# A Function of Lung Surfactant Protein SP-B

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The primary function of lung surfactant is to form monolayers at the alveolar interface capable of lowering the normal surface tension to near zero. To accomplish this process, the surfactant must be capable of maintaining a coherent, tightly packed monolayer that avoids collapse during expiration. The positively charged amino-terminal peptide SP-B1-25 of lung surfactant–specific protein SP-B increases the collapse pressure of an important component of lung surfactant, palmitic acid (PA), to nearly 70 millinewtons per meter. This alteration of the PA isotherms removes the driving force for "squeeze-out" of the fatty acids from the primarily dipalmitoylphosphatidylcholine monolayers of lung surfactant. An uncharged mutant of SP-B1-25 induced little change in the isotherms, suggesting that a specific charge interaction between the cationic peptide and the anionic lipid is responsible for the stabilization. The effect of SP-B1-25 on fatty acid isotherms is remarkably similar to that of simple poly-cations, suggesting that such polymers might be useful as components of replacement surfactants for the treatment of respiratory distress syndrome.

A mixture of lipids and proteins, commonly known as lung surfactant, lines the pulmonary air spaces of all mammalian species. Human lung surfactant contains at least three specific proteins, SP-A, SP-B, and SP-C, along with several lipid species, primarily dipalmitoylphosphatidylcholine (DPPC), with smaller fractions of anionic lipids such as phosphatidylglycerol (PG) and fatty acids {typically PA [$CH_3(CH_2)_{14}COOH$]} (*1*). The primary function of lung surfactant is to form monolayers at the alveolus air-water interface that are capable of lowering the normal surface tension (72 mN/m) to near zero (*1, 2*). To accomplish this reduction, surfactant must quickly absorb to the interface and be capable of maintaining a coherent, tightly packed monolayer that avoids collapse even under the high compressions that accompany expiration. Monolayer collapse results in the expulsion of surfactant material from the surface monolayer into a three-dimensional bulk phase, usually with an increase in surface tension (a decrease in the surface pressure) as the monolayer relaxes into the bulk phase.

Deficiency and inactivation of lung surfactant are contributing factors to a number of pulmonary diseases. A deficiency of surfactant at birth, often due to premature delivery, is responsible for the development of neonatal respiratory distress syndrome (*1, 3*). Inactivation of lung surfactant, perhaps as a result of pulmonary edema, is likely involved in the development of adult respiratory distress syndrome (*4–7*). As a consequence of the serious morbidity and mortality of these syndromes, surfactant replacement therapy is being investigated by a number of research groups and is increasingly used in the treatment of premature infants (*8*).

The major component of human lung surfactant, DPPC, is capable of forming monolayers that do not collapse at surface pressures ≥70 mN/m, which correspond to near zero surface tensions (*2*). However, DPPC by itself is a poor lung surfactant in that it forms rigid, multilamellar structures in solution that do not effectively form monolayers under physiological conditions (*9, 10*). The apparent role of the anionic PG and PA in natural lung surfactant is to fluidize and separate the normally rigid DPPC bilayers in solution to enhance dispersion of the surfactant and increase absorption to interfaces (*9, 10*). However, the monolayer collapse pressures of anionic lipids and fatty acids are typically much less than that of DPPC (usually 30 to 50 mN/m). Lower collapse pressures lead to the ejection of these components from simple anionic lipid–DPPC mixed monolayers under sufficient compression. This effect has led to the belief that anionic lipids and fatty acids are also ejected from lung surfactant monolayers, a phenomenon that has been referred to as "squeeze-out" (*10–12*). However, in addition to DPPC, anionic phospholipids, and fatty acids, lung surfactant contains specific proteins. One of these proteins, SP-B, a low molecular weight amphiphilic protein with reduced mass of 5 kD (*13, 14*), appears to be essential to surfactant function in vivo (*13*). It has several

