J. exp. Biol. (1979), 83, 325–333
[illegible] 3 *figures*
Printed in Great Britain

HAEMOLYMPH-VOLUME CHANGES IN *RHODNIUS PROLIXUS* DURING FLIGHT

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(*Received* 14 *February* 1979)

SUMMARY

Exhaustive flight in male *Rhodnius prolixus* Stål (Hemiptera: Reduviidae) can cause up to a 50% decrease in haemolymph volume. Insects urinate during the flight period which may account for this decrease. The percentage water in the haemolymph does not appear to change substantially during exhaustive flight.

INTRODUCTION

Very little information has been published on the adaptations of the insect excretory system during acute physical stress such as flight. Presumably the increased metabolic activity required for flight would impose a sudden load on the excretory system. Mordue (cited by Goldsworthy, 1976) claims that Malpighian tubule activity increases in locusts during flight. Since locust haemolymph volume is not changed during flight (Beenakkers, 1973), Goldsworthy (1976) suggested that both a diuretic hormone, acting on the Malpighian tubules, and an antidiuretic hormone, acting on the hindgut, were being simultaneously released. This results in the cycling of water through the Malpighian tubules and back into the haemolymph via the hind gut, thus allowing the locust to maintain its water balance while eliminating waste products.

Water flux during flight as a result of both enhanced excretory activity and, to some extent, evaporative cooling (Church, 1960*a*, *b*; Edney & Barrass, 1962; Makings, 1968; Farnworth, 1972*a*, *b*) must be compensated for to allow the insect to remain in water balance. In addition to the recycling of water released into the hindgut, this balance might also be achieved by the production of metabolic water or the extraction of water from a reserve such as stored food in the intestine. Failure to achieve a balance would be manifested in declining haemolymph volumes and perhaps tissue dehydration as well.

In the course of collecting haemolymph from *R. prolixus* that had been flown, we became aware of the increasing difficulty of obtaining samples from insects which had been flown for periods of progressively longer duration. We also observed that *R. prolixus* excreted both faecal matter and an increasingly clear, colourless urine during the flight period and that flight muscle from exhaustively flown insects was [illegible]void of haemolymph and appeared dehydrated.

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These observations led us to question whether *R. prolixus* was capable of maintaining its internal water balance during a protracted flight period and to determine the extent to which haemolymph volume was actually affected.

MATERIALS AND METHODS

Experimental insects

The insects used were from a strain of *R. prolixus* originally collected as eggs in the wild in Venezuela approximately 9 years ago. They were reared in the dark at 23–27 °C and 71–88% R.H. Nymphs were fed a blood meal every 40–45 days on New Zealand white rabbits.

All insects used in the flight experiments were virgin males fed on the fifth day following ecdysis and, with few exceptions, flown 15–20 days later (days 20–25 post-ecdysis). Under the present rearing conditions post-teneral development of adult males is completed by day 20 post-ecdysis (Gringorten & Friend, manuscript in preparation). In a few experiments where older insects were used, these were fed again and flown 15–20 days following the second blood meal. Care was taken to ensure that the experimental and control insects were as close in age as possible.

Stationary flight was stimulated in restrained insects by suspending them in a humid air stream. The term 'flight' throughout this communication refers to sustained and continuous wing beating with the insect held in a fixed position. 'Exhaustion' was defined as the point at which insects could no longer be stimulated to beat their wings and occurred after 2–3 h of flying. Further details on the flight stimulus have been described elsewhere (Gringorten & Friend, 1979).

Expressible haemolymph volume, water content and specific gravity (Figs. 1 and 2)

Haemolymph samples were collected in exact-volume Drummond Microcaps from an opening made in the pronotum. The maximum volume of haemolymph which could be squeezed out from insects was calculated from the length of the haemolymph sample and the known length:volume ratio of the Microcap. The specific gravity was determined by weighing samples of known volume, and the water content determined by measuring the loss in weight after oven-drying to a constant weight in the Microcap.

Total haemolymph volume (Fig. 3, Tables 1 and 2)

Total haemolymph volume was determined by isotope dilution using [carboxyl-^{14}C]-inulin (New England Nuclear Corp., specific activity 2·50 mCi/g). The inulin was dissolved in 1·157 g% NaCl adjusted to pH 7·2 with NaOH. This concentration of NaCl is isosmotic with adult *R. prolixus* haemolymph (Ramsay, 1952; Shaw & Stobbart, 1963).

