australian lungfish	$\alpha$	•			Sulfate	Mixture of C <sub>27</sub> alcohols	NC	0.034	0	0	26.2	15.8
	Arapaima gigas	$\beta$	•	•		Sulfate, taurine	C <sub>27</sub> alcohols, C <sub>27</sub> acids	NC	0.023	2.6	0.11	1.3	0.6
	Asiatic carp	$\alpha$	•			Sulfate	5 $\alpha$ -cyprinol	0.74	0.001	0			
	Electric eel	$\beta$			•	Taurine	C, unknowns	0.12	0.13	12	0.076	5.1	0.14
	Garpike	$\beta$			•	Taurine	C	-0.06	0.072	4.7	0.067	38.1	2.9
	California sculpin	$\beta$			•	Taurine	C, CDC	0.62	0.177	10.5	0.059		
	Pacific barracuda	$\beta$			•	Taurine	C	0.02	0.037	4.7	0.19		
	Giant marine toad	$\beta$	•	•		Taurine	C <sub>27</sub> alcohols, C <sub>27</sub> acids	NC	0.009	1.6	0.16	4.7	6.36
Reptiles	Galapagos tortoise	$\beta$		•		Taurine	Mixture of C <sub>27</sub> acids	0.88	0.072	4.8	0.07	1	3.34
	South American yellow-foot tortoise	$\beta$		•		Taurine	Mixture of C <sub>27</sub> acids	0.62	0.035	5.5	0.17	0.16	0.3
	Red-eared turtle	$\beta$		•		Taurine	C <sub>27</sub> tetraOH (22OH)	0.3	0.005	1.6	0.31	51.0	11.2
	Alligator snapping turtle (2)	$\beta$		•		Taurine	C <sub>27</sub> tetraOH (22OH)	0.52	0.166	2.05	0.012		
	Loggerhead turtle (2)	$\beta$		•		Taurine	C <sub>27</sub> tetraOH (22OH)	0.26	0.058	6.8	0.117	3.3	10.1
	Chinese alligator	$\beta$		•		Taurine	Mixture of C <sub>27</sub> acids	1.14	0.303	2.9	0.010	46.5	0.34
	Dwarf crocodile (2)	$\beta$		•		Taurine	Mixture of C <sub>27</sub> acids	1.1	0.005	7.15	1.59	4.4	2.6
	Parson's chameleon	$\alpha$			•	Taurine	alloC	-0.12	0.008	22.3	2.73	6.6	13.3
	Stump-tailed skink	$\alpha, \beta$			•	Taurine	C <sub>27</sub> , C <sub>24</sub> mixed acids	0.42	0.005	2.17	0.48	11.4	16.7
	Casque-headed iguana (2)	$\alpha, \beta$			•	Taurine	alloC, alloCDC, CDC	0.29	0.046	7.15	0.157	9.5	7.5
	Komodo dragon	$\beta$			•	Taurine	C <sub>27</sub> , C <sub>24</sub> mixed acids	0.32	0.012	12	1	2.3	1.3
	Chinese crocodile lizard (2)	$\beta$			•	Taurine	C <sub>27</sub> , C <sub>24</sub> mixed acids	0.41	0.025	2.7	0.115	1.6	0.5
	Russell's viper	$\beta$			•	Taurine	$\alpha$ OH-C, OH-DC	-0.26	0.004	0.57	0.13	3.0	2.1
	Death adder (2)	$\beta$			•	Taurine	C	-0.06	0.06	17.1	0.285	1.3	0.65
	Philippine habu viper	$\beta$			•	Taurine	C	-0.06	0.016	0.75	0.04		
	Green tree snake (2)	$\alpha, \beta$			•	Taurine	alloC, C	0.38	0.005	1	0.2	7.87	3.37
	Eastern indigo snake	$\beta$			•	Taurine	C, CDC	0.39	0.024	5.3	0.226	1.4	0.81
Rhinoceros viper	$\beta$			•	Taurine	$\alpha$ OH-C, $\alpha$ OH-DC, C	-0.16	0.032	1.4	0.043			

C, cholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; NC, not calculated because of the high proportion of bile alcohols; WHI, weighted hydrophobicity index.

<sup>a</sup>These values have been multiplied by 1,000.