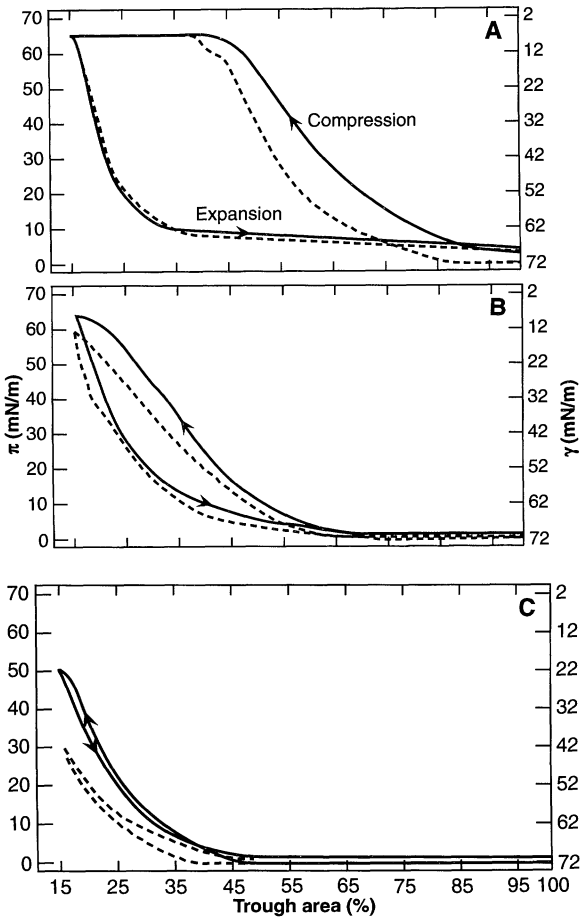


**Fig. 1.** Wilhelmy balance compression and expansion tracings of pressure-area curves for model lung surfactant dispersions (*2*) containing (**A**) DPPC:PG:PA:SP-B1-25, 68:21:9:3 w/w; (**B**) DPPC:PG:PA:SP-B1-25, 68:21:6:3 w/w; and (**C**) DPPC:PG:PA, 68:21:9 w/w. Displayed are the first (dashed trace) and fifth (solid trace) compression cycles of dispersions on 154 mM saline solution. The abscissa is displayed in percentage of trough area available to the surfactant monolayer. A dispersion of 25 μg in 10 μl of saline was applied to the surface of a Kim Ray Langmuir-Whilhelmy surface balance with 51.5 $cm^2$ of surface area at 25°C. The complete compression-expansion cycle took 90 s to complete. The DPPC:PG:PA:SP-B1-25 (68:21:9:3 w/w) dispersion raises the surface pressure immediately as the surface area is reduced, and the hysteresis between expansion and compression cycles obtained on the first compression is repeated on the fifth and subsequent compressions. The other tracings demonstrate diminished surface activity, especially a diminished hysteresis between compression and expansion cycles and a lower maximum surface pressure (higher minimum surface tension).

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positively charged residues, associates primarily with the negatively charged lipids and fatty acids in bilayers, and is accessible to the membrane surface (*9, 13–15*).

One role of SP-B is to alter the isotherms of fatty acid monolayers so that they resemble those of DPPC. In particular, the collapse pressure of the fatty acid monolayer is increased to nearly 70 mN/m and a highly compressible region of the isotherm is created at large areas per molecule. These changes in the fatty acid isotherm eliminate the driving force that causes squeeze-out of the fatty acid from the mixed surfactant monolayer and lead to lower surface tensions of the mixed lung surfactant. We see little change in the fatty acid isotherms when an uncharged mutant of SP-B is used, which confirms that stabilization occurs through a specific charge interaction between the negatively charged fatty acids and the positively charged protein. The effects of SP-B on the monolayer are virtually identical to those produced by simple polymer cations in solution (*16*). This close similarity is especially important because SP-B is the most expensive and difficult to obtain component of replacement surfactants for treatment of respiratory distress syndrome.

