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Mechanical Properties of Mineralized and Sclerotized Puparial Cuticles of the Flies *Musca autumnalis* and *M. domestica*

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ABSTRACT Mechanical properties of mineralized and sclerotized puparial cuticles of the face fly, *Musca autumnalis*, and the house fly, *M. domestica*, respectively, were determined. The thickness of mineralized cuticle is 55% greater than that of sclerotized cuticle (thickness = 41.7 and 26.8 μm, respectively) from puparia of similar diameter (2.4 to 2.8 mm). Nonetheless, breaking forces for both types of puparial cuticle are similar (load ultimate strength = 9.3–10.9 g). However, the mineralized puparia are considerably stiffer than sclerotized puparia (elastic modulus = 346.4 kg mm^{-1} and 711.5 kg mm^{-1}, respectively) and exhibit fracture at relatively lower load values (percentage total deformation at fracture = 3.5% and 11.0%, respectively). Differences in mechanical properties of puparial cuticle are probably due to the kinds and quantities of cuticular components: The mineralized puparia contain appreciably more inorganic components such as calcium, phosphorus and magnesium, while the sclerotized puparia consist of relatively more organic components such as proteins and phenolics. The evolutionary and functional significance of mineralization and sclerotization as mechanisms for puparial cuticle stabilization is discussed.

The higher Diptera harden and stabilize the last larval cuticle into a puparium as the protective covering for the pupal stage and developing adult. Two different mechanisms of cuticular strengthening have been reported for dipteran puparia, sclerotization and mineralization. The former is a complex mechanism involving diphenols and their oxidized or polymerized products such as quinones which are used for crosslinking or polyarenes which are used for denaturing structural proteins to achieve a stabilized cuticular matrix (Neville, '75; Andersen and Barrett, '76; Sugumaran and Lipke, '82; Peter et al., '84; Roseland et al., '85). Some researchers have proposed that dehydration of the cuticle involving impregnated diphenols may also contribute to stabilization in sclerotized cuticle (Fraenkel and Rudall, '40; Vincent and Hillerton, '79). Mineralization of puparial cuticle usually involves the incorporation of large quantities of calcium and magnesium salts (predominantly carbonates or phosphates) into the chitin-protein matrix for cuticular hardening (Weismann, '38; Fraenkel and Hsiao, '67; Gilby and McKellar, '76; Darlington et al., '83; Grodowitz and Broce, '83; Roseland et al., '85). Although the stabilization process for these two types of puparial cuticle appears to differ strikingly (Fraenkel and Hsiao, '67), Roseland et al. ('85) have demonstrated several similarities. For example, the covalent crosslinking of proteins by diphenols (i.e., sclerotization) occurs in mineralized puparia of face fly (*Musca autumnalis*) as well as in sclerotized puparia of house fly (*M. domestica*) and stable fly (*Stomoxys calcitrans*). However, the degree to which sclerotization of puparial cuticle occurs in the face fly is considerably less than that in the house fly. Puparia of house fly and stable fly also contain appreciable

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quantities of inorganic substances by weight, in excess of 2%. Inorganic components, however, are 20 to 50 times more prevalent in the mineralized puparial cuticles. Therefore, dipterans utilize both diphenols and minerals for puparial cuticle stabilization but to widely varying degrees.

Both mechanisms of puparial stabilization (mineralization and sclerotization) achieve the same end result, that is, the formation of a protective housing for the pupal stage using the last larval cuticle. Since the puparium serves similar functions for all dipteran species, regardless of the stabilization mechanism used, it would seem reasonable to propose that both puparial types would have similar physical characteristics. However, the mineralized puparial cuticle of *M. autumnalis* is substantially weaker per unit thickness than the sclerotized puparial cuticle of *M. domestica* and *S. calcitrans* (Roseland et al., '85). Nevertheless, face fly puparia attain similar cuticular mechanical strength by the incorporation of more material as reflected by the larger thickness of mineralized puparial cuticles relative to the sclerotized types.