TABLE 4. Biliary lipid composition and WHI values of birds

Species Name	C <sub>27</sub> Acids	C <sub>24</sub> Acids	Conjugation	Bile Acid Composition	WHI	Phospholipid-Bile Salt Ratio	Cholesterol-Bile Salt Ratio <sup>a</sup>	Cholesterol-Phospholipid Ratio	Campesterol-Cholesterol Ratio <sup>a</sup>	β-Sitosterol-Cholesterol Ratio <sup>a</sup>
Eastern emu (2)	•		Taurine	Mixed C <sub>27</sub> acids	0.25	0.094	11.8	0.126	1.06	2.08
Carmine bee eater	•		Taurine	Mixed C <sub>27</sub> acids	0.83	0.37	9.6	0.025	4.95	0.66
White-bellied go-away bird	•		Taurine	Mixed C <sub>27</sub> acids	0.14	0.029	2.56	0.09	0.54	1.18
Buton hornbill	•		Taurine	Mixed C <sub>27</sub> acids	1.22	0.44	52.9	0.120	2.2	
Andean condor	•	•	Taurine	Mixed C <sub>27</sub> and C <sub>24</sub> acids	0.75	0.062	0.17	0.003	1.48	0.98
California condor		•	Taurine	CDC, DC	1.15	0.119	1.44	0.012	0.91	
Golden eagle	•	•	Taurine	Mixed C <sub>27</sub> and C <sub>24</sub> acids	0.02	0.016				
Bali mynah		•	Taurine	C, CDC	0.62	0.029	1.5	0.05	94.6	32.0
Guam kingfisher	•	•	Taurine	C <sub>27</sub> triOH, C <sub>24</sub> triOH acids	0.09	0.35	1.1	0.002		
Zoe imperial pigeon		•	Taurine, glycine	CDC	0.57	0.20	4.5	0.02	14.9	15.8
African jacana		•	Taurine	CDC	0.36	0.018	2	0.12	51.2	6.3
New Guinea masked plover		•	Taurine	C, CDC	0.68	0.055	2.55	0.046	10.5	1.25
Crestless fireback pheasant		•	Taurine	CDC	0.31	0.041	3.5	0.08	1.76	0.55
Chicken		•	Taurine	C, CDC	1.01	0.067				
Gray wing trumpeter		•	Taurine	CDC	0.45	0.143	5.1	0.036	1.39	1.73
Stanley crane		•	Taurine	C	0.37	0.022	1.2	0.05	1.88	0.44
Guam rail		•	Taurine	C, CDC	0.05	0.015	2.7	0.18		
Yellow-billed duck		•	Taurine	CDC, αOH-CDC	0.22	0.338	18.1	0.054	3.69	2.07
Black neck swan (2)		•	Taurine	CDC, unknown triOH	0.46	0.053	6.2	0.117	2.11	1.72
Greater flamingo (2)		•	Taurine	CDC, αOH-CDC	0.19	0.021	2.35	0.112	4.2	2.55
Rockhopper penguin		•	Taurine	C, CDC, 16OH-CDCA	0.17	0.047	0	0	0.18	0.09

Abbreviations are as in Table 3.

<sup>a</sup> These values have been multiplied by 1,000. All bile acids had the 5β configuration.

TABLE 5. Biliary lipids and WHI values of diverse orders of mammals: ancient mammals, rodents, a lagomorph, cetaceans, marsupials, and insectivores

Order	Species Name	C <sub>27</sub> Alcohol	C <sub>24</sub> Acids	Conjugation	Major Bile Salts	WHI	Phospholipid-Bile Salt Ratio	Cholesterol-Bile Salt Ratio <sup>a</sup>	Cholesterol-Phospholipid Ratio	Campesterol-Cholesterol Ratio <sup>a</sup>	β-Sitosterol-Cholesterol Ratio <sup>a</sup>
Ancient mammals	Caribbean manatee (2)	•		Sulfate	Trichechols		0.524	4.45	0.01	0.6	1.6
	Pony	•	•	Taurine, sulfate	Complex mixture		0.064	52	0.8		
Rodents	Mountain paca		•	Glycine	Δ <sup>22</sup> -3αOH7oxo	-0.02	0.006	0	0		
	Nutria		•	Taurine, glycine	3αOH7oxo, UDC	-0.04	0.002	0	0		
	Bornean prevoat squirrel		•	Taurine	C, CDC, αOH-C	0.39	0.37	6.9	0.02	10.6	5.41
	Rat		•	Taurine, glycine	βMC, C, CDC, DC	0.21	0.11	14	0.13		
	Guinea pig		•	Glycine	CDC, 3αOH7oxo	0.33	0.011	3	0.15		
	Prairie dog		•	Taurine	C	0.41	0.19	26.1	0.08		
Mouse	Golden hamster		•	Taurine, glycine	C, MC, C, CDC, DC	0.38	0.11	26.5	0.24		
			•	Taurine, glycine	C, CDC, DC	0.52	0.039	11.8	0.3		
			•	Taurine, glycine	C, CDC, DC	0.52	0.039	11.8	0.3		
Lagomorph	Rabbit		•	Glycine	DC, C	1.01	0.09	17	0.2		
Marine mammals	Harbor seal		•	Taurine	αOH-CDC, CDC, C	0.04	0.09				
Cetacean	Bottle nose dolphin (2)		•	Taurine	C	0.42	0.608	12.1	0.02	2.09	28.8
	Dwarf sperm whale		•	Taurine	CDC, DC	0.82	0.067	0	0		
Marsupial	Bennet wallaby		•	Taurine	C, CDC, DC	0.58	0.11	3.06	0.03	114.7	14.26
	Parma wallaby		•	Taurine	C, CDC, DC	0.62	0.62	5.05	0.01	16.19	0
	Pademelon		•	Taurine	C, CDC, DC	1.02	0.215	5.99	0.03	62.15	22.48
	Northern koala (5)		•	Taurine	3αOH7oxo	0.21	0.062	3.7	0.05	0.58	4.27
Insectivore	Central African hedgehog		•	Taurine	C	0.42	0.19	5.36	0.03	3.35	0.46
	Aardvark		•	Taurine	C	0.72	0.72	64	0.07		

βMC, β-muricholic acid; MC, mixture of muricholic acid epimers. Other abbreviations are as in Table 3.

<sup>a</sup> These values have been multiplied by 1,000. All bile salts had the 5β configuration.

other than cholesterol were present in appreciable proportions in any species. Values are given for sitosterol-cholesterol ratios and campesterol-cholesterol ratios ( $\times 10^3$ ).

