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RESEARCH ARTICLE

How temperature influences the viscosity of hornworm hemolymph

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ABSTRACT

Hemolymph is responsible for the transport of nutrients and metabolic waste within the insect circulatory system. Circulation of hemolymph is governed by viscosity, a physical property, which is well known to be influenced by temperature. However, the effect of temperature on hemolymph viscosity is unknown. We used *Manduca sexta* larvae to measure hemolymph viscosity across a range of physiologically relevant temperatures. Measurements were taken from 0 to 45°C using a cone and plate viscometer in a sealed environmental chamber. Hemolymph viscosity decreased with increasing temperature, showing a 6.4-fold change (11.08 to 1.74 cP) across the temperature range. Viscosity values exhibited two behaviors, changing rapidly from 0 to 15°C and slowly from 17.5 to 45°C. To test the effects of large particulates (e.g. cells) on viscosity, we also tested hemolymph plasma alone. Plasma viscosity also decreased as temperature increased, but did not exhibit two slope regimes, suggesting that particulates strongly influence low-temperature shifts in viscosity values. These results suggest that as environmental temperatures decrease, insects experience dramatic changes in hemolymph viscosity, leading to altered circulatory flows or increased energetic input to maintain similar flows. Such physical effects represent a previously unrecognized factor in the thermal biology of insects.

KEY WORDS: *Manduca sexta*, Thermal biology, Plasma, Density, Insects

INTRODUCTION

Blood is a critically important connective tissue in the animal body, delivering necessary nutrients to organs and cells, protecting from foreign bodies, removing metabolic waste and repairing injury (Hefnawy, 1972; Woodcock, 1976). In insects, the blood and lymphatic fluid commingle as hemolymph within an open circulatory system (Chapman et al., 2013; Ichikawa, 2009), with flow governed by the geometry of the circulatory network and the physical properties of the hemolymph itself. These physical properties include viscosity and density, which are biologically important because they directly influence the energetic requirements of flow production and patterns of flow throughout the body within an open circulatory system (Vogel, 1993, 1994). Blood viscosity has been measured in many vertebrates, including mammals (Amin and Sirs, 1985; Windberger et al., 2003), fish (Brill and Jones, 1994; Macdonald and Wells, 1991; Wells and Baldwin, 1990), reptiles (Dunlap, 2006; Snyder, 1971; Wells et al., 1991), amphibians (Palenske and Saunders, 2003) and birds (Clarke and Nicol, 1993; Zhou et al., 1999). However, perhaps owing to the presence of an open system, blood viscosity is generally unstudied in invertebrates (Snyder, 1978) and is unknown in insects.

Changes in blood viscosity can lead to physiological problems related to circulation. For example, hyperviscosity syndrome in humans results in decreased cardiac output, which increases the chance of mucosal hemorrhage, visual abnormalities, neurological anomalies and cardiac failure (Gertz and Kyle, 1995). Such problems arise from changes in blood composition (specifically, cell count), but the viscosity of any fluid also depends on external physical factors including pressure, shear rate (for non-Newtonian fluids) and temperature. The temperature dependence of viscosity is well known, with a general trend of increasing viscosity as temperature decreases. For example, human blood viscosity increases by a factor of nearly five when measured from 37 to 0°C (Eckmann et al., 2000), a change which could, in theory, retard flow by a factor of 15 (Dormandy, 1971). However, as endotherms, most mammals actively thermoregulate, maintaining precise internal body temperatures (with fluctuations of ∼1°C; Dulceata, 2014), meaning that large changes in blood viscosity should never be experienced by mammals under most non-pathological conditions.

Insects, however, are poikilotherms, whose body temperature varies with the external environment. Temperature affects many aspects of insect physiology, such as activity (Casey, 1976; Mellanby, 1939), foraging (Guarneri et al., 2003), flight (Taylor, 1963), courtship calling (Sueur and Sanborn, 2003), mating (Katsuki and Miyatake, 2009) and territorial behavior (Wickman, 1985). Changes in activity owing to temperature can also lead to secondary effects, including changes in critical thermal minimum and chill injury (Overgaard and MacMillan, 2017), as well as life span (Ragland and Sohal, 1975). Despite its importance in insect physiology, the role of temperature-driven changes in hemolymph viscosity has never been considered. Hemolymph comprises up to 45.4% of the volume of the insect (Chapman et al., 2013; Hefnawy, 1972; Jones, 1964; Lee, 1961; Wharton et al., 1965), and it must be pumped by the heart and accessory pulsatile organs, and move throughout the hemocoel and into micro-sized spaces such as the long and thin antennae, legs and delicate wing veins (Chapman et al., 2013; Wirkner et al., 2013). Correspondingly, flow speeds can range by orders of magnitude (Lee and Socha, 2009), resulting in fluid behavior that spans from inertia-dominated to viscosity-dominated. This characteristic suggests that changes in hemolymph viscosity mediated by temperature could significantly affect the circulation of hemolymph throughout the open circulatory system of insects.