The fly puparium is an ideal structure for comparing mechanical properties of invertebrate cuticle because of similarities in shape and size of different species. In addition, it is formed specifically for the protection of the pupa, and the mechanical properties should be useful for understanding the function and evolution of puparia that possess different types of cuticle. Additional research is needed to characterize the mechanical properties of invertebrate cuticles. Little information is available which adequately describes the mechanical properties of insect cuticles in closely related species or in insect species which utilize mineralization as the primary hardening mechanism. The majority of this type of work has been done on many different species which rely on sclerotization for cuticular hardening (Hepburn and Ball, '73; Hepburn and Joffe, '74a,b; Hepburn and Levy, '75; reviewed by Hepburn and Joffe, '76). Most of the physical testing research on mineralized cuticles has involved crustacean species (Joffe et al., '75; Hepburn et al., '75; Wainwright et al., '76). In this report we characterize mechanical properties of the puparial cuticles of the face fly and house fly, two closely related species which differ in the manner of puparial hardening.

MATERIALS AND METHODS

Insects

Face fly and house fly puparia were obtained from the Kansas State University insect rearing facilities. Face flies were reared on fresh bovine dung, while house flies were grown on a modified Chemical Specialities Manufactures Association (CSMA) medium (Ralston Purina, St. Louis, MO). Both species were maintained at 25 ± 2°C, 70 ± 10% RH, and 18L:6D photoperiod. Puparia were cleaned by rapid washing and sonication in dilute detergent (Micro, International Corporation) followed by several rinses in deionized water. Cleaned puparia were stored at approximately 25°C and 50% RH.

Mechanical properties

Mechanical properties were determined on cleaned puparia selected for comparable diameters ranging from 2.4 to 2.8 mm. Cylindrically shaped pieces of cuticle were made by removing with forceps the posterior portion of empty puparia. Length, diameter, and thickness measurements were determined using a stereomicroscope fitted with an ocular micrometer. Previous studies of mechanical properties of biomaterials have primarily used rectangular pieces instead of whole structures. In order to test the mechanical properties of puparial cuticle in as natural a conformation as possible, a specially designed sample holder for the application of a uniform load on a cuticular cylinder was utilized (Fig. 1). The load which is basically compressive is applied using an Instron Tensile Tester (Roseland et al., '85). The holder is composed of two removable plates with a 1.39-mm depression bored through the length of both plates; 15% of the diameter (0.41 mm) was removed from each depression. The specimen is placed lengthwise in the depression. This alignment allows the application of a relatively uniform load across most of the top and bottom portions of the cylindrical puparia and also restricts the horizontal plane of fracture to the exposed lateral portions of the puparia (~30% of the total surface area). Fracture is caused by a combination of compressive and bending loads on the puparium.

Total displacement of the cylindrical cuticle is two times the deformation (d) of the cuticle since both halves are deformed equally. Actual load-displacement curves are illustrated by the solid lines in Figure 2. All curves examined had a gradually rising tail when the load was first applied. This is due to the initial alignment of the puparia in the sample holder depression and is known as settling. The settling contribution to the load-displacement curve was eliminated by extrapolating the linear portion of the load curve until it intersected the horizontal axis (Fig. 2, dashed line a). Relative loads along the curve were determined at three distinct

