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The silk cocoon of the silkworm, *Bombyx mori*: Macro structure and its influence on transmural diffusion of oxygen and water vapor

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ABSTRACT

The cocoon of insect larvae is thought to help conserve water while affording mechanical protection. If the cocoon is a barrier to water loss, then it must also impose a barrier to inward oxygen diffusion. We tested this hypothesis in pupae of the silkworm, *Bombyx mori*. The rate of water loss and oxygen uptake ($\dot{V}O_2$) at 25 °C was measured in control pupae in their naturally spun cocoon and in exposed pupae experimentally removed from their cocoon. Additional measurements included the oxygen diffusion coefficient, DO_2, of the cocoon wall and dimensions and density of the cocoon fibers. Water loss (as % body mass loss) in both control and exposed pupae was ~1%·day^{-1}, and was not significantly different between populations. Similarly, $\dot{V}O_2$ was statistically identical in both control and exposed pupae, at 0.22 ± 0.01 and 0.21 ± 0.02 mL g^{-1} · h^{-1}, respectively. The silk fiber diameter was significantly different in the outer fibers, 26 ± 1 μm, compared with 16 ± 1 μm for the inner fibers lining the cocoon. Inner fibers were also spun significantly more densely (20.8 ± 1.2 mm^{-1} transect) than outer fibers (8.3 ± 0.2). Mean DO_2 at 25 °C was 0.298 ± 0.002 cm^2 · s^{-1}, approximately the same as unstirred air. These data indicate that the cocoon, while creating a tough barrier offering mechanical protection to the pupa, imposes no barrier to the diffusion of oxygen or water vapor.

1. Introduction

The cocoon of most insect larvae is an intricate structure potentially serving numerous simultaneous functions. In the leek moth (*Acrolepiopsis assectella*) and the green lacewing (*Mallada signata*), the cocoon is generally thought to provide the pupa with protection against predation, biodegradation, and dehydration (Gauthier et al., 2004; Weisman et al., 2008). The innermost layer of the cocoon, the pelade, helps prevent abrasion of the waterproof external cuticle of the pupa during its frequent movements within the cocoon. Cocoon fibers also have both antifungal and antibacterial properties (Danks, 2004).

Among the most extensively studied cocoons are the silk cocoons of the silkworm (*Bombyx mori*). Silk fabric has been valued in numerous cultures for many millennia (Good, 1995; Kadolph, 2007). Accordingly, the production of silk in *Bombyx* has been extensively investigated. As typical of many holometabolous insects, the silkworm begins life as a larva, passing through five larval instars. At the end of the fifth larval instar, the decline of juvenile hormone allows the neurosecretory hormone, ecdysone, to initiate metamorphosis, marking the beginning of the prepupal stage. The prepupa wanders to locate a suitable place for cocoon formation, a process requiring approximately 5 days. The silk cocoon is spun around the contracted body of the pupa which uses silk strands secreted from labial glands analogous to salivary glands in other larval insects (Asakura et al., 2007). Silk strands themselves are polypeptide polymers composed of multiple components — microfilaments of insoluble proteins (fibroin), covered with a soluble adhesive protein (sericin) that provides structural support for the cocoon (Zhou et al., 2000; Hakimi et al., 2006). Other minor components include small proteins, lipids, and carbohydrates (Gauthier et al., 2004).

Silk fibers have been extremely well categorized as a material (see Hakimi et al., 2006; Xiao et al., 2009; Zhang et al., 2009 for an entry into the extensive literature). Less attention has been directed at the cocoon as opposed to the fibers that create it. Recent engineering analyses of the structural properties of the silk cocoon show that the pelade of the cocoon has higher values of Young's modulus, tensile strength, storage modulus, and loss modulus than the overall cocoon (Zhao et al., 2007). Unknown, however, is how the physical characteristics of the heterogeneously structured cocoon and its pelade (inner lining) actually influence the physiology of pupal *B. mori*. Does the cocoon present a tradeoff between mechanical protection on the one hand, and limited gas diffusion on the other? Given speculation that the insect cocoon could help reduce water loss (e.g. Danks, 2004; Gauthier et al., 2004), we hypothesized that the cocoon surrounding the pupa helps conserve water, but as a consequence also reduces oxygen diffusion into the cocoon, thus potentially limiting pupal oxygen consumption. To test this hypothesis, we measured oxygen

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consumption and water loss of the pupal stage of the silkworm, with and without their cocoons. The diffusion characteristics of the cocoon were also quantified, as was the microstructure of the cocoon using scanning electron microscopy.