Experimental investigations and surfactant replacement therapies that use SP-B are expensive because the protein is difficult to obtain from animal or human sources. Therefore, we have focused on the study of amphipathic peptides of the amino-terminal residues (1 to 25) of SP-B known as SP-B1-25, which contain four positively charged residues (*17*). Researchers have investigated other sequences of SP-B or even more simple amino acid sequences that mimic certain properties of SP-B (*17–25*). The surface behavior of model lung-surfactant mixtures [containing DPPC and PG (68:21 ratio w/w)] indicates that monolayers with low surface tensions, similar to those of natural lung surfactant, are obtained with 9% w/w PA and 3% w/w SP-B1-25 (*26, 27*) (Fig. 1A) and improve oxygenation in animal models (*17*). A lower percentage of PA and a deficiency of SP-B1-25 resulted in diminished surface activity of the surfactant (Fig. 1, B and C). This effect is not surprising because PA is a significant fraction of natural lung surfactant and is commonly added to both naturally derived and synthetic replacement lung surfactants to enhance performance (*18*).

Isotherms of monolayers of PA or arachidic acid (AA) [$CH_3(CH_2)_{18}COOH$] (Fluka, 99%) with various concentrations of SP-B1-25 show the dramatic effects of this peptide on surface phase behavior (Fig. 2, A and B). The fatty acid species and peptide were spread quantitatively from a solution (0.7 to 2 mg/ml) of $CH_3Cl$ (Fisher spectranalyzed) onto a clean water surface (Milli-Q, Millipore) in a commercial Langmuir trough with a surface area of 1000 $cm^2$ at 23° ± 0.5°C (Nima Technology, Coventry, United Kingdom). The monolayer area was compressed at various speeds from 0.005 to 0.05 $Å^2$ per molecule per second. We observed no changes in the isotherms over this range of compression rates. The collapse pressure is a kinetic effect and so depends somewhat on the speed of compression, the type of barrier, and the geometry of the Langmuir trough. However, quantitative comparisons can be made between different monolayers if these variables are held constant between experiments (*28*).