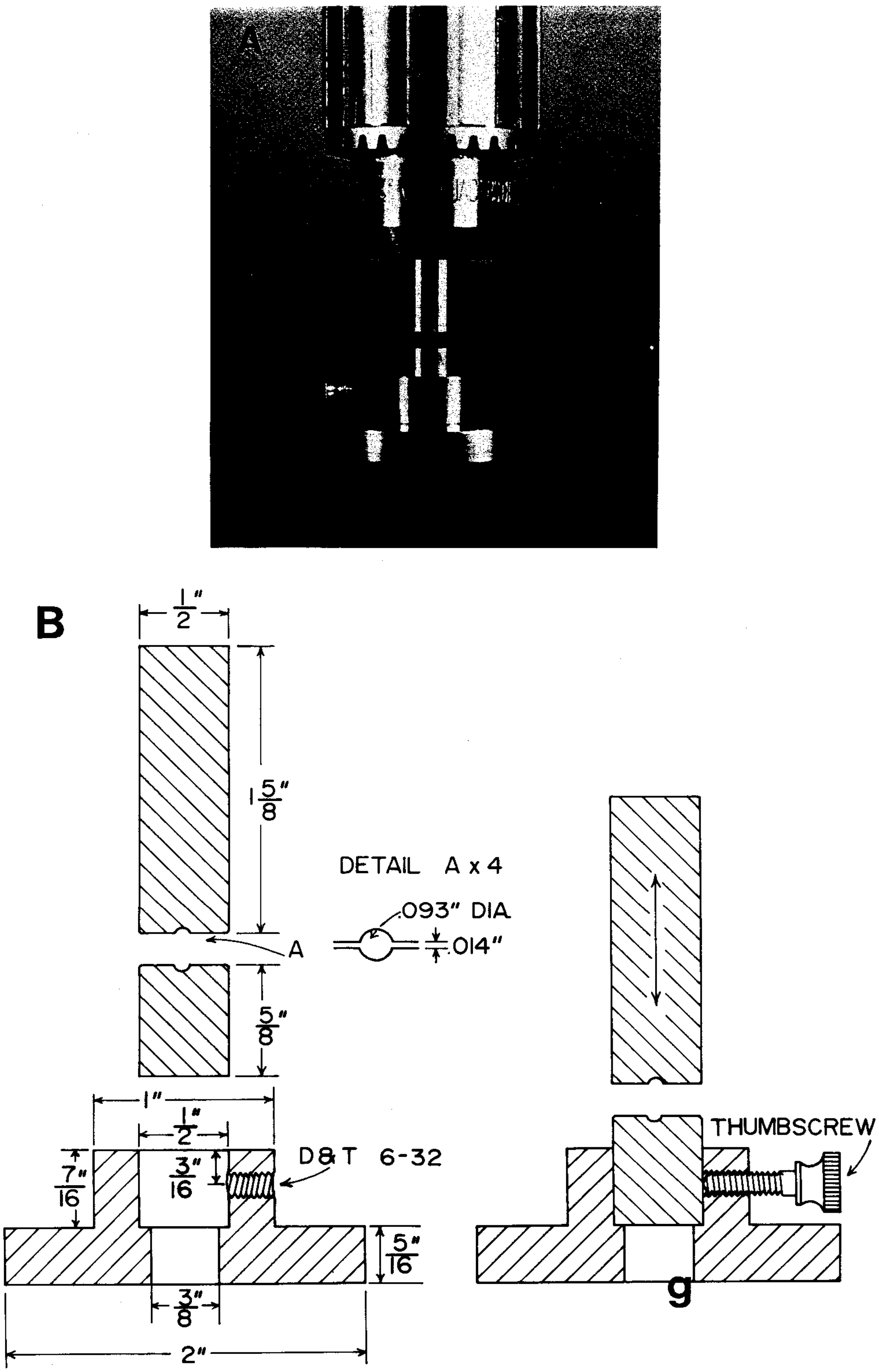

Fig. 1. Sample holder for the application of a uniform load on cylindrical puparial exuviae of face fly and house fly. To convert values to metric scale (cm), multiply by 2.54.

