Haem detoxification by an insect

Haem is involved in many biological reactions, including oxygen transport, respiration and photosynthesis. In the free state, however, haem can generate reactive oxygen species that can damage biological molecules. It can also disrupt the phospholipid bilayer of cell membranes[1]. In *Plasmodium* parasites, which are the aetiological agents of malaria disease, up to 80% of host-cell haemoglobin is digested[2], leaving the free haem group to be detoxified in the parasite's food vacuole by polymerizing it into a harmless dark-brown crystalline structure called malaria pigment or haemozoin[3]. Haem detoxification is also a challenge for blood-sucking insects, which digest several times their own weight of vertebrate blood during a blood meal. Here we show that haem polymerization into haemozoin is not exclusive to *Plasmodium*: it also occurs in the midgut of the blood-sucking insect *Rhodnius prolixus* (Hemiptera), an important vector of *Trypanosoma cruzi*, the causative agent of Chagas' disease.

Transmission electron microscopy (TEM) reveals that the lumen of the *R. prolixus* midgut contains large electron-dense aggregates (Fig. 1a) that are similar in appearance to the haemozoin granules found in *Plasmodium* parasites[4]. These aggregates can be visualized even in the absence of impregnation with electron-dense stains (Fig. 1b), and contain an abundance of iron (Fig. 1c). We conclude that they are derived from digestion of haemoglobin in the insect midgut. After extraction using the same protocol as for malarial haemozoin[5], this material gives a Fourier transform infrared (FTIR) spectrum very similar to that of malarial haemozoin[3], with distinctive peaks at 1,210 and 1,663 cm^{-1} (Fig. 1d). These peaks are not seen in the haemin spectrum and have been attributed to iron–carboxylate bonds in the haemozoin[3].

Adducts of haem and acetate anion give a haemozoin-like FTIR spectrum but, like unpolymerized haem, this derivative is soluble in a weakly alkaline solution, whereas haemozoin is virtually insoluble[6]. The material from the *R. prolixus* midgut is completely insoluble at pH 9.1, but it can be solubilized in 0.1 M NaOH as this breaks the iron–carboxylate bonds of haemozoin to produce monomeric haem. These results show that the pigment isolated from the *R. prolixus* midgut is haemozoin. To our knowledge, this is the first time haemozoin has been found in an organism that is not a malaria parasite.

The mechanism of haemozoin formation *in vivo* is controversial. The existence of a haem polymerase enzyme has been proposed on the basis that a crude extract from trophozoites can induce haem polymerization *in vitro*, although no such enzyme has ever been isolated. Also, preformed haemozoin can seed haemozoin formation even in the absence of protein[7], and it may be driven by phospholipids[8]. In *R. prolixus*, we found that the capacity to induce haem polymerization is associated with a particulate fraction from the midgut lumen that is composed mainly of perimicrovillar membranes, which are extracellular phospholipid-containing membranes that cover the epithelium microvilli and bleb to the intestinal lumen (Fig. 1a).

Haem polymerization in the midgut would be the first line of defence against the effects of releasing haem by haemoglobin digestion. Sequestration of haem into an insoluble form would lead to its elimination in the insect's faeces. Otherwise, in the absence of haemozoin formation, large amounts of haem could cross the midgut wall, potentially resulting in oxidative tissue damage.

The protective effect of haemozoin synthesis in *R. prolixus* is complemented by other mechanisms directed against haem toxicity. *R. prolixus* also has a haem-binding antioxidant protein[9] and unusually high titres of uric acid (a free-radical scavenger) in its haemolymph[10]. Building an array of defences against haem toxicity therefore seems to be an important trend in the evolution of blood-sucking insects.