2. Materials and methods

2.1. Animal husbandry

Fertilized eggs of the silkworm, *B. mori*, were obtained from Carolina Biological Supply (Burlington, NC, USA) and Mulberry Farms (Fallbrook, CA, USA). Upon arrival in the laboratory, the eggs were placed in Petri dishes and incubated in a transparent incubator at a temperature of 25 ± 0.5 °C with a relative humidity of 60%. The incubators were illuminated with an LD 12:12 photoperiod. Petri dishes were examined for hatched larvae each morning, since hatching (eclosion) occurs in the morning due to circadian rhythms of metabolism and eclosion hormone secretion (Fugo et al., 1985). Newly hatched instar I larvae, approximately 3 mm long and 0.4 mg in mass, were transferred into date-labeled Petri dishes, where they were subsequently raised through to pupation. All larval instars were fed an artificial silkworm diet obtained from Carolina Biological Supply in the afternoon and any food remaining in the Petri dishes was removed daily in the evening.

2.2. Body mass determination

The pupa within its cocoon forms an integral unit in the development of the silkworm. Non-living material associated with the pupa (i.e. cocoon + dehydrated prepupal skin) comprises 16.2 ± 1.0% of the mean body mass of undisturbed pupae (Blossman-Myer, 2007). The dehydrated prepupal skin inside the cocoon remaining from metamorphosis comprises a near-negligible amount of the total body mass (3.0 ± 0.1%) and was grouped with the cocoon mass.

2.3. Respirometry

Pupal $\dot{V}O_2$ was measured using closed system respirometry using the techniques described in detail by Blossman-Myer and Burggren (2009). Essentially, respirometers were constructed from transparent glass syringes (volume = 50 mL). Twenty-gauge needles were fastened securely onto each syringe and inserted into a rubber stopper. Sealed syringes were then gently placed in a water bath maintained at 25 ± 0.5 °C. Pupae from the incubator were placed in the gas-tight respirometers for 30 min to ensure that pupae were in a quiescent state and maintaining a standard metabolic rate.

The first of three successive standard $\dot{V}O_2$ measurements was then taken by allowing the pupa to respire in the syringe during a 4 h period. Oxygen consumption by the pupa created a maximum decrease in PO_2 of 5.0 mmHg during the measurement period. The oxygen level in a 0.5 mL gas sample injected directly from the syringe was measured with a thermostatted Clark-type oxygen electrode (Cameron Instruments Model PHM 72 Mk-2; ON, Canada). The electrode was calibrated with a two-point calibration using humidified atmospheric air and nitrogen prior to each experiment and after each measurement period throughout the day. The respirometer was refreshed with air at 25 ± 0.5 °C before a second measurement was made. Each pupa was measured in this manner three times. Mean standard $\dot{V}O_2$ was calculated from the average of the three readings for each pupa.

Mass-specific oxygen consumption, expressed as mL O_2 $g^{-1} \cdot h^{-1}$, was calculated using conventional closed respirometry calculations involving the PO_2 change in the syringe, syringe volume (after correction for animal volume), elapsed time and pupal body mass.

Hormonal activity has been linked to the light–dark cycle in silkworms (Fugo et al., 1985), and changes in light and/or dark have also been shown to alter $\dot{V}O_2$, heart rate and/or cause heart rate reversals in other insect species (Stusek et al., 2000; Kazuyuki et al., 2005). Thus, all of the incubators, holding chambers, and respirometers were transparent to admit ambient light (12 h light:12 h dark), and all measurements were made during daylight hours to control for normal circadian rhythms.

2.4. Cocoon influence on pupal $\dot{V}O_2$

Three groups of pupae (control, sham, and exposed) were subjected to $\dot{V}O_2$ measurements to determine if the cocoon was a limiting factor for oxygen consumption. The control group consisted of intact, undisturbed pupae inside their cocoon. Body mass in this group comprised the pupae and its surrounding cocoon, pre-weighed to the nearest mg. In the sham group, each pupa in its cocoon was pre-weighed to the nearest mg and then carefully extracted from its cocoon through a thin opening in the cocoon made with a razor blade. The exposed pupa was weighed to the nearest mg, placed back into its cocoon, and the cocoon opening sutured with black-braided non-absorbable silk (size 8-0). For consistency, body mass in this group comprised the pupa and its surrounding cocoon. In the final group the pupa in its cocoon was pre-weighed to the nearest mg and then carefully removed.