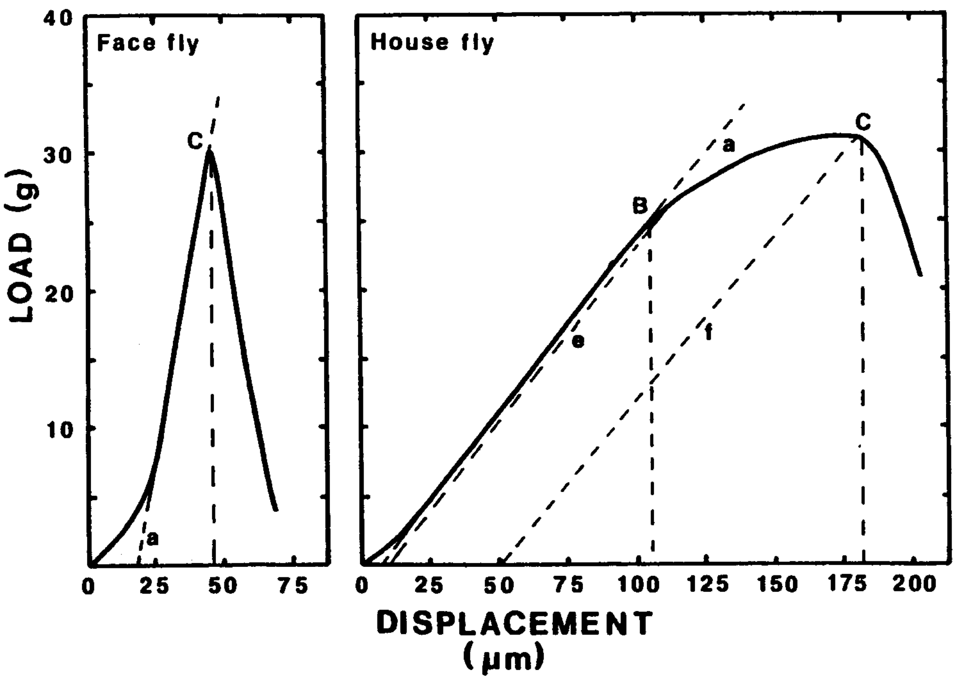

Fig. 2. Typical stress or load-displacement curves for exuviae of face fly and house fly illustrating the various measurements of mechanical properties.

points: 1) the proportional limit is the stress calculated according to the load at that point where the linearity of the load-displacement curve ends (Fig. 2, point B), 2) the ultimate strength is the stress calculated according to the highest load sustained by the cuticle (Fig. 2, point C) and 3) yield strength is the stress calculated according to the load at that point where the removal of the load will result in a permanent cuticular deformation of 0.2% of the diameter. The load to estimate yield strength was determined where the offset displacement (2d) is equal to 2×10^{-3} times the diameter. Graphically this is done by constructing a line parallel to the linear portion of the load-displacement curve but displaced by 2×10^{-3} d (Fig. 2, dashed line e). The load value at the intersection of the offset parallel line and the load-displacement curve is the value used to calculate the yield strength of the material. The following formula was used for determining the maximum bending stress in the load-displacement diagram (Timoshenko, '56): stress (g mm^{-2}) = $(0.318Pr/t/2)(12/Lt^3)$, where P = load (g), r = average cuticular cylinder radius (mm), t = cuticle thickness (mm), and L = length of cuticle cylinder (mm).

Note that for loading values beyond the proportional limit, stress calculated by the use of this formula is an approximation. The elastic modulus was determined using the values of P and displacement 2d at the proportional limit by the following formula: E = modulus of elasticity (g mm^{-2}) = $[Pr^3/2d][12/Lt^3]$, where 2d = total cuticle displacement (mm) to the proportional limit of load, d = deformation of cuticle, and L = length (mm). Note that the modulus of elasticity which is the stiffness of the material before it begins to yield is the slope of the linear portion of the stress-strain curve. Slope of the load-displacement curve was calculated by determining the relative load at the proportional limit and dividing by the corresponding displacement. Permanent set quantifies the total amount of nonlinear deformation of the cuticle by the application of a load. This was determined by constructing a line at the ultimate strength parallel to the linear portion of the load-displacement curve (Fig. 2, dashed line f). The corresponding displace-

TABLE 1. Mechanical properties of face fly and house fly puparia (mean ± standard error)