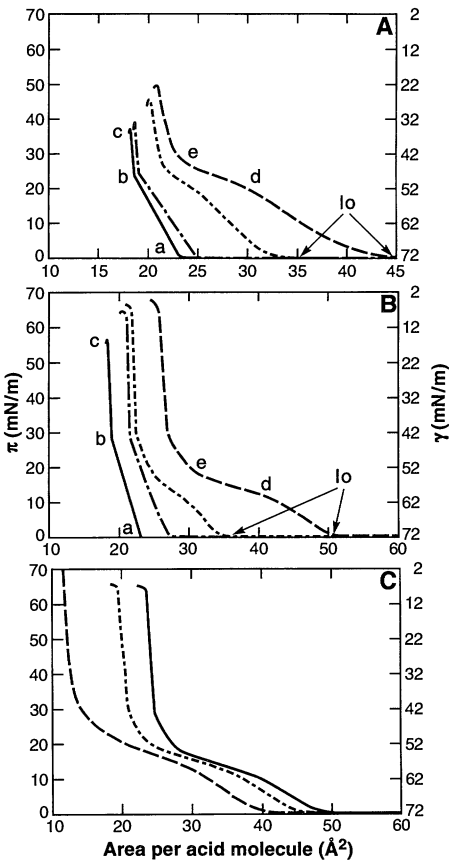


**Fig. 2.** Surface pressure versus area per fatty acid molecule isotherms for (**A**) PA and (**B** and **C**) AA as a function of peptide weight fraction. (A) Combined PA/SP-B1-25 on a pure water subphase and (B) AA/SP-B1-25 on a pure water subphase: 0% w/w peptide (solid trace); 2.0% w/w peptide (alternating solid and dashed trace; 0.21% mol/mol in PA, 0.25% mol/mol in AA); 10% w/w peptide (alternating dotted and dashed trace; 1.8% mol/mol in PA, 2.0% mol/mol in AA); and 20% w/w peptide (dashed trace; 5.4% mol/mol in PA, 5.7% mol/mol in AA). The isotherms of fatty acid alone showed two distinct phases: (segment a to b) a compressible liquid-like phase and (segment b to c) an incompressible solid-like phase (*29*). In addition to these phases, 10 and 20% w/w SP-B1-25 displayed a flattened region (segment d to e), typical of a two-phase coexistence region. Lift-off (lo) in the 10 and 20% SP-B1-25 isotherms occurs at a larger area per molecule than in pure fatty acid, indicative of a longer range of interaction between molecules in the presence of SP-B1-25. Isotherms were performed on a Nima trough that was thermostated at 23°C at compression speeds ranging from 0.005 to 0.05 $Å^2$/mol per second. (C) Isotherms of AA:SP-B1-25 (80:20 w/w) on a pure water subphase as a function of temperature: 23°C (solid trace), 31°C (dotted and dashed trace), and 37°C (dashed trace). The surface pressure of phase coexistence (the flattened, horizontal region in the isotherms) occurs at higher surface pressure with increasing temperature.

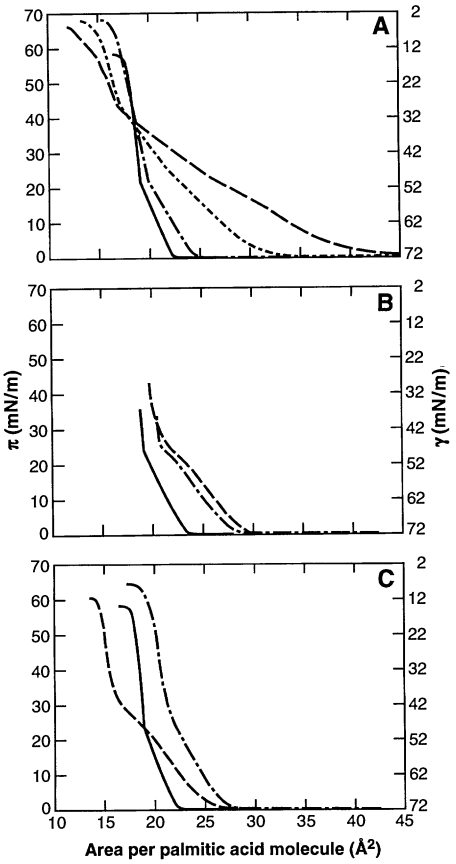


**Fig. 3.** Effects of ionic strength and peptide charge on monolayer behavior. (**A**) Isotherm of PA/SP-B1-25 on a $NaHCO_3$-buffered (pH 6.6, 154 mM) saline subphase, which shows a substantial increase in collapse pressure from pure water (55 mN/m compared with 37 mN/m) (see Fig. 2A). (**B**) Isotherm of PA using a neutral, mutant peptide (SP-B1-25m) with polar serine residues to replace positively charged lysine and arginine residues of SP-B1-25 on pure water subphase. (**C**) Isotherm of PA/SP-B1-25m on a $NaHCO_3$-buffered (pH 6.6, 154 mM) saline subphase: 0% w/w peptide (solid trace); 2.0% w/w peptide (solid and dashed trace; 0.23% SP-B1-25m mol/mol); 10% w/w peptide (dotted and dashed trace; 1.9% SP-B1-25m mol/mol); and 20% w/w peptide (dashed trace; 5.5% SP-B1-25m mol/mol).