The control, sham and exposed pupae were then placed individually in their respirometers, and $\dot{V}O_2$ then measured as described above.

2.5. Cocoon influence on pupal water loss

Undisturbed pupae in their cocoon (control) and exposed pupae (cocoon removed) previously held at 60% rh were placed on individual Petri dishes within a 1 L thermostatted (24 ± 1.0 °C) glass dehydration chamber held at a constant relative humidity of 33%. Pupae were preweighed (controls comprising cocoon plus pupa) immediately prior to placement in the chamber. The controls, exposed pupae, and empty cocoons were weighed to the nearest mg after 0.5, 2, 24, and 48 h in the dehydration chamber to determine body mass loss from initial readings. All mass losses were attributed to water vapor loss, assuming negligible mass change due to respiratory gas exchange different from 1 (i.e. an imbalance between oxygen consumption and carbon dioxide production).

Following this dehydration regime, the change in body mass and empty cocoon mass was calculated as both an absolute change and % change from initial values.

2.6. Cocoon oxygen diffusion coefficient

The diffusion characteristics of the cocoon wall were determined by measuring the rate of O_2 diffusion across 95 mm^2 pieces of cocoon wall cut from cocoons that had been spun 2–3 days previously to ensure that silk spinning had ceased and the pupal case was fully secreted around the metamorphosing silkworm. Cocoon thickness was measured to the nearest 10 μm. with a micro-caliper. The cocoon wall was then secured across the sole opening (63.5 mm^2) of a cylindrical 9.0 mL airtight plastic chamber containing an oxygen electrode (Microelectrodes, Inc. Bedford, NH, USA). The chamber was then repeatedly flushed with nitrogen gas (PO_2 = 0.0 mmHg). Flushing was stopped and the subsequent rate of rise in PO_2 in the chamber from inward O_2 diffusion into the chamber across the cocoon wall from the gas surrounding the chamber was then measured over time using Chart software (ADI Instruments). Gas surrounding the chamber was carefully controlled, allowing diffusion rates across the cocoon wall to be determined over a range of O_2 gradients corresponding to PO_2 (O_2%) gradient of 40 (5.5), 60 (8.3), 80 (11.1), 100 (13.9), 120 (16.6), 130 (18.0) and 140 (19.4) mmHg (%O_2). To ensure the accuracy of the O_2 diffusion calculation, diffusion rates were calculated twenty times for each O_2 gradient. From the rate of PO_2 rise in the chamber and the volume of the chamber, the amount of O_2 (MO_2) diffusing into the diffusion chamber could be readily determined. Then, from the surface area of the diffusion pathway

(A), the O_2 concentration gradient (ΔCO_2) across the cocoon wall and the thickness of the cocoon wall (E), the O_2 diffusion coefficient (DO_2, $cm^2 s^{-1}$) could be calculated thusly.

$$DO_2 = \dot{M}O_2 \cdot \frac{1}{A} \cdot \frac{1}{\Delta CO_2 / E} \quad (1)$$

From the O_2 diffusion coefficient, the O_2 permeability coefficient, D_2/E ($mM\ O_2 \cdot s^{-1} \cdot cm^2 \cdot \%\ \Delta O_2$) could also be calculated:

$$\frac{DO_2}{E} = \dot{M}O_2 \cdot \frac{1}{A} \cdot \frac{1}{\Delta CO_2} \quad (2)$$

2.7. Cocoon structure

A 3 mm × 3 mm section of the cocoon wall was removed and placed directly into a Hitachi tabletop scanning electron microscope (SEM) (model TM-1000) for examination and image collection. No prior specimen preparation is required for examination of materials in this instrument. From captured cocoon images, silk fiber densities of the inner cocoon surface (the pelade) and outer cocoon surface were calculated from the number of fibers transecting a 500 µm reference bar at 60 to 180 times magnification. Silk fiber diameters (outer and inner) and cocoon wall thickness were calculated to the nearest µm.

2.8. Analyses and statistics

Mean $\dot{V}O_2 \pm 1$ standard error (s.e.m.) was calculated for control, sham and exposed pupae, and was expressed as mass-specific $\dot{V}O_2$. A one-way ANOVA was performed on these groups to determine significant treatment effects.