	Face fly	House fly
Diameter (mm)	2.7 ± 0.0	2.6 ± 0.0[a]
Thickness (μm)	41.7 ± 0.0	26.8 ± 0.0[a]
Density (g mm^{-2})	2.6 ± 0.1	2.2 ± 0.2[b]
Load (g)		
Proportional limit	10.7 ± 1.2	7.6 ± 0.5
Modulus of fracture	10.9 ± 1.0	9.3 ± 0.7
Stress (kg mm^{-2})		
Proportional limit	7.9 ± 1.0	19.2 ± 2.4[a]
Modulus of fracture	8.1 ± 0.9	23.6 ± 3.0[a]
Yield Strength		
Load (g)	—[c]	7.9 ± 0.6
Stress (kg mm^{-2})	—[c]	20.2 ± 2.6
Stiffness (kg mm^{-1})		
Load-displacement	1.1 ± 0.1	0.3 ± 0.1[b]
Elastic modulus (kg mm^{-2})	346.4 ± 42.1	711.5 ± 133.6[d]
Total deformation at fracture (%)	3.5 ± 0.2	11.0 ± 0.7[a]
Permanent set (%)	0.10 ± 0.02	0.90 ± 0.3[a]
Decrease in stress at 25μm displacement after attaining ultimate strength (%)	68.0 ± 5.6	39.0 ± 3.9[e]

[a]Prob. > F (df = 1,40) = 0.0001.
[b]Prob. > F (df = 1,10) = 0.0038.
[c]There are no yield strength values for the face fly exuviae.
[d]Prob. > F (df = 1,40) = 0.02.
[e]Prob. > F (df = 1,33) = 0.0002.

ment was then divided by the puparium diameter and multiplied by 100. Percentage total deformation characterizes the total amount that the structure can be deformed before failure occurs, and this was determined by taking the displacement at the ultimate strength (Fig. 2, point C), dividing by the diameter, and multiplying by 100. Note that the area under the load-displacement curve is the work done to break the cuticle. Density was also determined by calculating the weight of puparium per unit volume.

Scanning electron microscopy

Specimens were randomly selected after load application for examination of fractures using scanning electron microscopy (SEM). Specimens were prepared by air drying and subsequently coated with gold-palladium for viewing with an ETEC U-1 Auto Scan electron microscope.

RESULTS

Cuticular strength

Face fly and house fly puparia differed in most of the physical and mechanical properties examined. Face fly puparia were approximately 1.5 times thicker than house fly puparia of similar diameter (Table 1). There were also significant differences in density ($p < 0.05$): house fly puparia had a mean density of 2.2 g mm^{-3}, while face fly puparia were about 1.2 times denser. The load applied to reach the ultimate strength or load modulus of fracture of the puparia was not significantly different ($p > 0.05$) for the two species, but this measurement did not take into account the size, shape, or thickness of the puparium being tested. When the load data were transformed into stress values (force per unit area), large differences were observed between face fly and house fly puparia. For example, the mean strength or stress modulus of fracture was only 8.1 kg mm^{-2} for face fly puparia, which was nearly three times lower than that observed for house fly puparia (23.6 kg mm^{-2}). There was also a significant difference ($p < 0.05$) between the load and stress values observed at the proportional limit and modulus of fracture (ultimate strength) for the house fly. In this species, load curves deviated from linearity with only a gradual change in slope occurring after the proportional limit was reached. However, the proportional limit and modulus of fracture values for face fly puparia were equal. Therefore, most face fly puparia exhibited a linear relationship between stress and deformation (Hookean behavior), in contrast to house fly puparia. However, a few face fly puparia tested (5%) did exhibit a minor deviation from linearity in their load-displacement curves. Another measure of ductile (non-Hookean) behavior of house fly puparia was reflected in percentage permanent set values. House fly puparia had a relatively high mean percentage permanent set of 0.90%, while face fly puparia had a lower value of only 0.10%. Most of the face fly puparia tested, 16 out of 20, exhibited no percentage permanent set before fracture, while the other four samples had values ranging from 0.16 to 0.36%. Yield strength values for house fly puparia were approximately 15% less than both the corresponding stress and load modulus of fracture values. Therefore, the cuticle of the face fly has a brittle nature similar to that of concrete while the cuticle of the house fly has a plastic nature similar to that of epoxy cement.

Stiffness

Puparia from the two species were quite different in their stiffness. House fly puparia had a 2-fold higher mean modulus of elasticity than the face fly puparia (Table 1). This difference is further illustrated when considering that house fly puparia were deformed 11% of their diameter before fracture occurred, while face fly puparia had a relatively low value of only 3.5%.