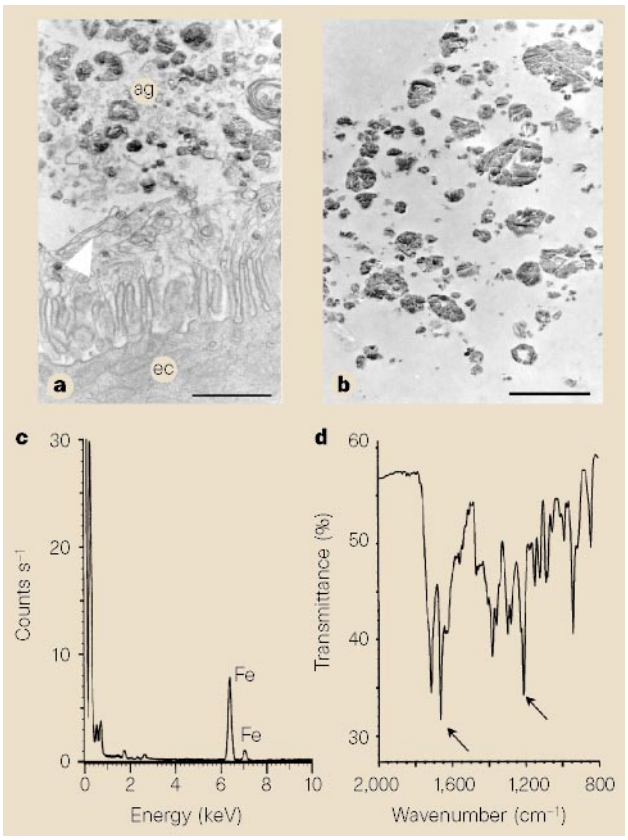

Figure 1 Haemozoin in *R. prolixus* midgut. **a,** TEM of a cross-section of *R. prolixus* midgut stained with uranyl acetate and lead citrate, showing electron-dense aggregates in lumen (ag) and epithelial cells (ec). Arrowhead indicates perimicrovillar membranes. **b,** TEM with no staining, showing luminal electron-dense aggregates. **c,** X-ray microanalysis of crystals in the intestinal lumen on nylon grids. **d,** FTIR spectrum of *R. prolixus* pigment from midgut contents 4 days after a blood meal. Arrows indicate peaks characteristic of haemozoin. Midgut contents were obtained by shaking dissected midguts in 0.15 M NaCl. Tissue was discarded and the suspension centrifuged. The insoluble pigment was purified and remaining solids were washed and dried. KBr pellets were prepared from dried samples and spectra were acquired for 32 cycles with a FTIR spectrometer. Scale bars: 0.9 μm (**a**) and 0.6 μm (**b**). Further experimental details are available from the authors.

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'Raise the stakes' evolves into a defector

To understand how cooperation can evolve by reciprocal altruism when individuals can make variable investments, Roberts and Sherratt[1] have introduced a new strategy, 'raise the stakes' (RTS), for a continuous version of the iterated 'prisoner's dilemma'. An individual investing I bears a cost I, while the recipient gets a benefit kI. For $k>1$, this generalizes the standard prisoner's dilemma[2–5]. Over R alternating encounters[6,7], RTS is defined as follows: on the first move, invest a, subsequently raise your investment by $2b$ (or b) if your partner's previous investment bettered (or equalled) your last move, otherwise match your partner's last move. This strategy is denoted by $\sigma=(a,b)$. Roberts and Sherratt[1] reported that the strategy $\sigma=(1,1)$ performs well in computer simulations against various alternative strategies but did not consider how a population of RTS strategies with different a and b values evolves. We find that selection within RTS populations always acts to lower the values of a and b, hence RTS cooperation is not a robust phenomenon.

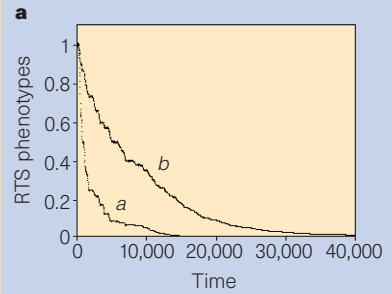

Figure 1 Simulation of the evolution of RTS strategies in the game studied in ref. 1. **a,** Changes in the population mean values of the RTS parameters a and b (starting values, $a=1$ and $b=1$). **b,** Change in the mean payoff. In this simulation, $k=2$, $R=20$ (the same as in all figures in ref. 1).