Here, we report the first measurements of viscosity of insect hemolymph, and specifically address its temperature dependence.

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We measured hemolymph viscosity of *Manduca sexta* larvae across a physiologically relevant range of temperatures spanning from 0 to 45°C (Casey, 1976). To further characterize its properties, we also measured density and varied shear rate at a single temperature. Considering the importance of the circulation of hemolymph, viscosity of the hemolymph and possible temperature-mediated changes in viscosity may represent an important factor in the physiology of insects.

MATERIALS AND METHODS

Animals

Manduca sexta (Linnaeus 1763) larvae were obtained from an established colony at the University of Washington, and shipped to Virginia Tech overnight in individual containers within an insulated box. The larvae of this species were chosen for their short, well-studied life cycles, and because their large size enabled us to obtain sufficient hemolymph for testing (approximately 700 µl per animal). They were reared individually at room temperature (21–23°C) and given access to food *ad libitum* (Hornworm Diet, Carolina Biological Supply Company, NC, USA) until they were at least 8 g in mass and had reached the fifth instar of their developmental cycle.

Experimental set-up

Viscosity measurements were conducted with a cone and plate viscometer (DV-II+ Pro, Brookfield Engineering, MA, USA) (Fig. 1). Trials were performed with either a CP-40 or CP-51 spindle (Brookfield Engineering), which are designed for different ranges of viscosity. Because viscosity values may depend on shear rate (e.g. mammalian blood is shear-thinning; Eckmann et al., 2000), we chose spindle rotation speeds to produce reliable measurements and keep shear rates as consistent as possible (Table S1). The CP-40, referred to hereafter as the low-viscosity spindle, was used to measure viscosity values from 0.51 to 5.12 centipoise (cP; 1 cP=1 mPa s); it was operated at 60 rpm, producing shear of 450 s^{-1}. The CP-51, referred to hereafter as the high-viscosity spindle, was used to measure viscosity values from 4.05 to 40.45 cP; it was operated at 120 rpm, producing shear of 460 s^{-1}.

Temperature was controlled to the nearest 0.1°C using a chiller/heater circulator (RE 206, Lauda, Germany), which provided a constant flow of aqueous glycol through an internal chamber of the viscometer cup. We chose 0–45°C as the temperature range for measurements based on the lower (0°C) and upper (45°C) critical lethal limits for *M. sexta* (Casey, 1976), with trials performed at 5°C intervals. Additional trials were conducted at 17.5°C to provide further resolution on an apparent change in behavior in viscosity from 15 to 20°C. At each temperature, we recorded data from five samples, with one sample per individual.

To minimize oxygen-induced clotting (Movie 1), the viscometer was placed within a sealed environmental chamber (Cleatech Critical Laboratory Supplies, CA, USA), which was flooded with dry nitrogen gas (Airgas, VA, USA). Oxygen concentrations within the chamber were kept below 2%, verified with an oxygen sensor (Expedition X O2 Analyzer, OxyCheq, FL, USA). Humidity within the chamber was maintained at 60–80% using a small humidifier (Ultrasonic Cool Mist, Pure Enrichment) and measured with a hygrometer (Traceable, Fisher Scientific, NH, USA).

Experimental protocol

To extract a sample, larvae were first anesthetized by placing them in the environmental chamber and exposing them to nitrogen gas for 5 to 10 min. While still within the chamber, a small incision was made through the ventral exoskeleton of the animal between the second and third sets of prolegs. Hemolymph was dripped onto parafilm (Pechiney Plastic Packaging, WI, USA), and 0.7 ml was transferred to the cup of the viscometer via pipette. Because the incision site was close to the gut and fat bodies, some samples were contaminated by brown gut contents or white fat and were not tested. The viscometer required a minimum of 0.5 ml of fluid for measurements; we chose to use a slightly greater amount (0.7 ml) to account for the removal of potential contaminants, such as small bubbles that formed when placing the hemolymph within the cup. The time from incision to the start of the viscosity measurement was less than 2 min. Following each trial, any visible changes in the sample that may have occurred during testing were observed and recorded, such as the formation of thrombi (clots) (Fig. S1).