Changes in body mass from the baseline due to water loss were calculated for the control and exposed pupae. Percentage change data were arcsine transformed prior to statistical testing. A two-way ANOVA was performed to assess mass loss by group, effects of time or an interaction of group and time. An independent t-test was performed between the groups (i.e. control and exposed) at each time period.

Paired t-tests were performed on the fiber density and fiber diameter groups (outer and inner). An alpha level of 0.05 was adopted for all statistical decisions.

Table 1
Characteristics of the inner and outer silk fibers forming the cocoon of the silkworm, *Bombyx mori*.

	Outer silk fibers	Inner silk fibers of pelade	n	Significance level of differences between inner and outer fibers (P)
Silk fiber diameter (µm)	26 ± 1*	16 ± 1	8	<0.0001
Density index (silk fibers/1 mm transect)	8.3 ± 0.2	20.8 ± 1.2	8	<0.0001

* Mean ± s.e.m. presented. $n = 8$ for all parameters.

3. Results

3.1. Cocoon structure

The silk cocoon is an elaborately spun structure that weighs a mean of 0.15 ± 0.01 g ($n = 7$), or approximately 13% of the total mass of the pupal–cocoon complex. The cocoon's mean wall thickness is 553 ± 40 µm ($n = 8$). Silk fibers are spun individually from the paired silk glands, but upon close examination these individual "brins" fuse upon extrusion into a single strand (arrow in left panel, Fig. 1).

The cocoon itself is heterogeneous in structure, consisting of two distinct types of silk fibers. Most of the thickness of the cocoon wall is composed of large-diameter "outer" fibers with a mean diameter of ~26 µm, laid down in a criss-crossing and somewhat random overlapping fashion at a density of ~8 fibers/mm transect (Fig. 1; Table 1). The smaller diameter inner fibers (~16 µm diameter) form a more tightly woven pelade lining of the cocoon wall. Fibers in the pelade are

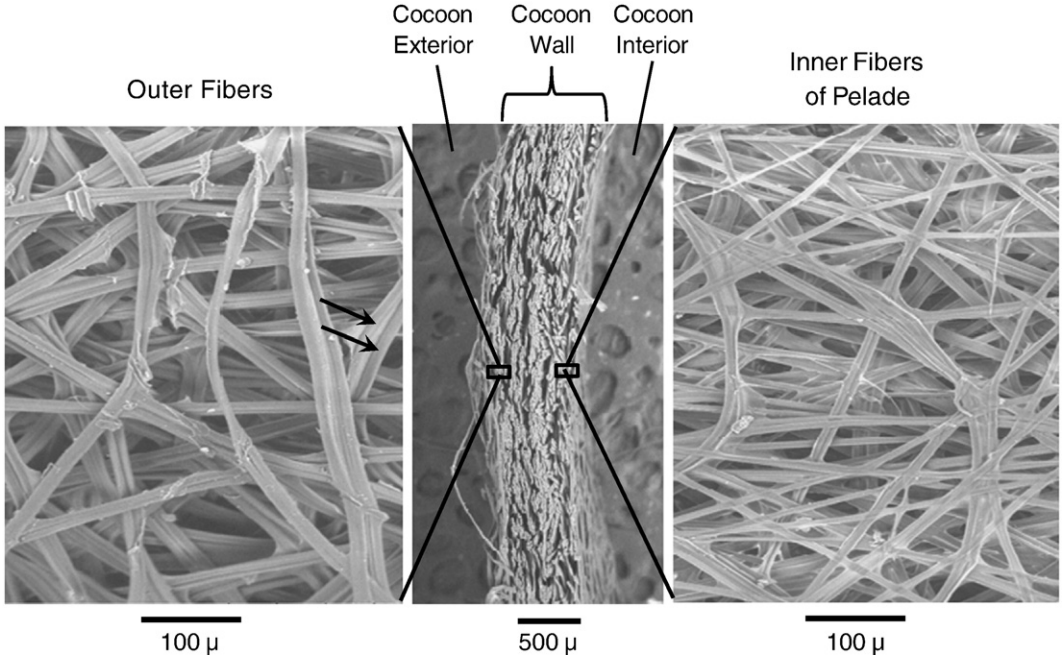

Fig. 1. Structure of the cocoon of the silkworm, *Bombyx mori* revealed by scanning electron microscopy. Center panel shows entire cocoon wall in cross section. The dimpled surface to either side of the wall itself is the stage used for microscopy. The outer large-diameter silk fibers and inner small diameter silk fibers of the pelade are detailed in the left and right panels, respectively. Each fiber consists of the fused strands exuded from the pair of silk glands. Arrows in the left panel point to the individual "brins" fused into a common strand. Both outer and inner fibers are crisscrossed during the spinning process, with the thinner inner fibers laid down at a much higher density than the larger outer fibers. Table 1 provides quantitative information on fiber densities and diameters.