Assuming that mutations are small and rare, evolution in a population of RTS strategies can be understood analytically by using adaptive dynamics[8]. If the population consists of individuals using the strategy $\hat{\sigma}=(\hat{a},\hat{b})$, then the vector field $\xi=\{[\partial S(\sigma,\hat{\sigma})/\partial a]_{\sigma=\hat{\sigma}},\ [\partial S(\sigma,\hat{\sigma})/\partial b]_{\sigma=\hat{\sigma}}\}$ determines the direction that optimizes the increase in payoff of a mutant strategy $\sigma=(a,b)$ (ref. 8), where S is the payoff from an iterated interaction. S, and hence ξ, can be calculated analytically and it can be shown that evolution acts to lower the (a,b) parameters of the population. This yields the general prediction that the (a,b) parameters in a population of RTS strategies evolve to zero under natural selection.

This prediction is verified by evolutionary simulations. Consider a population of RTS strategies, with new mutants introduced at a certain rate. In every generation, each strategy plays against all the others and their frequencies in the next generation are calculated using standard game dynamics[8]. Any strategy whose frequency falls below a given threshold is eliminated. A typical simulation (for parameter values used in ref. 1) is shown in Fig. 1. As predicted, the (a,b) parameters evolve to zero. Extensive simulation has confirmed the analytical result for all parameter values studied (including extreme cases, such as $k=100$, $R=1{,}000$).

Thus, in general, RTS evolves under natural selection into an unconditional defector (a=0, b=0). The lack of robustness of RTS arises because, although it is essential from an evolutionary perspective to allow the strategies $\sigma=(a,b)$ to vary continuously (as mutations can, in principle, result in arbitrary changes in a and b), the definition of RTS is discontinuous. From a biological viewpoint, the discontinuous nature of RTS is unrealistic as it is implausible that two strategies that are arbitrarily close would have qualitatively different behaviour.

Although reciprocal altruism with variable investments is an important approach to understanding the evolution of cooperation, our results indicate that new strategies are required to give a satisfactory theoretical account of this process. We have found, both analytically and by simulation, that investment strategies based on an individual's payoff in the previous round (see those used to study mutualism in ref. 9), rather than on the partner's investment, are evolutionarily robust and show how intraspecific cooperation can emerge with variable investments. We believe that these payoff-based strategies represent a more fertile area for future research than RTS strategies.

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Sherratt and Roberts reply — Killingback and Doebeli argue that our cooperative strategy 'raise the stakes'[1] (RTS) can be continually undermined by selection for less generous strategies. They suggest that the "lack of robustness of RTS" arises from our use of a discontinuous strategy. However, this cannot be the case because the instability they report was in their reformulation of our model in continuous terms. Whether a continuous model is "essential" is debatable. Discontinuous strategies can be more realistic, particularly when resources are not infinitely divisible, hence our notion of a minimal non-zero investment of one unit.

We have also considered the relative success of rare, mutant continuous RTS strategies, but our analyses show that the mean initial investment parameter a will always evolve upwards. Simulations confirm this. Therefore, after trying to replicate their approach, we can find no evidence that even the continuous form of RTS-based cooperation can be eroded in the way they suggest. From this, we cannot exclude the possibility that they have misinterpreted the way RTS operates.

Killingback and Doebeli appear to agree that cooperation can thrive in variable investment systems and that successful strategies would tend to exhibit some initial build up of 'trust'. However, they claim that a strategy that depends on responding to the payoff would be more stable, which we question for two reasons. First, payoff dependency can lead to unnecessary investment in a sucker. Second, in a recent payoff-dependent model[2], negative payoffs always resulted in the end of cooperation, whereas RTS can rebuild relationships. These sources of instability are highlighted by the need for spatial structuring before payoff-dependent mutualism could evolve[2]. Such assumptions are not required when cost-dependent mutualistic strategies are considered (unpublished data). (Further details are available from T.N.S.)

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