Between each trial, the spindle and cup were cleaned using Kimwipes (KimTech Science Brand, Kimberly-Clark Professional, GA, USA) and distilled water. We also performed a control experiment between each trial, wherein we measured the viscosity of 0.7 ml of distilled water and compared the measurements with published values. If our control measurements deviated from published values by more than 5%, the cleaning procedure and control trial were repeated.

In addition to testing whole hemolymph, we measured the viscosity of the plasma portion of the hemolymph using separate

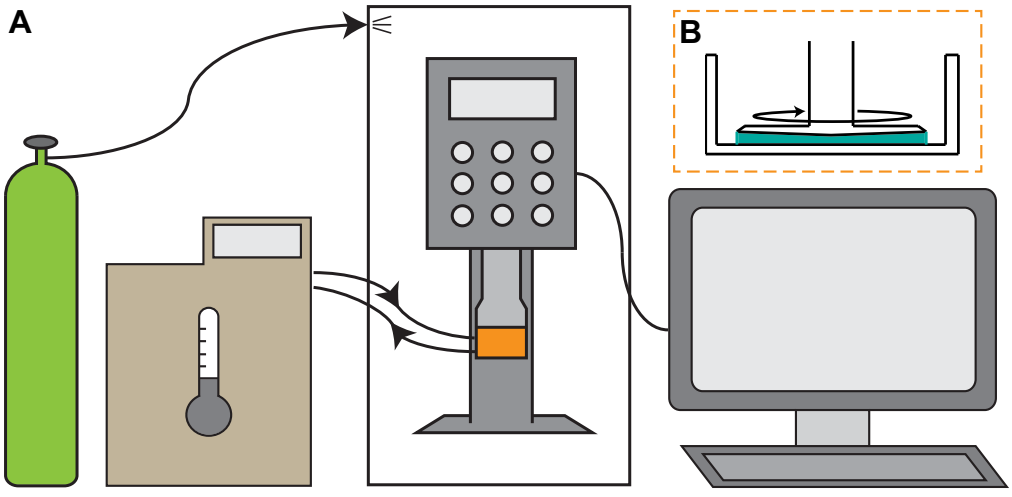

Fig. 1. Experimental set-up. (A) Diagram showing (left to right) the nitrogen gas tank, the temperature control circulatory bath, the environmental chamber with viscometer, and the computer. (B) Inset in top right corner shows the cross-section of the viscometer cup and spindle with blue-green hemolymph shown in the gap between (gap size is not to scale).

trials and specimens. To obtain the plasma, we first extracted a hemolymph sample using the same method as previously described. Each hemolymph sample was centrifuged (Mini Centrifuge Mini-10k+, Miulab, China) for 5 min at 5600 ***g*** two times, separating the supernatant after each round. The final supernatant, which we consider to be the plasma, was isolated via pipette and tested in the viscometer. Removal of cells was verified via cell counting (Vi-Cell Cell Counter, Beckman Coulter, USA) from five 1 ml samples of 9:1 dilutions of whole hemolymph and plasma with an anticoagulant [98 mmol l^{-1} NaOH, 186 mmol l^{-1} NaCl, 1.7 mmol l^{-1} EDTA, 41 mmol l^{-1} citric acid, pH 6.8 (Mead et al., 1986)]. The average cell volume was $7.05\times10^{6}\pm1.64\times10^{6}$ cells ml^{-1} for whole hemolymph and $3.90\times10^{5}\pm2.2\times10^{5}$ cells ml^{-1} for plasma, indicating that centrifugation removed 94.5% of cells.

Data collection and processing

During each trial, viscosity data were recorded at 2 Hz. The raw instantaneous viscosity values over time were processed using a custom MATLAB code to identify durations when the viscosity was steady. We quantified such periods by calculating a sliding window standard deviation of the raw viscosity values over a 75 s period centered on each data point, and then considered those points whose standard deviation was less than 0.05 cP as steady. The representative viscosity value for each sample was calculated as the average of all steady values for its trial. If the viscosity was not found to be steady, the trial was not included for analysis. In total, 96 trials were performed with whole hemolymph, and 55 trials yielded steady values. For plasma, 67 trials were performed, and 50 trials yielded steady values. See Fig. S1 for representative traces and Fig. S3 for all trials.