**Biliary lipid classes.** Conjugated bile acids ( $C_{24}$  and  $C_{27}$ ) were determined by reverse-phase HPLC using a C18 column and a methanol phosphate buffer system (29), as described (30). Retention times for some natural taurine and glycine conjugated bile acids relative to taurocholate are provided in **Tables 1, 2**. In some cases, bile acid peaks identified by HPLC were fractionated using thin-layer chromatography. The  $C_{24}$  conjugated bile acids were deconjugated by alkaline hydrolysis (1 N NaOH, 130°C, 4 h), isolated by solvent extraction, and examined by GC-MS, as described (30). Bile alcohol sulfates ( $C_{27}$ ) are not detected by the HPLC method, which quantifies bile acids based on the absorbance of the amide bond at 205 nm (29), but were estimated semi-quantitatively by electrospray mass spectrometry or GC-MS after solvolysis.

In 35 samples, phospholipid classes were measured using TLC to separate individual classes, followed by elution and phosphorus determination using the method of Rouser, Fleischer, and Yamamoto (25). Reference compounds (individual molecular species) were obtained from Avanti Polar Lipids (Alabaster, AL). In addition, a subset of samples was used for the quantification of phospholipid classes by HPLC separation followed by detection using an evaporative light-scattering detector. The HPLC system consisted of an LKB low-pressure mixer, a model 2248 pump (Pharmacia, Uppsala, Sweden), a Rheodyne injector, and a Vorex MKIII light-scattering detector (Alltech, Deerfield, IL). To per-

form phospholipid isolation, lipid classes were separated by HPLC using a 4  $\mu$ m lichrosphere normal-phase silica column purchased from Merck (Darmstadt, Germany) as the stationary phase. The light-scattering detector used compressed air as the nebulizing gas. Peaks were integrated using the EZChrom Chromatography Data System (Scientific Software, San Ramon, CA). This software was also used to calculate molar amounts of phospholipid classes from the constructed (nonlinear) calibration curve. Peaks eluting from the column were collected using a flow splitter between the column and the detector and were collected manually. The procedure used was validated previously (31, 32).

In some samples, the identities of PC and sphingomyelin classes were further confirmed by HPLC followed by MS. For on-line MS, the flow rate of the gas chromatograph was adjusted to 1 ml/min and a flow splitter was inserted between the column and the mass spectrometer. The flow into the mass spectrometer was set to  $\sim 0.1$  ml/min.

A Sciex 4000 QTRAP mass spectrometer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), fitted with an electrospray ion source operated at atmospheric pressure, was used to identify the components eluting from the column. Air was used as nebulizer gas and nitrogen as curtain gas. The capillary voltage was set to 5.5 kV, and the decluster potential (cone voltage) was set to 35 V.

### Data analysis

Biliary lipid composition was determined in 112 samples from 89 species. Phospholipid classes were determined in 35 species,

TABLE 6. Biliary lipids and WHI values of carnivores, bovids, and primates

Order	Species Name	Conjugation	Major Bile Acids	WHI	Phospholipid-Bile Acid Ratio	Cholesterol-Bile Acid Ratio <sup>a</sup>	Cholesterol-Phospholipid Ratio	Campesterol-Cholesterol Ratio <sup>a</sup>	$\beta$ -Sitosterol-Cholesterol Ratio <sup>a</sup>
Carnivores	Sumatran tiger (2)	Taurine	C, CDC, DC	0.51	0.244	0.238	0.001	2.61	1.5
	Cat (domestic)	Taurine	C, CDC, DC	0.49	0.16	3.8	0.03		
	Arabian wildcat	Taurine	C, CDC, DC	0.81	0.033	1.8	0.055	4.28	2.71
	African civet (4)	Taurine	C, CDC, DC	0.92	0.176	12.7	0.23	6.38	1.28
	Guiana bush dog	Taurine	C, CDC	0.49	0.261	14.2	0.054	25.3	15.7
	Red panda	Taurine	C, CDC	0.41	0.147	4.4	0.023		
	Kinkajou	Taurine	CDC, DC	0.81	0.527	27.2	0.051	0.32	0.8
	Malayan sun bear	Taurine	CDC, DC	0.92	0.113	21.5	0.19	2.74	2.68
	Dog	Taurine	C, CDC, DC	0.71	0.19	8.5	0.044		
Bovids	Hippopotamus	Taurine, glycine	C, CDC, DC	0.91	0.25	14.7	0.001		
	Nubian ibex	Taurine	C, CDC, DC	0.81	0.29	15	0.039		
	Javan banteng (2)	Taurine, glycine	C	0.71	0.21	3.05	0.0145	1.16	
	Central Chinese goral	Taurine	C	0.79	0.423	11.3	0.027	0.11	0.25
	White-tailed gnu	Taurine	C	0.52	0.049	1.8	0.036	9.64	0.92
	Slender horn gazelle	Taurine	C, DC	0.99	0.19	31.8	0.167	1.19	0.5
	Black-faced impala	Taurine	C	0.53	0.166	15.1	0.09		
	Ellipsen waterbuck (2)	Taurine, glycine	C	0.72	0.109	7	0.064		
	Gemsbok (2)	Taurine, glycine	C	0.61	0.103	37	0.222		
	Sheep (domestic)	Taurine	C, DC, CDC	0.82	0.11	29.8	0.27		
	Cattle (domestic)	Taurine, glycine	C, DC, CDC	0.93	0.1	25	0.25		
	Lowland wisent	Taurine, glycine	C, CDC, DC	0.91	0.323	9.3	0.028	0.17	0.08
	European wild boar (2)	Glycine	HC, HDC, C	0.02	0.1385	24.9	0.181	0.6	0.17
	Pig (domestic)	Taurine, glycine	HC, HDC, C	0.69	0.25	66	0.26		
	Siberian musk deer	Taurine, glycine	C, CDC	0.49	0.229	12.2	0.053	3.24	
Primates	Mantled howler	Taurine, glycine	C, CDC, DC	0.99	0.045	10.7	0.236		
	Squirrel monkey	Taurine	C, CDC	0.91	0.28	23.1	0.083		
	Pygmy marmoset	Taurine	C, alloC, CDC	0.62	0.286	24.9	0.087	2.18	2.86
	Gueraza	Taurine, glycine	C, CDC, DC	0.78	0.054	2.1	0.04	59.7	46.4
	Rhesus macaque	Taurine	C, CDC, DC	0.97	0.1	18.3	0.19		
	Lion-tailed macaque (4)	Taurine, glycine	C, CDC, DC	1.23	0.141	26.8	0.022	4.56	4.18
	Douc langur (2)	Taurine, glycine	C	0.48	0.066	2.75	0.042	2.28	2.54
	Mainland drill (2)	Taurine, glycine	C, CDC, DC	0.52	0.097	28.6	0.295	2.42	5.14
	Human	Taurine, glycine	C, CDC, DC	0.24	0.3	66	0.2		