Another measurement which showed that house fly puparia were significantly more flexible than face fly puparia was the 3-fold lower slope associated with the load-displacement curve of the former (Fig. 1, Table 2). House fly puparia required less force than the face fly puparia to attain an equal deformation or displacement. However, face fly puparia did not withstand an ultimate stress value as high as that observed for the house fly puparia. Fracture of the former occurred at a 3-fold lower stress. In addition, face fly puparia exhibited a more distinct failure point than house fly puparia, as demonstrated by the relatively rapid drop in strength with further deformation after the ultimate strength was reached. For example, face fly puparia had a 68% drop in stress with a 25-μm further deformation while house fly puparia, in contrast, had a mean drop of only 39%. These data represent an almost 2-fold difference in stress reduction after the ultimate stress point was reached. From the difference in areas under the load-displacement curves, it is clear that the house fly cuticle requires 5-fold more work to reach the fracture point than does the face fly cuticle.

Fracture morphology

Differences in cuticular mechanics were further evident after SEM examination of fracture surface morphology of puparial cuticle. Both types of puparia were generally characterized by having failures perpendicular to the applied load (Fig. 3). Internal fracturing for each type of cuticle appeared somewhat different. For example, face fly puparia had numerous failures throughout the longitudinal section with no regard to lamellae orientation. The fracture surface was relatively uniform and had distinct areas where breakage appeared to occur simultaneously. This is indicative of a brittle-type failure mechanism. In contrast, the apparent house fly endocuticle (upper portion of cuticle in Fig. 3b) had rather uneven fractures running throughout and parallel to the lamellae of the cuticle, which is typical of a progressive failure of a flexible material. However, the apparent house fly exocuticle had a relatively uniform fracture surface with no delaminations. The apparent exocuticle of the house fly fractured in a brittle manner similar to the fracture of the entire surface of the face fly puparium.

DISCUSSION

The mechanical properties of sclerotized puparia of the house fly and mineralized puparia of the face fly reflect differences in biochemical composition, in particular the presence of greater quantities of organic components in the former and of inorganic components in the latter. Several studies have documented large quantities of calcium, phosphorus, and magnesium in the puparial cuticle of face fly (Fraenkel and Hsiao, '67; Darlington et al., '83; Grodowitz and Broce, '83; Roseland et al., '85). The preponderance of inorganic components in certain biomaterials has been associated with low deformability, low breaking stress, and brittleness (Joffe et al., '75; Hepburn et al., '75; Hepburn and Joffe, '76). We found this to be the case for the puparial cuticle of muscid flies. The flexibility of the mineralized puparia is substantially reduced relative to the sclerotized puparia. This was demonstrated by a lower modulus of elasticity and percentage total deformation at fracture for the former cuticle. In addition, significantly lower fracture force was associated with the mineralized cuticle.

The fracture surface patterns of the puparia also indicated differences in chemical