Generally, viscosity values equilibrated within 2 to 15 min and were run for at least 3 min once they appeared steady. Trials were not run for longer than 25 min total, yielding a total range of 9–25 min across trials. Plasma trials took less time to equilibrate on average and had a range of 3–23 min in total trial length. In some trials, the measured viscosity increased rapidly for anywhere from a few seconds to a few minutes. The exact cause of these fluctuations is unknown, but they may have been due to the formation of one or more clots, which were found free-floating in some samples post-test. Many of the whole hemolymph trials and only a few of the plasma trials showed the formation of such clots, which ranged in size from 0.4 to 8.6 mm (Fig. S1).

Varying shear rate

In a second set of trials, we tested whole hemolymph and plasma at multiple shear rates, controlled by varying the rotation speed of the spindle. Trials were begun at 15 or 30 rpm with 15 or 30 rpm increases until reaching 120 or 135 rpm, representing a 112.5 to 1012.5 s^{-1} range of shear rates. For some trials, the lowest or highest shear rate measured viscosity values outside of the instrument's range and were unable to be measured (Table S1). A representative viscosity was determined for each shear rate using the same technique described previously. All trials were performed at 25°C using the low-viscosity spindle.

Density measurements

The density of whole hemolymph at room temperature was calculated by measuring the mass and volume of additional samples. Hemolymph was extracted as before, allowing the fluid to collect onto parafilm. A 0.3 ml syringe with needle was first weighed, then loaded with 0.05–0.29 ml of hemolymph depending on the amount that was obtained from the animal. In total, 99 larvae were tested ranging in age from 17 to 37 days at room temperature (22°C). As a control, the density of distilled water at 22°C was also measured (n=10) using the same method.

Statistical analysis

RStudio (Foundation for Open Access Statistics) was used for all statistical analyses. One-sample t-tests and paired t-tests were used for comparisons of values. A linear model (LM) was used to test for the effect of one variable upon another. A linear model with logarithmic transform was used to fit an exponential model to the viscosity versus temperature data, as is regularly used to parameterize the temperature dependence of liquid viscosity (Reynolds, 1886). For the trials with varying shear rate, each trial did not yield a value at every measurable shear rate, so a linear mixed model (LMM) was used both with and without shear rate considered; an ANOVA compared these two models to test for an effect of shear rate on viscosity values.

RESULTS

Effect of temperature on viscosity of whole hemolymph

As temperature increased from 0 to 45°C, the average viscosity of whole hemolymph decreased from 11.1 to 1.7 cP (LM: P<0.0001; Fig. 2), representing a 6.4-fold change across temperature. There appeared to be a steeper decline in viscosity at lower temperatures (0–15°C; 11.1 to 5.1 cP) than at higher temperatures (17.5–45°C; 3.5 to 1.7 cP). To quantify this difference, a continuous piecewise exponential model with a single break point was fit to the data [LM with a logarithmic transform of viscosity following Muggeo (2008), R^2=0.92; Fig. S2], yielding the following relationship:

$$\mu_w = \begin{cases} 11.8e^{-0.0663T} & \text{for } T \leq 22.7°C \\ 4.10e^{-0.0196T} & \text{for } T > 22.7°C \end{cases}, \quad (1)$$

where μ_w is the viscosity of whole hemolymph (cP) and T is the temperature (°C). The viscosity of whole hemolymph was greater than that of water at all temperatures (paired t-test: P<0.005; Fig. 2).

The relative viscosity of whole hemolymph compared with water shows a distinct change between high and low temperatures, but follows a different trend than seen in whole hemolymph (Fig. 3). At low temperatures (0–17.5°C), relative viscosity decreased with increasing temperature with a slope of $-0.17°C^{-1}$ (LM: P<0.0001), but at high temperatures (17.5 to 45°C), temperature was not correlated with relative hemolymph viscosity (LM: P=0.344). On average, the viscosity of whole hemolymph at high temperatures (17.5 to 45°C) was 2.90±0.46 (mean±s.d.) times the viscosity of water.

Effect of temperature on viscosity of plasma

Similar to whole hemolymph, as temperature increased from 0 to 45°C, the viscosity of plasma decreased from 3.6 to 1.1 cP (LM: P<0.0001; Fig. 2), representing a 3.3-fold change. The viscosity of plasma was less than the viscosity of whole hemolymph at all temperatures (paired t-test: P=0.006). An exponential model fit to the data (LM with a logarithmic transform of viscosity, R^2=0.94; Fig. S2) yielded:

$$\mu_p = 3.25e^{-0.0249T}, \quad (2)$$

where μ_p is the viscosity of plasma (cP). The viscosity of plasma was also greater than that of water at all temperatures measured (paired t-test: P<0.0001; Fig. 2). Plasma viscosity relative to water decreased with increasing temperature by only $-0.006°C^{-1}$, but this was