HC, hyocholic acid; HDC, hyodeoxycholic acid. Other abbreviations are as in Table 3.

<sup>a</sup> These values have been multiplied by 1,000. All bile acids had the 5 $\beta$  configuration.

including 4 additional species. Values for individual sterols (determined by GC-MS) were obtained in 60 species. Values for biliary lipid composition in 15 species were taken from the literature (33–47). Species were classified by taxonomic class, order, family, and genus (see supplemental Tables 1–4). Species are denoted by their common English names in the tables providing biliary lipid composition.

Bile salts were divided into five classes according to the nature of the side chain (22): type I, only C<sub>27</sub> bile alcohols; type II, C<sub>27</sub> bile alcohols and C<sub>27</sub> bile acids; type III, only C<sub>27</sub> bile acids; type IV, C<sub>27</sub> bile acids and C<sub>24</sub> bile acids; type V, only C<sub>24</sub> bile acids. Two possible additional categories—the combination of C<sub>27</sub> bile alcohols and C<sub>24</sub> bile acids, or the simultaneous occurrence of all three bile salt classes—were not found in any of the species examined.

Data were expressed as ratios of biliary lipids rather than as absolute amounts to correct for differences in biliary concentration in the gallbladder. Data were expressed as coupling coefficients (i.e., phospholipid-bile salts, cholesterol-bile salts, and cholesterol-phospholipid ratios) (48). Because the cholesterol-bile salt ratio was very low in many species, data are expressed as the cholesterol-bile salt ratio  $\times 10^3$ . The phospholipid-bile salt ratio is a measure of the canalicular phospholipid secretion induced by bile salt secretion, and the cholesterol-bile salt ratio is a measure of the canalicular cholesterol secretion induced directly or indirectly by bile salt secretion. For both PC and cholesterol, post-canalicular fluxes (absorption and secretion) could, in principle, modify the measured ratios. The cholesterol-phospholipid ratio is a measure of vesicle and/or mixed micelle composition. Plant sterols were expressed as sterol-cholesterol ratios  $\times 10^3$ .

Bile samples were obtained from two independent animals in 20 species, and in these cases, the lipid values were averaged. Interanimal variation was determined by calculating percentage difference from the mean. It averaged 44% for phospholipid-bile salt ratios and 46% for cholesterol-bile salt ratios, indicating that interanimal variation was considerable. However, 11 of these 20 samples were obtained from nonmammalian species in which phospholipid-bile salt and cholesterol-bile salt ratios were very low.

A weighted hydrophobicity index (WHI) was calculated for each sample containing only bile acids and for the few samples that contained bile alcohols when the relative retention time (RRT) of the bile alcohol sulfate was known. The hydrophobicity index (HI) of each bile acid was based on the integrated area of its HPLC peak and its RRT. When the natural logarithm of the RRT was plotted against the HI values published by Heuman (49), the following equation was obtained:  $HI = -0.09 + 0.905 \ln RRT$ . All peaks  $>5\%$  of total peak area were considered bile acids, even if their RRTs did not correspond to any known bile acid. Peaks that were  $<5\%$  of total peak area were not considered. Because the RRT of glycine conjugated bile acids is influenced by the pH of the solvent buffer, the masses of all glycine conjugated bile acids were assigned to their corresponding taurine conjugated bile acid. Glycine conjugates were present only in one bird (pigeon) and in some mammals (rodents, bovids, primates). RRT values for common natural bile acids and their corresponding HI values are given in Tables 1, 2. WHI values were not calculated for most samples containing an appreciable proportion of C<sub>27</sub> bile alcohols (by mass spectrometry), as these are not detected by the HPLC method used. The weighted average for each bile sample was calculated as described by Heuman (49).

Cholesterol saturation was calculated using the equations developed by Thomas and Hofmann (50), which are based on the original measurements of Hegardt and Dam (51) and Carey and Small (52). This calculation is based on the unvalidated assumption that the solubility relationships in model systems simulating human bile hold for biliary lipids in all vertebrates; nonetheless,

this value provides some indication of the extent to which a bile sample is saturated with cholesterol.

## Statistical analysis

Figures were constructed and correlation coefficients were calculated using Sigmaplot™ software (Systat Software, Inc., Point Richmond, CA). Differences in lipid ratios between mammals and nonmammals were tested for statistical significance using the Mann-Whitney nonparametric test.