Insects were injected through a foreleg, which had been amputated at the distal end of the femur, with approximately 19 nCi [^{14}C]inulin in 5 μl saline delivered from a 25 μl Hamilton syringe. The wound was sealed with beeswax-colophony and the haemolymph sampled after a 30 min equilibration period.

The effects of flight on total haemolymph volume were investigated by two different methods. The first was to establish a relationship between live weight and haemolymph

volume in a group of control insects and then use this relationship to estimate the pre-flight haemolymph volumes of insects which were injected with [^{14}C]label after being flown.

The second method was to inject insects prior to the flight period, measure pre- and post-flight concentrations of the label in the same insect, and attempt to measure and correct for possible losses of label from the haemolymph compartment during the flight period. With this method, haemolymph was sampled from the legs, and an additional half-hour 'recovery' period, measured from the end of the initial equilibration period, was allowed before the flight was initiated. The insects were flown in a fume hood and any excreta produced were collected in 20 ml glass counting vials.

Radioactive counting

The total activity of [^{14}C]inulin injected into insects was determined by placing 5 μl of the radioactive solution used for the injection in counting vials and subsequently treating them in a similar manner as the haemolymph and excreta samples.

Standards and samples were solubilized in 0·15 ml double deionized-distilled water followed by the addition of 1 ml Protosol (New England Nuclear Corp.). The solutions were incubated at 45–50 °C for 24 h and then dissolved in 13·5 ml cocktail (0·3% glacial acetic acid + 0·8 g% PPO in toluene).

Haemolymph samples demonstrated no colour quenching. Quench curves were derived with *n*-[1-^{14}C]hexadecane solutions (Amersham Corp., specific activity $1{\cdot}130 \times 10^6$ dpm/g) quenched with chloroform. Excreta samples imparted a variable amber colour to counting solutions, depending on the amount of faecal matter present. Quench curves for excreta samples were therefore derived with [^{14}C]hexadecane solutions which were quenched with a dried, pulverized gut (mid- and hind gut) of unlabelled insects (fed 3 days prior to dissection).

Counting was done on a Beckman LS 350 counter. The effect of quenching on the background was determined by counting quenched cocktail solutions prior to adding [^{14}C]hexadecane.

Control insects

These consisted of three types: insects sampled after their removal from rearing jars ('untreated controls'); insects exposed to the air stream in the same manner as experimental insects but inhibited from flying by taping their legs to a piece of paper ('air-stream controls'); and one insect which was allowed prolonged incubation of the [^{14}C]inulin label (4·5 h) while at rest, in the dark, in a loosely capped counting vial kept in the fume hood (approximately 22 °C and 37% R.H.), ('resting control').

RESULTS

Expressible haemolymph volume, water content and specific gravity

The quantity of haemolymph which could be obtained from flown insects depended very much on the duration of the flight period. Fig. 1 shows that the volume of haemolymph which could be sampled from exhaustively flown insects declined by over 80% of that in unflown insects. The haemolymph volume in air-stream controls

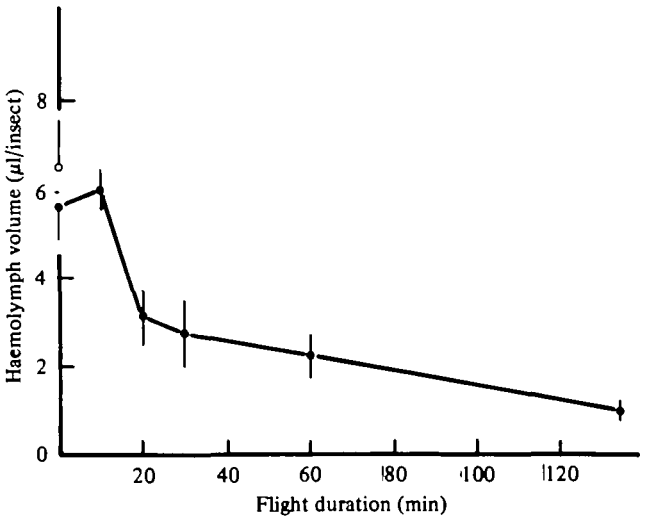

Fig. 1. Relationship between expressible haemolymph volume and duration of flight in male *R. prolixus*. The points are the means ± 1 S.E. of from three to nine insects. The point at 134 min represents the median flight duration of seven exhaustively flown insects (range 106–161 min). The open point at 0 min represents a group of insects which were exposed to the air stream for 3 h but not flown ('air-stream controls').