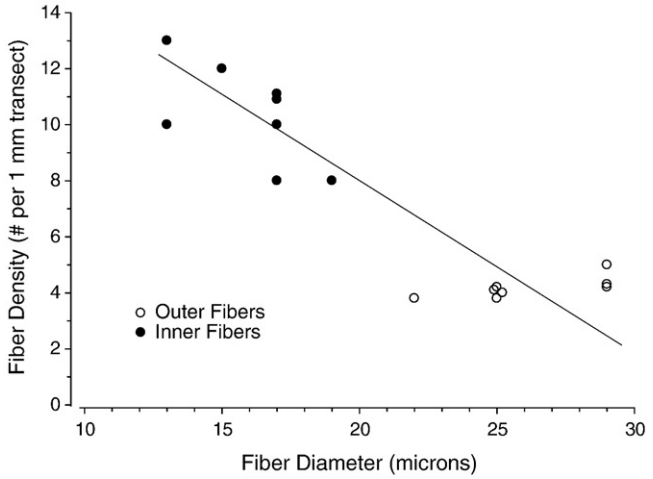

Fig. 2. Relationship between density and diameter in silk fibers from the inner and outer regions of the cocoon of *Bombyx mori*, determined from scanning electron micrographs. $n = 7$ for both groups.

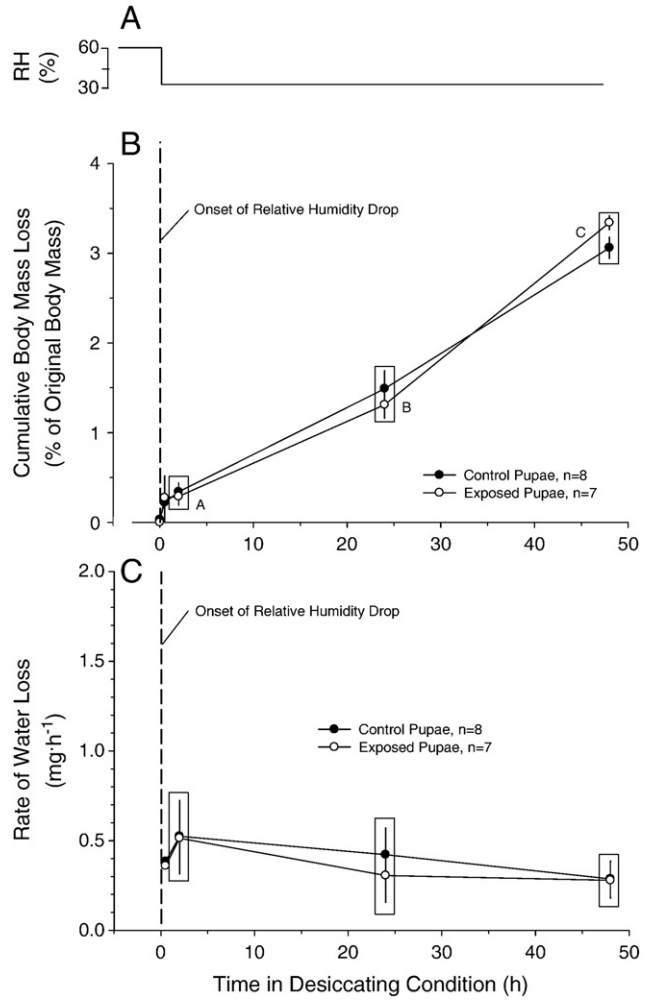

Fig. 3. Body water loss over time in control and exposed pupae previously acclimated to 60% relative humidity and then introduced into a constant desiccating relative humidity of 33% at 24 °C. A) Relative humidity. B) Cumulative body mass loss. C) Rate of water loss. Means ± 1 s.e.m., n values are shown. Letters indicate values in statistically identical groupings, while boxes enclose statistically identical means. See text for additional details.