## RESULTS

### Biliary phospholipids

Figure 1 shows the phospholipid-bile salt ratios by vertebrate class expressed in both linear coordinates (left panel) and logarithmic coordinates (right panel). Median values are indicated by horizontal lines for each class, and the data used in the figure are provided in Tables 3–6, which also provide data on the major types of bile salts that were present. The phospholipid-bile salt ratio is low in cartilaginous fish and amphibians but has a broad range of values in all other vertebrate classes. With the exception of rodents, mammals tended to have higher phospholipid-bile salt ratios than nonmammalian species. The phospholipid-bile salt ratio tended to be lower in bile containing C<sub>27</sub> bile alcohols alone (class I) or in bile containing C<sub>27</sub> bile alcohols and C<sub>27</sub> bile acids (class II) than in the remaining three bile salt classes (Fig. 2).

In Table 7, the individual phospholipid classes of 35 vertebrates are summarized. The phospholipids in Table 7 and Tables 3–6 were measured using differing methodologies, so the phospholipid-bile salt ratios are not always identical. Those species with low levels of biliary phospholipids, such as the Komodo dragon, tended to have biliary phospholipids enriched in sphingomyelin, as determined using HPLC-MS (Fig. 3 and supplementary Figs. I and II).

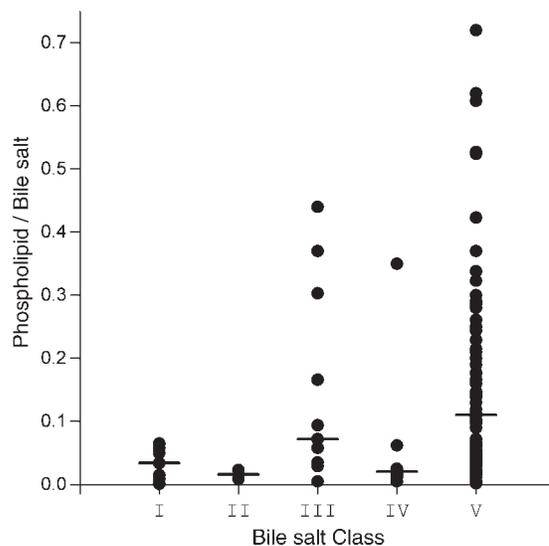


Fig. 2. Relationship between the type of bile salt present and the phospholipid-bile salt ratio. Bile salt class I, C<sub>27</sub> bile alcohols; class II, C<sub>27</sub> bile alcohols and C<sub>27</sub> bile acids; class III, C<sub>27</sub> bile acids; class IV, C<sub>27</sub> bile acids and C<sub>24</sub> bile acids; class V, C<sub>24</sub> bile acids.

TABLE 7. Bile salt classes, phospholipid-bile salt ratios, individual phospholipid classes, and WHI values for a spectrum of vertebrates

Order	Species	Bile Salt Class	Phospholipid-Bile Salt Ratio	Phospholipid Classes					WHI
				PC	SM	PI	PS	PE	
Reptiles	Chinese alligator	III	0.76	82.4	9.0	0.0	3.4	5.1	1.14
	Chinese crocodile lizard	IV	0.08	78.0	22.0	0.0	0.0	0.0	0.41
	Komodo dragon	IV	0.012	37.1	48.1	0.0	8.1	5.1	0.32
	Loggerhead sea turtle	III	0.11	20.7	79.3	0.0	0.0	0.0	0.26
Cartilaginous fish	Cow-nose ray	I	0.058	68.1	24.9	0.9	3.8	2.3	-0.05
Bony fish	Australian lungfish	I	0.034	40.8	59.2	0.0	0.0	0.0	NC
	Electric eel	V	0.13	37.1	48.1	0.0	8.1	0.0	0.12
	Garpike	V	0.072	73.3	21.4	1.5	2.4	1.5	-0.06
Birds	Black neck swan	V	0.071	76.7	11.3	0.0	4.3	0.0	0.46
	Golden eagle	IV	0.016	45.0	50.9	4.1	0.0	0.0	0.02
	Grey-winged trumpeter	V	0.78	96.0	0.0	0.0	4.0	0.0	0.45
	Rockhopper penguin	V	0.047	56.1	22.2	0.0	0.0	21.7	0.17
	Buton hornbill	III	0.62	68.1	1.3	1.3	17.1	0.0	1.22
	Yellow-billed duck	V	0.018	67.6	13.7	0.0	18.7	0.0	0.22
	Caribbean greater flamingo	V	0.18	86.9	13.1	0.0	0.0	0.0	0.19
	Chicken	V	0.067	78.2	0.0	0.0	0.0	29.2	1.01
	African pygmy falcon	V	0.031	8.9	1.7	0.0	89.5	0.0	0.88
Mammals	Caribbean manatee	I	0.65	75.8	12.8	0.0	11.4	0.0	NC
	Capybara	V	0.36	79.8	20.2	0.0	0.0	0.0	0.97
	Rat (Sprague-Dawley) <sup>a</sup>	V	0.18	100.0	0.0	0.0	0.0	0.0	0.21
	Guinea pig <sup>a</sup>	V	0.004	100.0	0.0	0.0	0.0	0.0	0.33
	Queensland koala	V	0.07	53.2	29.4	0.0	15.6	1.8	0.21
	South African aardvark	V	0.72	95.0	1.2	2.0	2.8	0.0	0.72
	Harbor seal	V	0.09	92.2	1.8	2.9	0.0	0.0	0.04
	Sumatran tiger	V	0.6	96.0	0.0	0.0	4.0	0.0	0.51
	Dog <sup>a</sup>	V	0.04	94.5	0.0	0.0	0.0	5.5	0.71
	River hippopotamus	V	0.62	100.0	0.0	0.0	0.0	0.0	0.91
	Nubian ibex	V	0.29	100.0	0.0	0.0	0.0	0.0	0.81
	Sheep <sup>a,b</sup>	V	0.047	95.1	5.9	0.0	0.0	0.0	0.82
	Ox <sup>a</sup>	V	0.1	100.0	0.0	0.0	0.0	0.0	0.93
	Pig <sup>a</sup>	V	0.1	100.0	0.0	0.0	0.0	0.0	0.69
	Lion-tailed macaque	V	0.19	83.6	5.2	1.6	8.8	0.8	1.23
	Douc langur	V	0.07	83.9	11.3	0.5	4.3	0.0	0.48
	Mainland drill	V	0.29	96.9	3.1	0.0	0.0	0.0	0.52
Human <sup>a</sup>	V	0.3	95	0	0	0	5	0.24	

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

<sup>a</sup> Values published by Alvaro et al. (33).