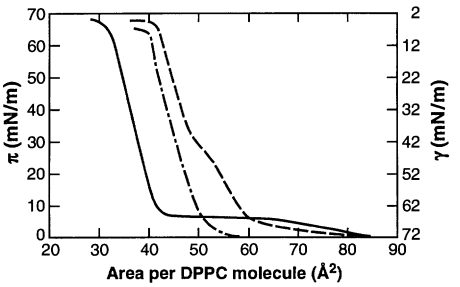


**Fig. 4.** Isotherms of DPPC/SP-B1-25 on a $NaHCO_3$-buffered (pH 6.6, 154 mM) saline subphase: 100% DPPC (solid trace); DPPC:PA (68:8) (dashed and dotted trace); and DPPC:PA:SP-B1-25 (68:8:3 w/w) (dashed trace).

The isotherm of the fatty acid alone showed two distinct condensed phases: the first was a compressible liquid-like phase (Fig. 2A, segment a to b) and the second was a less compressible, solid-like phase (segment b to c) followed by collapse (point c) at about 37 mN/m for PA. [For discussions of the liquid expanded, liquid condensed, and solid condensed monolayer phases, see (*29*).] For AA, four carbons longer than PA, the collapse pressure was about 55 mN/m (Fig. 2B). As SP-B1-25 was added, the collapse pressures of both monolayers were raised significantly, even for 2% w/w SP-B1-25. These pressures finally reached about 52 mN/m for PA (Fig. 2A) and 70 mN/m for AA (Fig. 2B) as the percentage of SP-B1-25 was increased to 20% w/w. The area per fatty acid molecule at collapse also increased, which indicates that the water-soluble peptide was retained in the monolayer under compression. We could not obtain an isotherm of pure SP-B1-25 because of its solubility in water. The onset of the first pressure increase (known as lift-off and labeled as lo in Fig. 2, A and B) occurred at successively larger surface areas as the fraction of SP-B1-25 was increased, which indicates that there was a longer range of interaction between the molecules in the monolayer. The isotherms of 10 and 20% w/w SP-B1-25 in fatty acids displayed a flattened, somewhat horizontal region (segment d to e) typical of a two-phase coexistence region that is also observed in DPPC isotherms (*29*).

As the temperature was increased, the surface pressure at which this coexistence region occurred increased (Fig. 2C for AA), which is typical for both phospholipids and fatty acids (*16*, *29*). The isotherms shifted to lower areas per molecule at higher temperatures, indicating either an increased solubility of SP-B1-25 and fatty acids with temperature or a conformational change in the protein. The isotherms of the combined fatty acid/SP-B1-25 are also strikingly similar to those of fatty acids on a subphase containing polyethyleneimine, a simple, cationic polymer gegenion [see (*16*)]. The polyethyleneimine also induced a higher collapse pressure and a two-phase coexistence region in the isotherms of the fatty acid monolayer. This result leads us to believe that the positively charged SP-B1-25 interacts specifically with the negatively charged fatty acids and that these electrostatic interactions are primarily responsible for the observed effects on the isotherms.

Physiological conditions were mimicked and the effects of charge and ionic strength were tested by the measurement of isotherms of the fatty acid/SP-B1-25 monolayers on $NaHCO_3$-buffered saline solutions (pH 6.6, 154 mM) (Fig. 3A). Perhaps because of the decreased solubility of fatty acids in salt solutions or the screening of the repulsive interactions between the charged carboxyl groups, fatty acid films on saline collapsed at surface pressures significantly higher than fatty acid films on pure water (55 mN/m on saline versus 37 mN/m on pure water) (Fig. 2A). However, the collapse pressures of PA monolayers on saline increased by roughly the same amount as those on water, from about 55 to 68 mN/m with the incorporation of ≥2% w/w SP-B1-25. Again, as the percentage of SP-B1-25 was increased, lift-off occurred at successively larger surface areas and a coexistence region was created at large areas per molecule. At 20% w/w SP-B1-25, the monolayer was more compressible at high surface pressures but could be compressed to 68 mN/m before collapse. This result suggests that the fatty acid–peptide complex may be more soluble in saline than in pure water. The compressibility also suggests that the remaining, primarily hydrophobic part of the SP-B protein might be necessary to retain the protein in the monolayer under physiological conditions; the full SP-B protein is insoluble in water or saline (*17*). The AA monolayers with SP-B1-25 showed similar behavior. Isotherms of monolayers of PA on pure water (Fig. 3B) or $NaHCO_3$-buffered saline (Fig. 3C) containing a mutant peptide of SP-B1-25 (SP-B1-25m) in which each cationic residue was replaced by neutral but polar serine residue were unaffected, aside from a small increase in the collapse pressure at ≥10% SP-B1-25m. Thus, the charged amino acid residues appear to be essential for the specific interactions between SP-B1-25 and fatty acid responsible for the alterations in the isotherms.