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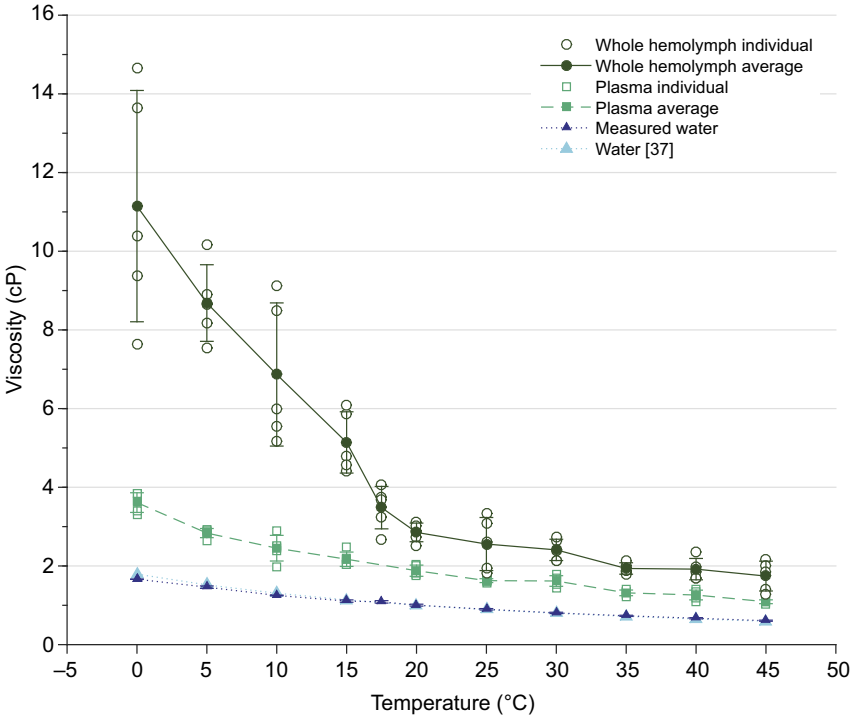

Fig. 2. Temperature dependence of viscosity for whole hemolymph and plasma of *Manduca sexta* larvae. Known values of water (from Kestin et al., 1978) are shown for comparison. Filled points represent the mean value for each temperature, with the individual trials shown as non-filled points. Error bars represent standard deviation of the mean. The quantitative fits for each trend are provided in Eqns 1 and 2.

significant (Fig. 3, LM: $P<0.001$). On average, plasma was 1.92±0.18 (mean±s.d.) times the viscosity of water at all temperatures.

Effect of shear rate on viscosity

For both whole hemolymph and plasma, as shear rate increased, viscosity decreased (LMM with ANOVA: $P<0.0001$ for both; Fig. 4). The average change in viscosity over shear rate was -1.9×10^{-3} cP s for whole hemolymph and -7.6×10^{-4} cP s for plasma.

Density of whole hemolymph

The density of whole hemolymph at 22°C was 1.02±0.03 g ml^{-1}, which was significantly greater than the density of water at that temperature [0.9977705 g ml^{-1} (Kell, 1975); one-sample *t*-test: $P<0.0001$]. Density increased with age (LM: $P=0.022$) and mass (LM: $P=0.003$) of the animal (Fig. S4).

Validation of methods

Viscosity values for water trials for each temperature were within 2.46±1.74% of literature values (Kestin et al., 1978), which was not a significant difference (paired *t*-test: $P=0.2$). As expected, viscosity of water did not vary with shear rate. Density values of distilled water yielded an average density of 0.995±0.016 g ml^{-1}, which was not significantly different from the literature value at 22°C [0.9977705 g ml^{-1} (Kell, 1975); one-sample *t*-test: $P=0.59$].

