Overview: Haemolymph coagulation in response to an immune elicitor is measured as the retention of a dye (Amaranth Red) injected into the insect haemocoel at the same time as an immune challenge (LPS) or vehicle (Ringer). If the dye is retained at the site of haemolymph coagulation less will be available to freely diffuse through the open haemocoel and it will therefore be more dilute compared to injection of the same volume of dye when coagulation is not occurring.

Materials:
Amaranth Red (Sigma)
Insect Ringer solution (NaCl, CaCl₂, KCl, and NaHCO₃; recipe follows)
Lipopolysaccharide (LPS)
Insect anesthetic (such as CO₂)

Special lab equipment needed:
Plate reader/spectrophotometer
96-well plates

Methods
Making a calibration curve
A 2% solution of Amaranth Red (Sigma) was made up in Insect Ringer solution (128 mM NaCl, 18 mM CaCl₂, 1.3 mM KCl, 2.3 mM NaHCO₃), and a second solution was made up in Insect Ringer supplemented with LPS (0.5 mg/ml). 5 µl of Amaranth solution was diluted in 25, 50, 100, 200, 300 and 400 µl in Insect Ringer or LPS (0.5 mg/ml in Ringer), respectively. A 5 µl aliquot of these dilutions was added to 195µl LPS/Ringer and the optical density at 520nm (OD₅₂₀) was measured photometrically on a plate reader (Versamax, Molecular Devices, Sunnyvale, California, USA). The OD was plotted against dilution volume in order to construct standard curves for LPS/Ringer dilutions.

Measuring clotting in vivo
Experimental cultures were assigned to two treatments: ‘Ringer’ and ‘LPS’. Cockroaches were anaesthetized for 30 seconds with CO₂ then injected with 5 µl Amaranth in Ringer, or Amaranth in Ringer + LPS, respectively. After a set period of time (5, 15, 30, 45 and 60 minutes after injection), individuals were briefly CO₂ anaesthetized and a 5 µl sample of haemolymph was taken from an anterior leg severed at the femural-tibial joint. Three consecutive 5 µl samples were taken from each individual. Each sample was added to 195 µl of Ringer/LPS solution in a 96-well plate and the OD₅₂₀ measured. The mean OD₅₂₀ for the three measures for each individual was converted to dilution of the dye in insect haemolymph, based on the standard curves, in order to obtain a relative measure of in vivo coagulation between treatments. Twenty cockroaches were used for each treatment at each time point. Some individuals failed to produce three 5 µl haemolymph samples: these individuals were excluded from analyses.

References