<sup>b</sup> Includes 20.1% lysoPC.

As the ratio of phospholipid to bile salts increased, biliary phospholipids became predominantly PC. This is illustrated in **Fig. 4**, where the phospholipid-bile salt ratio is plotted in relationship to the proportion of PC in bile.

The relationships between phospholipid-bile salt ratios and the calculated WHI values are shown in **Fig. 5**. In the scatterplot, values from animals having bile salts of classes I–IV and class V (only C<sub>24</sub> bile acids) are distinguished. There was a positive but weak relationship between phospholipid-bile salt ratios and the WHI values.

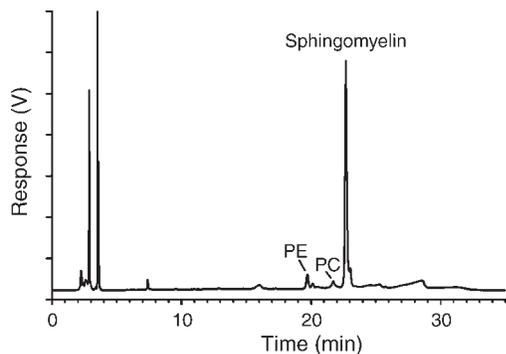
### Biliary cholesterol and plant sterols

The cholesterol-bile salt ratios for the bile are plotted using both linear and logarithmic coordinates (**Fig. 6**). Cholesterol was absent from bile in five species: the Australian lungfish, rockhopper penguin, mountain paca, nutria, and the Bennett wallaby. However, because cholesterol was identified in the bile of other closely related species, multiple samples of the biles of these animals are needed to confirm this finding. Median values were lower in fish, reptiles, amphibians, and birds than in most but

not all mammals. The cholesterol-bile salt ratio was significantly higher in mammals than in nonmammals ( $P < 0.001$ ).

Cholesterol-bile salt ratios would be predicted to be low in species with low phospholipid-bile salt ratios, because the major determinant of cholesterol solubility in model systems is the phospholipid-bile salt ratio (52). However, when cholesterol-bile salt ratios were plotted relative to phospholipid-bile salt ratios (**Fig. 7**), no general relationship between the two variables was apparent. **Figure 8** shows cholesterol-bile salt ratios in relation to bile salt class. The levels vary over a much wider range in the animals having only C<sub>24</sub> bile acids (class V). Some species possessing C<sub>24</sub> bile acids, such as the dog (48), had high phospholipid-bile salt ratios and little cholesterol.

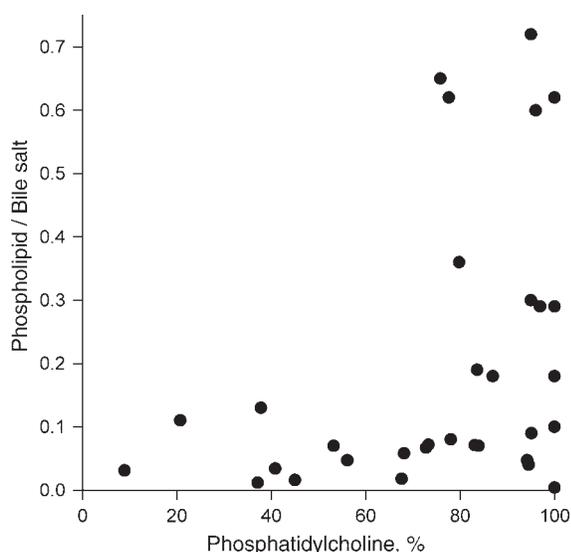
Biliary cholesterol saturation was calculated using the method of Thomas and Hofmann (50), based on Hegardt and Dam (51) and Carey and Small (52). Most samples were highly unsaturated as a result of low cholesterol-bile acid ratios (data not shown). Plant sterols were trace constituents in the bile of all species.



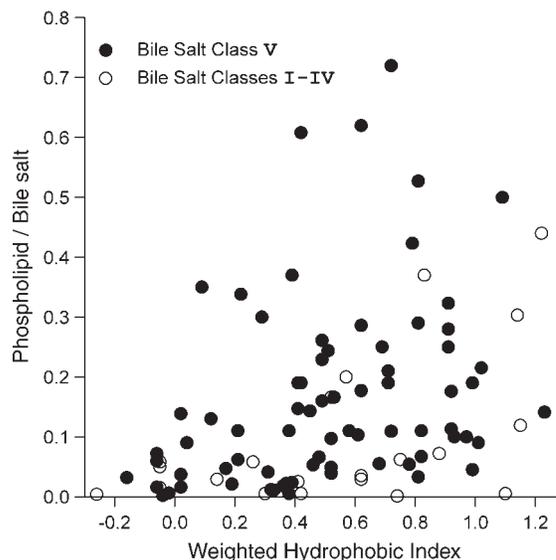
**Fig. 3.** HPLC evaporative light-scattering detection separation of Komodo dragon bile, enriched in sphingomyelin. The sample corresponds to that of MS analysis in supplementary Figure I. PC, phosphatidylcholine; PE, phosphatidylethanolamine.