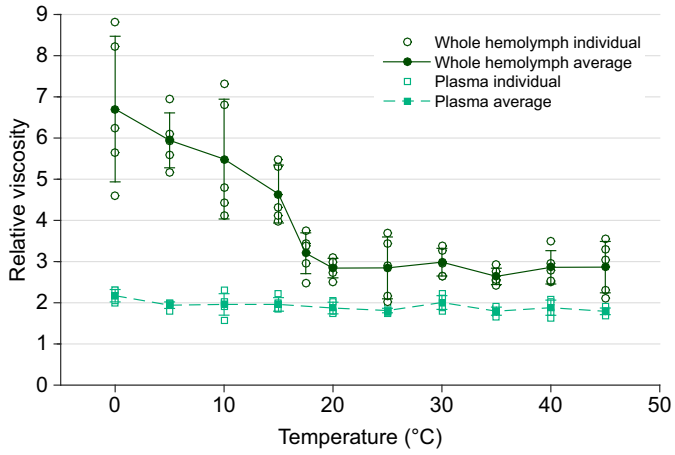

Fig. 3. Viscosity relative to water versus temperature for both whole hemolymph and plasma in *Manduca sexta* larvae. Filled points represent the mean value for each temperature, with the individual trials shown as unfilled points. Error bars represent standard deviation of the mean. Relative viscosity of plasma appears to remain consistent with temperature, as does relative viscosity of whole hemolymph temperatures higher than 17.5°C. At low temperatures (<17.5°C), relative viscosity of whole hemolymph increases with decreasing temperature.

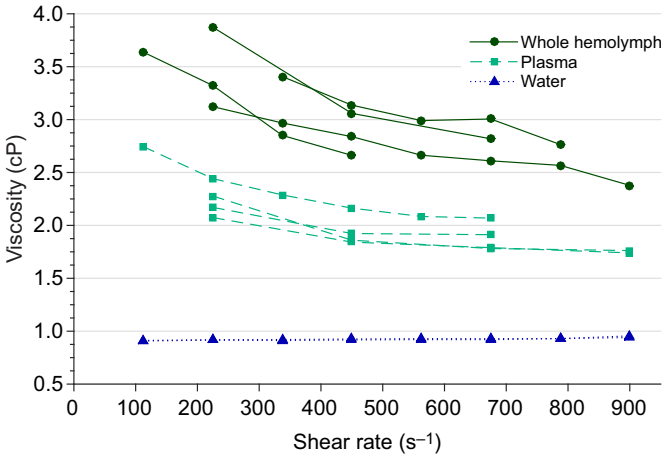

Fig. 4. Viscosity versus shear rate for whole hemolymph and plasma of *Manduca sexta* larvae. Water, shown for comparison, does not vary with shear rate. Each connected set of points represents a single trial of a single sample, in which shear rate was changed incrementally over time. For some trials, the lowest or highest shear rate measured viscosity values outside of the instrument's range and were unable to be measured (Table S1).

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DISCUSSION

In this study, we show that the viscosity of whole hemolymph and plasma of *M. sexta* larvae decreases as temperature increases. This general trend has also been observed in the blood of many other species across taxonomic groups, including mammals [humans (Eckmann et al., 2000; Guard and Murrish, 1975), penguins (Clarke and Nicol, 1993; Guard and Murrish, 1975), seals (Guard and Murrish, 1975), ducks (Guard and Murrish, 1975), dogs (Palenske and Saunders, 2002), horses (Palenske and Saunders, 2002) and rats (Palenske and Saunders, 2002)], amphibians [toads (Palenske and Saunders, 2002)] and fish (Macdonald and Wells, 1991). Hemolymph viscosity changes more rapidly in response to temperatures lower than 15°C, a behavior that is similarly found in human blood (Eckmann et al., 2000; Snyder, 1971), which contains far more cells. This low-temperature effect did not occur in plasma, suggesting that the presence of cells or other large particulates are responsible for this behavior. In human blood, decreasing temperature increases the aggregation of red blood cells (Neumann et al., 1987), which increases blood viscosity (Chien et al., 1967). Similarly, the deformability of red blood cells decreases with lower temperature (Eckmann et al., 2000; Neumann et al., 1987), which also increases blood viscosity (Eckmann et al., 2000; Neumann et al., 1987; Woodcock, 1976). Changes in cell deformability with temperature occur in other animal cell types as well (Lichtman and Kearney, 1970; Petersen et al., 1982), suggesting that this mechanical behavior is a characteristic of the cell's lipid bilayer membrane. Insect hemocytes may respond similarly to decreases in temperature, potentially explaining the steep incline in viscosity at low temperatures observed in the hemolymph of *M. sexta* larvae.