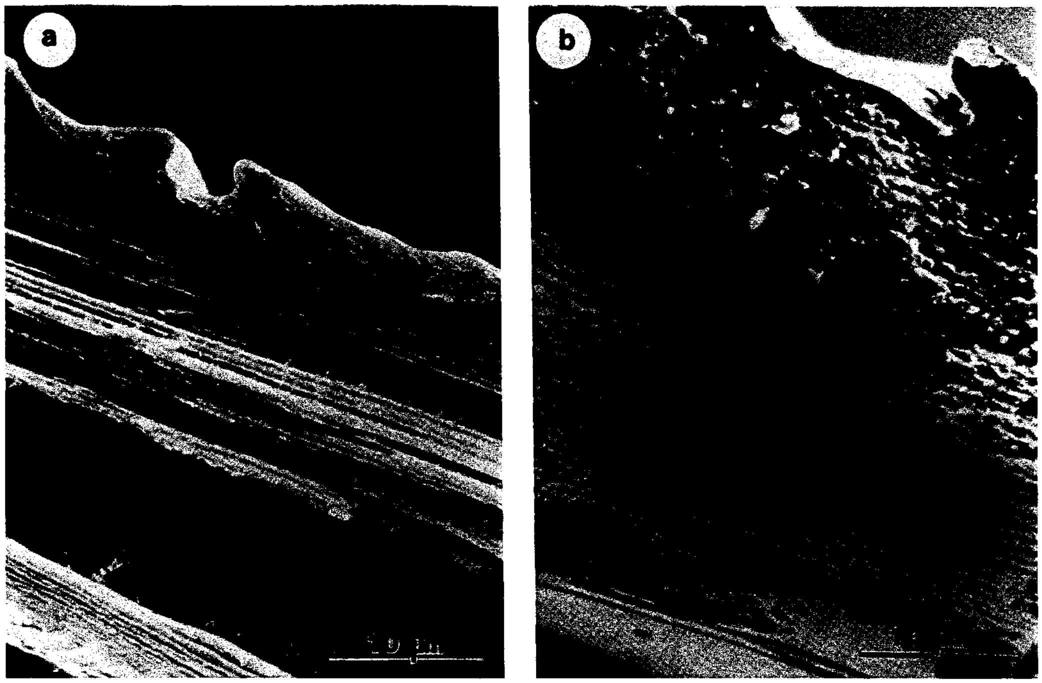

Fig. 3. Longitudinal fracture surface of face fly (a) and house fly (b) puparial exuviae.

nature. Mineralized puparial cuticle had fractures that were not oriented with the lamellae, suggesting that catastrophic failure was initiated rather quickly and proceeded rapidly. This pattern of failure is in accord with a brittle material-type failure, where the material on attaining its ultimate strength, can no longer deform uniformly and subsequently shatters (Joffe et al., '75; Hepburn et al., '75; Hepburn and Joffe '76). On the other hand, the fracture surface for sclerotized puparial exuviae was always aligned with lamellar orientation. In this case, there are both brittle and tensile failures occurring in different regions of the exuviae (i.e., apparent exo- and endocuticle). The exocuticle exhibits brittle failure as indicated by the relatively uniform fracture surface. The endocuticular region has a more plastic failure since there are numerous delaminations and the fracture surface is irregular. The nonuniform fracture surface indicates that lamellae fail individually. In addition, there appeared to be only limited interaction among lamellae because of the numerous delaminations. This type of fracture pattern is characteristic of more ductile materials (Hepburn and Joffe, '76). In addition, substantially greater energy is needed to break house fly puparia. This is indicated by the larger area under the load-displacement curve for the house fly puparium relative to that of the face fly. This area represents the total energy needed for fracture (Timoshenko, '56).

Little information is available that defines the relationship between submatrix components in arthropod cuticles and their mechanical properties. Although the presence of inorganic components in mineralized puparial cuticle may account for the overall mechanical properties, other chemical factors may be important. Organic components may strengthen the mineralized matrix substantially. Joffe et al. ('75) suggested that the protein phase in the cuticle of a crustacean, *Scylla serrata*, reinforces the inorganic submatrix. Weiner ('84) suggested that one of the possible functions of the organic matrix is to control mineral salt crystallization during the formation of mineralized cuticle.

The main reason that mechanical properties of the organic phase of biological materials have not been characterized is the difficulty in removing inorganic submatrix components while leaving the organic component intact (Hepburn and Joffe, '76). However, several researchers have found a significant relationship between cuticular mechanical properties and the quantity of extractable protein. A decrease in fracture force generally occurs with the extraction of matrix proteins (Hackman '72; Joffe et al., '75). Roseland et al. ('85) found 2- to 3-fold less amino acid or protein levels in the mineralized puparial cuticle of the face fly relative to the sclerotized puparial cuticles of the house fly and stable fly. A major amino acid in sclerotized cuticle, beta-alanine, was undetected in mineralized cuticle. Reduced amino acid levels and the absence of beta-alanine in face fly puparial cuticle have also been reported by Bodnaryk ('72). Jacobs ('85) demonstrated that injection of beta-alanine increased the puncture resistance of *Drosophila* wings. Roseland et al. ('87) found that the rate of strengthening and pigmentation of *Tribolium* cuticle is apparently dependent upon the availability of beta-alanine and the catecholamine, N-beta-alanyldopamine. Covalent incorporation of catecholamines appears to be important in determining the mechanical properties of insect cuticles by changing the solubility of the cuticular proteins. Such insoluble proteins in arthropod cuticles are thought to be crosslinked via quinonoid metabolites derived from catecholamines (reviewed by Neville, '75; Brunet, '80; Lipke et al., '83). The levels of extractable diphenols are approximately two orders of magnitude higher in house fly and stable fly puparial cuticles than in the mineralized cuticle of the face fly (Roseland et al., '85). More importantly, the covalent incorporation of diphenols is also significantly higher for sclerotized puparial cuticles. House fly puparial cuticle has 150 times more covalently incorporated diphenols than does face fly cuticle (Roseland et al., '85).