## DISCUSSION

To the best of our knowledge, this is the first extensive survey of biliary lipid composition in vertebrates. The chemical analysis of bile acids and alcohols in bile was initiated more than a century ago, but the analysis of biliary phospholipids and cholesterol did not attract the interest of lipid biochemists until more recently. Here, we show that the dominant organic constituent of bile in most cartilaginous fish, reptiles, and some birds is bile salt (conjugated bile acids or bile alcohol sulfates). Most fish and reptiles have low phospholipid-bile salt ratios, whereas there is a wide range of phospholipid-bile salt ratios in birds and mammals. No relationship was found between the type of bile salt— $C_{27}$  bile alcohol,  $C_{27}$  bile acid, or  $C_{24}$  bile acid—and the phospholipid-bile salt ratio, except that animals that synthesize only  $C_{24}$  bile acids tended to have higher phospholipid-bile salt ratios. Thus, biliary phospholipid secretion appears to have evolved before side chain oxida-



**Fig. 4.** Relationship between the percentage of PC in biliary lipids and the phospholipid-bile salt ratio. In samples having a high phospholipid-bile salt ratio, PC is the dominant biliary lipid.

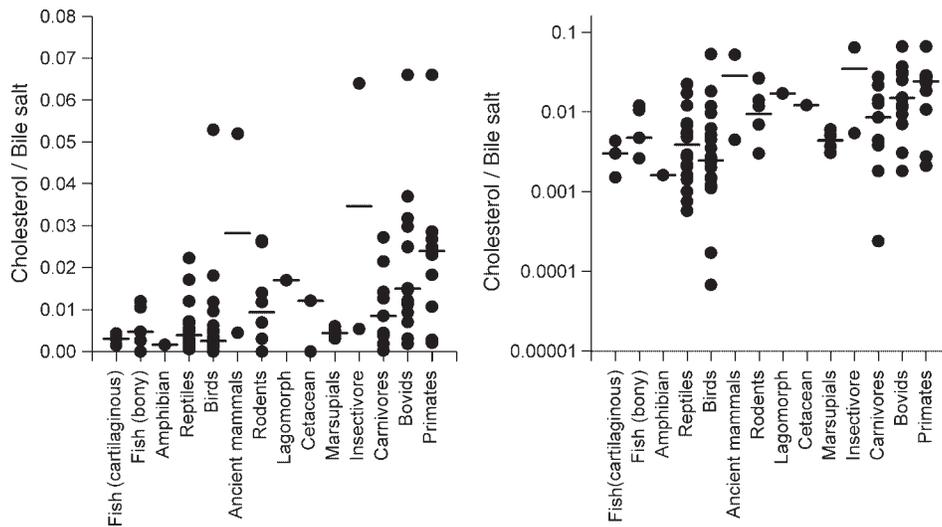


**Fig. 5.** Relationship between the weighted hydrophobic index of bile samples and the phospholipid-bile salt ratios. Open circles denote values for species having bile salt classes I–IV; closed circles denote values for species having bile salt class V ( $C_{24}$  bile acids). (For definitions of bile salt classes, see legend to Fig. 2.) For bile salt classes I–IV, the correlation coefficient was 0.55; for species having bile salt class V, the correlation coefficient was 0.29; for all bile salts, the correlation coefficient was 0.34.

tion (from  $C_{27}$  to  $C_{24}$ ) of bile salts. Cholesterol was present in the bile of almost all species sampled, and both the phospholipid-bile salt ratios and cholesterol-bile salt ratios tended to be higher in mammals than in nonmammals.

Because phospholipids have a cytoprotective effect in bile (53), we expected the phospholipid-bile salt ratio to correlate with the WHI values. Although there was a weak but significant positive relationship between the phospholipid-bile salt ratio and the WHI values, some species had bile with a high WHI value and a low phospholipid-bile salt ratio, raising the question of how the biliary tract epithelium is protected from the cytotoxicity of the bile salts in such animals. Several mechanisms can be envisioned. First, the relationship between cytotoxicity and HI values has been established for only a few  $C_{24}$  bile acids, and it is possible that this relationship may not extend to other bile acids and bile alcohols. Second, it is also possible that some of the HPLC peaks may not have been bile acids. Third, for mammals such as the guinea pig, bile secretion appears to be driven by bicarbonate and bile acid concentrations are low, mitigating bile acid cytotoxicity (16). In other species, such as rabbits, bile contains quite hydrophobic bile acids (30) yet has a low phospholipid-bile salt ratio; the rabbit biliary tract epithelium is highly resistant to the cytotoxic effects of bile acids. Finally, in some species, biliary phospholipids contain a high proportion of sphingomyelin, which is more potent than PC in decreasing the cytotoxicity of mixed micelles (54).

A survey of biliary lipid composition provides no direct information on canalicular transporters mediating biliary lipid secretion. It is reasonable to postulate that in sam-

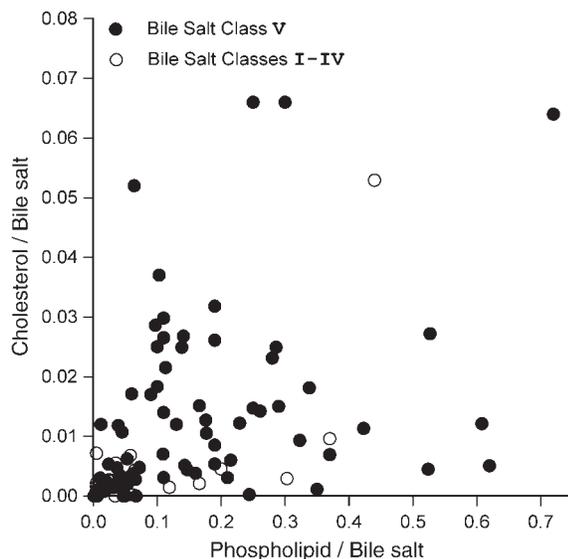


**Fig. 6.** Cholesterol-bile salt ratios shown in different vertebrate classes using a linear scale (left) and a logarithmic scale (right). Median cholesterol-bile salt ratios are indicated by horizontal lines for each class. The cholesterol-bile salt ratio was significantly higher in mammals than in nonmammals ( $P < 0.001$ ).