The whole hemolymph viscosity values found here, irrespective of temperature, are similar in magnitude to values reported in other animals (Clarke and Nicol, 1993; Dunlap, 2006; Guard and Murrish, 1975; Macdonald and Wells, 1991; Palenske and Saunders, 2002; Wells et al., 1991). In most cases, direct comparisons cannot be made, because measurements have been conducted at different shear rates and temperature. When compared with the few viscosity measurements that have been made at the same shear rate (450 s^{-1}) and temperature (30°C), the insect hemolymph viscosity (range=2.1–2.7 cP) is found to be close to that of both crocodiles (average=2.89 cP; Wells et al., 1991) and the western fence lizard (range=2.7–5.9 cP, with higher values at greater hematocrit; Dunlap, 2006). The differences are likely due to the percentage of cell mass within the blood, which is directly correlated with blood viscosity across species (Snyder, 1971; Woodcock, 1976). The hematocrit percent of insect hemolymph is <5% (Clark and Jones, 1980), which is considerably less than the 18.7–50.8% measured in crocodiles and the western fence lizard (Dunlap, 2006; Wells et al., 1991). The removal of cell mass from blood to obtain plasma yields a lower viscosity in many organisms (Windberger et al., 2003), as is also seen here with insect whole hemolymph compared with plasma.

Viscosity of both whole hemolymph and plasma of *M. sexta* larvae was also found to be significantly affected by shear rate, showing a decrease in viscosity with increasing shear rate. This behavior indicates that hemolymph acts as a shear-thinning, non-Newtonian fluid, similar to the whole blood of all species in which shear rate has been tested [penguins, chickens, horses, pigs, dogs, cats, rats, cattle, sheep, rabbits, mice and humans (Clarke and Nicol, 1993; Eckmann et al., 2000; Windberger et al., 2003)]. Human plasma has historically been considered Newtonian (Gertz and Kyle, 1995), but recent evidence suggests it is non-Newtonian (Brust et al., 2013), a result that we also found with insect hemolymph plasma. In many animals, blood viscosity is shear-rate dependent only at low shear rates (<45 s^{-1}) (Eckmann et al., 2000); at high shear rates (>45 s^{-1}), viscosity is nearly independent of shear rate. Unfortunately, owing to experimental limits, we were unable to measure viscosity at a shear rate lower than 112.5 s^{-1} (Table S1), but we can roughly estimate the shear rates that might be experienced within the insect. The insect circulatory system operates at the microscale with very low Reynolds numbers (ratio of inertial to viscous forces; Vogel, 1994), with the fastest flows likely occurring in the heart; thus, highest shear rates should be found here. Few exact values are known, but taking the grasshopper heart as an example, the maximum shear rate can be estimated. Assuming a maximum velocity of 9.5 mm s^{-1} with a 0.5 mm diameter of the heart (Lee and Socha, 2009), shear rate can be calculated to be approximately 152 s^{-1} at the inner surface of the dorsal vessel. This estimation assumes Poiseuille flow, in which the shear rate of a fluid flowing in a pipe is equivalent to eight times the ratio of the linear fluid velocity to the diameter of the pipe (Vogel, 1994). Although no detailed velocity profiles of flow in the heart are available to test for Poiseuille flow, this assumption likely represents a lower bound on shear rate (compared with other possibilities such as plug flow). In addition, the pumping in both the dorsal vessel and the accessory pulsatile organs involves time-varying velocities (Lee and Socha, 2009), which must result in varying and potentially higher shear rates. This analysis suggests that viscosity of hemolymph, as a non-Newtonian fluid, should change throughout the stroke cycle as well.

Changes in viscosity of hemolymph, owing to either temperature or shear rate, may influence any aspect of physiology that depends on circulation. The viscosity of a fluid is a measure of its resistance to deforming motion; essentially, how fast a fluid changes shape. In order to produce a faster shape change or fluid movement, more force must be applied to the fluid, which requires more mechanical work and, thus, the investment of metabolic energy. Similarly, the more viscous the fluid, the more energy is needed to simply maintain the same speed of deformation. If energy investment remains constant, then flow rates must change with temperature. Volume flow rate is inversely proportional to the viscosity of the fluid, assuming laminar flow through a rigid pipe with no entrance or branch point nearby (Vogel, 1993). Our results show a 6.4-fold increase in hemolymph viscosity as temperature decreases from 45 to 0°C, which would indicate, all else being equal, a 6.4-fold decrease in volume flow rate of hemolymph within the insect. This decreased flow of hemolymph throughout the insect could retard the circulation of nutrients, removal of metabolic waste, and other necessary functions of the circulatory system. Changes in viscosity might also induce changes in flow patterns as Reynolds number changes, meaning that flow of hemolymph through the smallest structures such as antennae, legs and various internal organs, including the dorsal vessel and auxiliary pumps, could become particularly limited. In humans, for example, many of the dangerous symptoms of hyperviscosity (increased viscosity) of the blood are due to decreased flow in the smallest capillaries, which experience the smallest Reynolds numbers. This can lead to pre-mortem lividity owing to the inability of deoxygenated hemoglobin to flow away from the skin (Gertz and Kyle, 1995), decreased cognitive function owing to lowered cerebral blood flow (McMullin et al., 2005), as well as eyesight impairment owing to decreased retinal blood flow (Gertz and Kyle, 1995). However, in contrast to vertebrate species, insects largely decouple gas exchange from circulation: most transport of oxygen occurs within the tracheal system, not the