The surface behavior of mixed lipid monolayers containing DPPC, PA, and SP-B1-25 on $NaHCO_3$-buffered saline (pH 6.6, 154 mM) is shown in Fig. 4. The mixtures were spread quantitatively from a solution of $CH_3Cl$ methanol (8:1 v/v). The large coexistence region of pure DPPC is easily observed, and the monolayer collapsed at 68 mN/m. A film of DPPC and PA (68:8 w/w) did not display a coexistence region and began to collapse at 62 mN/m. At higher PA fractions, the film collapses at lower pressure, ~45 mN/m for a 50:50 ratio w/w (*11*). A film containing DPPC, PA, and SP-B1-25 (68:8:3 ratio w/w) showed two distinct compressible phases. The first was at a surface pressure similar to that of the coexistence region of pure DPPC, and the second was at a surface pressure similar to that of the coexistence region of the PA/SP-B1-25 complex. This result suggests that DPPC and the PA/SP-B1-25 complex may undergo partial phase separation at the air-water interface at low surface pressures but not at higher surface pressures.

Our results show that the positively charged residues of SP-B interact electrostatically with fatty acids in a way that leads to distinctive changes in the monolayer isotherms: increased collapse pressures and the formation of a two-phase coexistence region. These changes make the fatty acid isotherms resemble those of DPPC, the major component of lung surfactant, and remove the driving force for squeeze-out of the PA from the DPPC films. Consequently, the mixed monolayers have higher collapse pressures and lower surface tensions. Uncharged mutants of SP-B1-25 had little effect on the fatty acid monolayers. The effect of SP-B1-25 on fatty acid monolayers is identical to that of simple poly-cations. Effective replacement surfactants could possibly include simple polymers or amino acid sequences instead of SP-B.

In general, monolayer collapse is poorly understood; the dramatic effects of fatty acid chain length, ionic strength, and cationic proteins on monolayer collapse were not predicted by current theories (*28*). However, our observations here help explain our earlier work (*17*, *25*) and that of Cochrane and Revak (*19*) and Venkitaraman, Hall, and Notter (*23*), who observed greater enhancements of surface activity for monolayers with amino acid sequences that contained cationic residues than for those that were primarily hydrophobic or anionic. Above all, important features of the isotherms of charged fatty acids can be dramatically altered by interactions with charged proteins or polymers incorporated in the monolayer or present in solution. These interactions might be the origin of surfactant inhibition by charged soluble proteins such as albumin or fibrinogen that are believed to be responsible for the onset of adult respiratory distress syndrome.

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tween the bare water surface tension (72 mN/m) and the measured surface tension. The collapse pressure, $\pi_c$, of a monolayer is the highest surface pressure obtainable before the monolayer "collapses," or ejects material into a bulk phase. The minimum surface tension of a monolayer film, $\gamma$, is the bare water surface tension minus the collapse pressure (72 mN/m − $\pi_c$). For a general discussion of monolayer film behavior including collapse, see A. W. Adamson, *Physical Chemistry of Surfaces* (Wiley, New York, 1990), chap. IV.
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26. The peptides SP-B1-25 and SP-B1-25m were synthesized by the solid-phase method of Merrifield, with the use of a *tert*-butyloxycarbonyl strategy or by Fmoc strategies (UCLA Peptide Synthesis Facility) (*17, 27*). In each sequence (*30*), the charged residues are indicated with a "+":