Table 2
Influence of cocoon presence on mass-specific $\dot{V}O_2$ of pupae of the silkworm, *Bombyx mori*.

Experimental condition	Body mass*	$\dot{V}O_2$ ($mL \cdot g^{-1} \cdot h^{-1}$)*	n
Control (pupa within intact cocoon)	0.768 ± 0.025	0.22 ± 0.01	43
Sham (pupa removed from cocoon, then returned)	0.767 ± 0.0282	0.24 ± 0.02	22
Exposed (pupa removed from cocoon)	0.727 ± 0.0266	0.21 ± 0.02	24

* Mean ± s.e.m.

laid down at a density about 2.5 times greater than that of the outer figures (Fig. 2; Table 1).

3.2. Cocoon influence on pupal water loss

Pupae were quite resistant to water loss when moved from a humidity of 60% to a desiccating humidity of 33%, with 48 h of desiccation producing a body mass loss of only ~3% (Fig. 3B). A two-way ANOVA showed a significant effect of time ($P<0.001$) but no significant difference between the control and exposed groups at any time ($P = 0.48$), nor a statistically significant interaction between group and time ($P = 0.39$). Multiple comparison procedures (SNK) separated the following mean mass loss relative to time as: A: 0.00 h–2.00 h; B: 2.00 h–24.00 h; C: 24.00 h–48.00 h.

The rate of water loss over time was relatively constant at about ~0.5 mg h^{-1}, and not significantly different between control and exposed pupae (Fig. 3C).

3.3. Cocoon influence on pupal $\dot{V}O_2$

Mass-specific $\dot{V}O_2$ for the control animals (pupa in cocoon), was 0.22 ± 0.01 $mL \cdot g^{-1} \cdot h^{-1}$, which compares favorably with the value of 0.21 ± 0.01 $mL \cdot O_2 \cdot h^{-1}$ reported for the pupae of *B. mori* in a recent study (Blossman-Myer and Burggren, in press). Sham operated and exposed groups also had mass-specific $\dot{V}O_2$ s in the range of 0.21 to 0.24 $mL \cdot g^{-1} \cdot h^{-1}$ (Table 2). Indeed, there were no significant differences ($P = 0.18$) between the three populations. Thus, the presence or absence of the cocoon had no impact on pupal oxygen consumption.

3.4. Cocoon oxygen diffusion properties

Fig. 4 shows the diffusion characteristics of the cocoon wall. The diffusion coefficient, DO_2, was determined for 7 different O_2 concentration gradients ranging from 5.5 to 19.4%. Because DO_2 is expressed per unit ΔCO_2, it should be independent of ΔCO_2, and indeed was, as

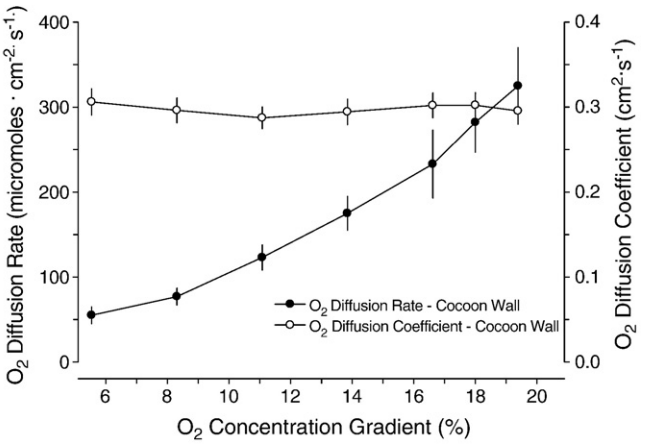

Fig. 4. O_2 diffusion rate and O_2 diffusion coefficient of the cocoon wall of *Bombyx mori*. Mean values ± 1 s.e.m. are shown. $n = 19$ for each mean point plotted.

evident in Fig. 4. Mean DO_2 at 25 °C was thus 0.298 ± 0.002 $cm^2 \cdot s^{-1}$ ($n = 19$ different cocoons).