hemolymph. Thus, hyperviscosity of hemolymph in insects should produce a smaller or negligible negative effect on the animal's metabolic rate and ability to function than it would in animals with coupled respiratory and circulatory systems. Regardless of these potential effects, the physics of flow dictate that at low temperatures, insects must compensate physiologically to maintain the same circulatory flow; if not, they must experience altered flows of hemolymph throughout the hemocoel.

In our experiments, hemolymph was extracted at room temperature before its temperature was altered for viscosity measurements, so we did not subject the insects themselves to changing temperatures. Many insects have evolved a variety of mechanisms to respond to and survive temperature extremes and variability that directly affect the hemolymph composition. Upregulation of heat shock proteins facilitates correct protein folding and degradation of non-functional proteins, which could be inhibited owing to heat exposure (Sørensen et al., 2003). Expression of heat shock proteins has been observed in several insect species including *Cataglyphis* ants (Gehring and Wehner, 1995) and adult *Drosophila* flies (Dahlgaard et al., 1998). In response to cold, insects have generally adopted three main strategies: (1) keep bodily fluids below their normal melting point (freeze avoidance), (2) survive ice formation (freeze tolerance) or (3) depress the melting point of their bodily fluids (cryoprotective dehydration) (Sinclair et al., 2003). Freeze avoidance can involve increased glycerol production, purging of water and synthesis of antifreeze proteins to lower crystallization temperatures (Holmstrup et al., 2002; Zachariassen, 1985). Freeze tolerance involves specific methods to avoid ice formation within cells. It is possible that such physiological responses may affect hemolymph composition and, subsequently, viscosity. In fact, when allowed to acclimate to different temperatures, American bullfrogs do not exhibit changes in blood viscosity with changing temperature (Palenske and Saunders, 2003). The possibility of such effects in insects suggests a direction for future exploration.

As a first study of viscosity in insect hemolymph, we chose to focus on one species and developmental stage. Insects vary not only in size and morphology across species, but they can also vary considerably within species owing to life cycle stage, environmental factors and availability of resources. Variation in hemolymph viscosity across species and developmental stages may arise because of differences in water content, cell properties and plasma protein concentrations, suggesting a large number of new research questions related to viscosity. For instance, how does hemolymph viscosity vary in pupae or adults? Has the response of hemolymph viscosity to temperature been influenced by evolutionary pressures? Do insects that experience near-constant temperatures have a different hemolymph viscosity response to temperature than those that experience more varying temperatures? Hemocyte numbers in *M. sexta* change daily throughout the larval stage (Beetz et al., 2008) as the larvae prepare for pupation, which suggests that hemolymph viscosity may change through development as well. Fifth-instar *M. sexta* larvae were used in this study for consistency and because of their large volume of hemolymph, which was required for our methods. Measuring the hemolymph viscosity in many other insect species and developmental stages, particularly those with smaller body size and/or hemolymph content, will require different methods. One promising candidate is magnetic rotational spectroscopy, which can measure viscosity in a much smaller volume of liquid (Tokarev et al., 2013); however, this method has not yet been applied successfully to hemolymph.

These findings provide the first quantification of insect hemolymph viscosity and density, as well as measuring the response of viscosity to changes in temperature and shear rate. Understanding these values and relationships helps to reveal potentially important factors for the role of hemolymph in insect physiology.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.N.G., E.G., J.J.S.; Methodology: M.C.K., M.N.G., E.G., J.J.S.; Software: M.C.K., M.N.G.; Validation: M.C.K., J.J.S.; Formal analysis: M.C.K., M.N.G.; Investigation: M.C.K., M.N.G., E.G., J.J.S.; Resources: J.J.S.; Data curation: M.C.K.; Writing - original draft: M.C.K., J.J.S.; Writing - review & editing: M.C.K., M.N.G., E.G., J.J.S.; Visualization: M.C.K., J.J.S.; Supervision: M.C.K., J.J.S.; Project administration: M.C.K., J.J.S.; Funding acquisition: J.J.S.

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Data availability
Data from this study are available at https://github.com/TheSochaLab/How-temperature-affects-the-viscosity-of-hornworm-hemolymph

Supplementary information
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