SP-B1-25 ($M_w$ = 2929)
+ ++ +
FPIPLPYCWLCRALIKRIQAMIPKG
SP-B1-25m ($M_w$ = 2709)
FPIP LPYCWLCSALISSIQAMIPSG
1 25

The crude peptides were purified by C4-column (Vydac, Hesperia, CA) reversed-phase high-performance liquid chromatography (HPLC) with a mixture of water, acetonitrile, and 0.1% trifluoroacetic acid. Solvents from HPLC and ion-pairing agents were removed from the purified peptides by vacuum centrifugation, and the expected molecular masses of each peptide were obtained by fast atom bombardment mass spectrometry or electrospray mass spectrometry (UCLA Center for Molecular and Medical Sciences Mass Spectrometry). Quantitative amino acid composition for the peptides was determined at the UCLA Protein Microsequencing Facility.
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30. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; S, Ser; W, Trp; and Y, Tyr.
31. We thank D. K. Schwartz, R. Viswanathan, and J. Israelachvili for helpful discussions. Supported by Office of Naval Research grant N00014-90-J-1551, National Science Foundation grant CTS90-15537, and National Institutes of Health (NIH) grant GM 47334 (J.A.N.Z.); NIH grant HL 40666 (A.J.W.); a Parker B. Francis Foundation fellowship and March of Dimes grant FY92-0360 (R.B.); Research Center in Minority Institutions award G 12 RRO 302607 and R01 HL 40666 (R.B. and A.J.W.); a University of California President's Dissertation Year fellowship (M.L.L.); and the National Consortium for Graduate Degrees for Minorities in Engineering and Sciences (A.M.B.).

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# Tough Times at La Brea: Tooth Breakage in Large Carnivores of the Late Pleistocene

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**One million to two million years ago, most of today's large, predatory mammals coexisted with larger extinct species, such as saber-toothed cats and giant running bears. Comparisons of tooth fracture frequencies from modern and Pleistocene carnivores imply that predator-prey dynamics and interspecific interactions must have been substantially different 36,000 to 10,000 years ago. Tooth fracture frequencies of four Rancho La Brea species—dire wolf, coyote, saber-toothed cat, and American lion—were about three times that of extant carnivores. Consequently, these findings suggest that these species utilized carcasses more fully and likely competed more intensely for food than present-day large carnivores.**

During the late Pleistocene in North America, the species richness of large carnivores and their presumed prey was much greater than at present. There were 56 herbivore species larger than 30 kg and, of those species, 29 (52%) exceeded 300 kg, the size of a moose (*Alces alces*) or larger. At least seven species, such as the mastodon and mammoth, were larger than any extant New World herbivore (*1*). By contrast, 11 herbivores larger than 30 kg (3 of them >300 kg) exist in North America today; even in Africa only 13% of the herbivore species exceed 300 kg (*1*). If Pleistocene herbivores existed at population densities comparable to extant species of similar body size and formed sizable herds as do living zebras (*Equus* spp.) and bison (*Bison bison*), then levels of prey availability would have been comparable to or greater than those of East Africa today. The rich array of large Rancholabrean carnivores is consistent with this hypothesis; for example, 15 species coyote-sized or larger existed in North America during the Pleistocene, whereas today there are 7 (*1*). Because the majority of extant large carnivore species originated more than 500,000 years ago, most of their history has been spent under Pleistocene rather than present-day conditions of predator-prey diversity. Evidence concerning levels of food availability and interspecific competition in the Pleistocene might provide some understanding of the behavior and morphology of living carnivores.

When food is limited, carnivores are likely to feed more rapidly, guard their kills more aggressively, and more completely consume their prey, often ingesting bone in the process (*2, 3*). All these activities involve the risk of tooth breakage, an event carnivores are expected to avoid given the importance of teeth for

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