4. Discussion

Bombyx mori, like many holometabolous insects, spins a complex cocoon as part of the pupation process. Under microscopic examination, the cocoon appears as a heterogeneous structure with respect to both silk fiber diameter and density (Iizuka et al., 1983; Zhao et al., 2007; Asakura et al., 2007). The outer cocoon layer is less dense and the individual silk fibers forming the outer cocoon are of larger diameter than the inner pelade of the cocoon. The pelade has higher Young's modulus, tensile strength, storage modulus and loss of modulus compared with the outer layers of the cocoon, and contributes significantly to the toughness and mechanical protection to the pupa afforded by the cocoon (Zhao et al., 2007). How the pelade is created during cocoon formation is not well understood, and may relate to the mechanics of cocoon creation. The coarser outer layer is spun first as the prepupa makes wide figure-eight movements to form the outer cocoon surface. As silk production and spinning continues, however, the emerging cocoon physically restricts the movements of the prepupa and limits maneuverability, as evident in the smaller magnitude spinning motions. This mechanical limitation of the prepupa within its cocoon might explain the production of the final inner, denser silk layer. The silk fibers spun late in the process are not only denser but also smaller in diameter (Fig. 2), perhaps due to decreased expulsion pressure in liquid silk from the silk glands as they begin to empty.

Irrespective of the reason for the cocoon heterogeneity, the cocoon as spun forms a dense sheath around the pupa. Insect silk comes in many forms due to its diverse protein structure (Iizuka et al., 1983; Sutherland et al., 2010), yet all silks seem to be characterized by a remarkable mechanical toughness (e.g. Asakura et al., 2007; Hakimi et al., 2007; Xiao et al., 2009). Indeed these properties have lead polymer chemists to attempt to replicate its characteristics in synthetic fibers used in everything from textiles to scaffolding for skin grafts (Hardy and Scheibel, 2009; Zhang et al., 2009)). The cocoon spun by *Bombyx* doubtlessly provides mechanical protection during the period of metamorphosis (e.g. Hakimi et al., 2006; Zhao et al., 2007). Unanswered, however, is the question of whether the cocoon of pupal *B. mori* also helps in preserving water vapor, as Gauthier et al. (2004) and Weisman et al. (2008), for example, have previously suggested as a function for cocoons of other insect species. If cocoons serve this purpose, then the exposed pupae of *B. mori* should have experienced a more rapid mass loss than the intact pupae when placed in a desiccating environment. Yet, the pattern and rate of water loss over a 48 h period is statistically identical in both the intact control pupae and the exposed pupae (Fig. 3). This refutes the idea that the pupal cocoon protects pupa from desiccation, an idea suggested by Gauthier et al. (2004) for the leek moth, *A. assectella*. However in more extreme desiccation situations than used in our experiments on silkworms–e.g. with very low humidity and/or when exposed to air currents–the cocoon might provide some modest degree of desiccation protection.

Does the mechanical protection afforded by the cocoon (without forming a barrier to water diffusion) occur at the price of restricted respiratory gas exchange? Control, sham and exposed pupa showed no significant differences in mass-specific $\dot{V}O_2$ within the four-hour measurement period (Table 2). While we cannot rule out compensatory changes by the intact pupae (e.g. increase in tracheal ventilation) that might offset oxygen diffusion limitation posed by the cocoon walls, the most parsimonious interpretation of these data is that the cocoon does not provide any significant barrier to O_2 diffusion. This is also compatible with both the high DO_2 of the cocoon wall (approximating the value of O_2 moving through an air pathway) as well as ultra structural observations of the cocoon fibers, the open weave of which yields numerous large pathways for gas diffusion.

Physiological evidence ($\dot{V}O_2$, rates of water loss), then, suggests that the cocoon has a very high diffusion coefficient and, indeed, this was evident from the DO_2 values obtained, which are very close to those for O_2 in an unobstructed air pathway. The diffusion coefficient of a gas increases with increasing temperature and, to a much lesser extent, decreases with increasing humidity. Extrapolation of published chemistry handbook values of DO_2, corrected for temperature and humidity, indicates a value of 0.221 $cm^2 s^{-1}$ for oxygen moving through an air pathway at 25 °C and 50% relative humidity, about 35% lower than our measured value of 0.298 $cm^2 s^{-1}$. To put this apparent discrepancy in context, both of these values are >7200× higher than for pure water (see Dejours, 1975) and the alveolar wall of mammal lungs (Federspiel, 1989) and about ~960 times higher than for the chitin that covers the body and lines the trachea.

In conclusion, the cocoon of *B. mori* provides structural protection, and perhaps camouflage, both of which would help survival and avoidance of predation. However, the data from this study do not fully support our original hypothesis, because the cocoon does not obstruct the exchange of respiratory gases between the pupa and its environment.

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