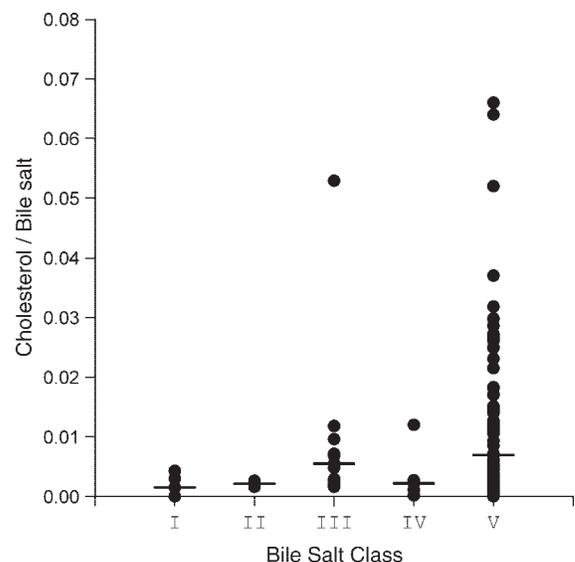
ples having a high phospholipid-bile salt ratio that a canalicular phospholipid transporter such as *Abcb4* for PC might be present. *Abcb4* has been identified in all vertebrate species for which total genome sequence is available, but not in any invertebrates. Whether there is a canalicular transporter for sphingomyelin in those species having sphingomyelin as the dominant biliary phospholipid is not known.

Cholesterol was detected in the bile of almost all species. A low cholesterol-bile salt ratio could result from ei-

ther little cholesterol being secreted into canalicular bile or cholesterol being absorbed in the biliary tract, or both. There is good experimental evidence for the absorption of cholesterol by cholangiocytes (55) or by gallbladder epithelial cells (56). In mammals, ABCG5 and ABCG8 are the major conduits for the transport of both animal-derived (cholesterol) and plant sterols. The plant sterols detected in the bile of these animals almost certainly reflect dietary intake. We had hoped that these data would provide additional insights into the evolutionary origin of ABCG5 and ABCG8, but this does not appear to be the case, and it now seems that genome sequencing of early vertebrates will be more informative. No ortholog of ABCG5 or ABCG8 is apparent in the genome sequence of the protochordate



**Fig. 7.** Relationship between phospholipid-bile salt ratio and cholesterol-bile salt ratio. Open circles denote values for species having bile salt classes I-IV; closed circles denote values for species having bile salt class V ( $C_{24}$  bile acids). For bile salt classes I-IV, the correlation coefficient was 0.68; for bile salt class V ( $C_{24}$  bile acids), the correlation coefficient was 0.34; for all bile salt classes, the correlation coefficient was 0.43. For definition of bile salt classes, see legend to Fig. 2.



**Fig. 8.** Relationship between cholesterol-bile salt ratios and type of bile salt present. For definition of bile salt classes, see legend to Fig. 2.

*Ciona intestinalis*, but ABCG5 and ABCG8 are present in head-to-head orientation in bony fish and amphibians. These data suggest that ABCG5 and ABCG8 arose early in the vertebrate lineage. Sequencing of a gnathostome such as lamprey and an elasmobranch such as shark will help to further elucidate the evolutionary origin of these proteins.

Given the unknown extent of absorption of bile salts and cholesterol in the small intestine, it is impossible to calculate from the measurement of biliary lipids per se what fraction of cholesterol is eliminated from the body by biotransformation to bile salts compared with what fraction is eliminated as cholesterol. Such can only be determined by the sterol balance technique that has been widely used in the human but relatively little used in other vertebrate species. However, the extremely low cholesterol-bile salt ratio in reptiles, some fishes, and some birds strongly suggests that in these species most cholesterol is eliminated from the body by conversion to bile salts. In the human, in contrast, the direct biliary secretion of cholesterol results in the majority of cholesterol being eliminated from the body in the chemical form of cholesterol rather than bile acids.

Studies of biliary bile salt composition of vertebrates during the past two centuries have shown the remarkable biodiversity in the chemical structure of bile salts. The present study extends these studies by showing that in vertebrates, biliary phospholipids also differ considerably in proportion and type. 

The authors thank the Pathology Laboratory of the San Diego Zoo; Sea World in San Diego and Coral Gables, FL; Dr. G. A. D. Haslewood (now deceased), Professor of Biochemistry at Guy's Hospital (London UK); Dr. Valentine Lance (Department of Biology, San Diego State University), and Dr. H. Johnston (Veterinarian of the County of San Diego) for kindly providing the animal bile samples; and Drs. Karin Nibbering, Willem Renooij, and Martin de Smet (Utrecht, The Netherlands) for criticism and helpful discussions. This work was partly supported by the Zoological Society of San Diego and by National Institutes of Health Grants DK-64891 (to A.F.H.), HL-2098, and HL-72304 (to H.H.H.).

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