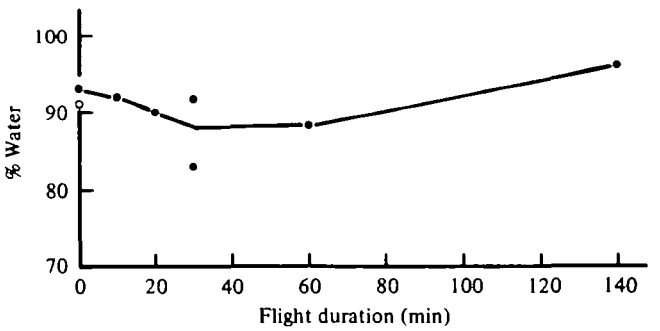

Fig. 2. Effect of flight on the percentage water in the haemolymph of male *R. prolixus*. Each point is a single insect except for the closed point at 0 min which represents the mean ± 1 S.E. of four insects (92·9 ± 0·2 %). The point at 140 min represents an exhaustively flown insect. The open point at 0 min represents a 3 h air-stream control.

was not significantly different from that of the unflown insects indicating that the air stream itself had no drying effect.

The percentage water in the haemolymph did not appear to change substantially during an exhaustive flight; it remained at an average of 91·0% (Fig. 2). The specific gravity of haemolymph in both resting and exhaustively flown insects is 1·03.

Total haemolymph volume

The formula for calculating total haemolymph volume from the radioactivity of the sample is:

$$V_H = \frac{(DPM_T - DPM_E)V_S}{DPM_S} - V_I,$$

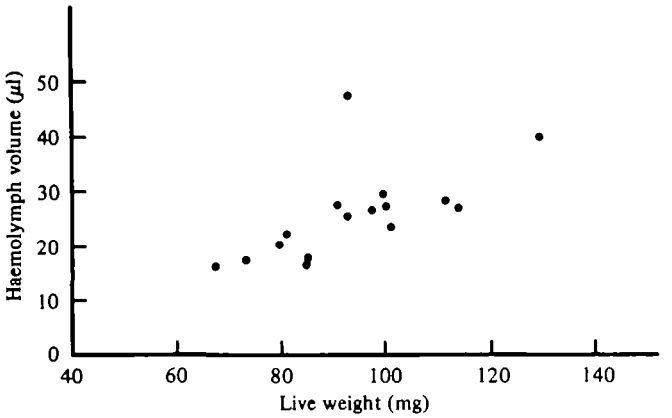

Fig. 3. Relationship between haemolymph volume and live weight in male *R. prolixus*. Each point is a single insect. The equation of the regression line (excluding the outlier) is: haemolymph volume = 0·343 × live weight −7·780.

where V_H = total haemolymph volume (μl); V_S = volume of haemolymph sampled (μl); V_I = volume of saline injected (5 μl); DPM_T = total radioactivity injected (disintegrations per minute); DPM_E = radioactivity sequestered from the haemolymph by other tissues and estimated by the amount excreted; DPM_S = radioactivity of the haemolymph sample.

The subtraction of the injected saline volume in the formula was based on the assumption that this volume was retained in the haemolymph compartment during the course of the experiment and not eliminated. This was probably a safe assumption since the saline was isosmotic with the haemolymph, and none of the control insects (untreated, air-stream or resting controls) excreted during the course of the experiment.

Only the flown insects excreted and these only during the actual flight period. Substituting the radioactivity recovered in the excreta for DPM_E in the formula was an underestimation of sequestration since it did not include residual activity in the Malpighian tubules or hind gut, or possible uptake by other tissues (Loughton & Tobe, 1969). However, evidence is presented below that such sequestration occurred very little, if at all, in control insects. The estimation of DPM_E by excreted activity in flown insects leads to an overestimation of their haemolymph volumes.