Although there are major differences in mechanical properties between the muscid puparial cuticle types, sclerotized and mineralized cuticles appear to be similar in overall strength and perhaps function. Similarly sized house fly and face fly puparia differ significantly in thickness, with the face fly puparial cuticle being substantially thicker. However, the relative brekaing force is not statistically different for these two species. Thus, both sclerotized and mineralized cuticles can sustain the same load before breakage occurs, but the mineralized species uses more material to do so.

The mechanical properties of the mineralized puparia of the face fly differ from those characterized for other mineralized arthropod cuticles, namely crustacean cuticles. For example, Joffe et al. ('75) and Hepburn et al. ('75) documented the presence of low stress discontinuities in the stress-strain behavior of *S. serrata* and *Penaenus mondon* cuticles. The discontinuity is caused by a failure of the mineral submatrix at substantially lower stress values leaving the chitin and protein components to support the additional loads. We did not observe discontinuities in the stress-displacement behavior of face fly puparial exuviae. Instead, complete failure occurred at lower stress values relative to the sclerotized type. The chitin-protein submatrix does not appear to be able to support the additional load after failure of the brittle inorganic phase. The entire cuticle matrix i.e., protein, chitin, catecholamines, and minerals, apparently fails simultaneously. The lack of discontinuity suggests that mineralization in muscid cuticle differs significantly from crustacean mineralization. For example there is a major difference in the mineral form. Crustacean cuticle is composed primarily of calcium carbonate in a weakly crystalline form of calcite. The minerals are secreted throughout the cuticle and are aligned with the chitin-protein fibers (reviewed by Roer and Dillarman, '84). Face fly puparial cuticle is composed mainly of calcium and magnesium phosphates, with only minor quantities of carbonates (Darlington et al., '83; Grodowitz and Broce, '83). The calcium and magnesium salts are in an almost entirely amorphous form (Grodowitz and Broce, '83). Another possible reason that complete failure of face fly puparial cuticle occurs at low stress is that there may be a strong and intimate interaction between the inorganic and organic phases. This is supported by the fact that extraction of face fly puparial cuticles with a metal ion chelating buffer, such as EDTA, causes substantial loss of both minerals and amino acids (proteins) from the cuticle (unpublished data).

In spite of the fact that there are substantial data available on the mechanical and chemical properties of various types of insect cuticle, the evolutionary advantage of cuticle mineralization over sclerotization in species such as the muscid flies is unknown. There is evidence suggesting that the mineralized puparial cuticle and not the sclerotized cuticle protects flies against parasitism, even though the former cuticle appears to be physically inferior to the latter. For example, while the house fly has a number of pupal parasites, only a few hymenopteran parasites have been identified for the face fly (Blickle, '61; Hayes and Turner, '71) with only low population numbers of parasites able to emerge unaided from the mineralized puparia. Successful laboratory culture of *Muscidifurax raptor* on face fly can be attained only by mechanically cracking the puparia to aid in the emergence of the wasps (Burton and Turner, '68). However, almost 100% parasite emergence occurs from house fly puparia without any mechanical assistance required (Hayes and Turner, '71). These observations on puparial parasitism suggest that both biochemical and mechanical properties of the cuticle determine whether parasite emergence can occur and that mineralized cuticle affords better protection than sclerotized cuticle.

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