Two haemolymph samples were taken for each measurement of radioactivity. In most cases (20 out of 24), the activity calculated in the second sample was fractionally higher than the first. This varied from just a few *DPM* up to 165 *DPM* (maximum 8% increase) and may have been due to the small extent to which the haemolymph had dried in the wound before the second sample could be taken. The second samples, therefore, were used only to check the validity of the activity in the first samples and were not averaged with the first in calculating the haemolymph volumes.

The relationship between total haemolymph volume and live weight is illustrated in Fig. 3. Two groups of insects were used to investigate this relationship: one was fed on day 5 post-ecdysis and sampled on days 21–25 post-ecdysis; the second was fed on day 5, again on day 32, and sampled on days 47–50 post-ecdysis. Because

Table 1. *Haemolymph volumes in exhaustively flown male* R. prolixus

Insect	Flight duration (min)	Pre-flight live weight (mg)	Estimated pre-flight volume (μl)	Actual pre-flight volume (μl)	Post-flight volume (μl)
1	207	115·1	31·7	—	19·6
2	151	111·2	30·4	—	23·4
3	153	94·8	24·7	—	15·2
4*	152	99·9	26·5	27·4	14·4
5*	158	84·9	21·3	16·8	9·2
Mean	—	101·2	26·9	—	16·4
S.E.	—	5·5	1·9	—	2·4

* Injected with [^{14}C]inulin prior to flight to determine haemolymph volume.

their age and feeding history differed, it was necessary to establish, before the data could be pooled, that the two groups did not exhibit any marked differences in either their mean live weights or mean haemolymph volumes. The two groups exhibited no significant differences in either category when subjected to *t*- tests ($P > 0{\cdot}60$ in both cases). The two groups were therefore combined into one data set for deriving the relationship in Fig. 3.

The point at the top of the growth in Fig. 3 could be statistically rejected from the sample as an outlier by an *r* test (Dixon & Massey, 1957; $P < 0{\cdot}005$). This was based on the magnitude of its deviation from a computer-derived best-fit curve through all the points (observed–expected volume), the expected volume being determined from the equation of the curve.

When the outlier was excluded, a linear regression computed for the remaining points in the sample resulted in the equation:

$$\text{haemolymph volume } (\mu\text{l}) = 0{\cdot}343 \times \text{live weight (mg)} - 7{\cdot}780,$$

with a correlation coefficient of 0·892.

The results of exhaustive flight on total haemolymph volume are given in Table 1. Pre-flight volumes were estimated from the initial live weights and the linear regression described above. Insects 4 and 5 were injected with [^{14}C]inulin prior to flight so that their actual pre-flight volumes could be determined. The post-flight volumes averaged 39% less than the estimated pre-flight volumes, a decrease which was statistically significant ($P < 0{\cdot}001$) as determined by an *F* test on the ratio of residual variances:

$$F = \frac{(\text{observed–expected volume})^2_{\text{controls}}/(N_1 - 2)}{(\text{post-flight–estimated pre-flight volume})^2_{\text{flight}}/N_2},$$

where N_1 and N_2 are the sample sizes and $(N_1 - 2)$ and N_2 are the degrees of freedom in the control and flown groups, respectively. Insects 4 and 5 exhibited an actual mean decrease of 47%. Excreted radioactivity in these two insects amounted to less than 0·3% of the total activity injected.

When the experiment was repeated with insects exposed to the air stream for 3·5 h, but prevented from flying, no significant changes were observed in their average haemolymph volumes (Table 2, $P > 0{\cdot}10$). The apparently large post-

Table 2. *Haemolymph volumes in male* R. prolixus *exposed to a humid air stream for* 3·5 *h* (*'air-stream controls'*)

Insect	Pre-treatment live weight (mg)	Estimated pre-treatment volume (μl)	Actual pre-treatment volume (μl)	Post-treatment volume (μl)
1	77·0	18·6	—	17·2
2	83·0	20·7	—	19·5
3	95·5	25·0	—	33·5
4*	129·4	36·6	39·9	40·0
Mean	96·2	25·2	—	27·6
S.E.	11·7	4·0	—	5·5

* Injected with [^{14}C]inulin prior to exposure to determine haemolymph volume.

treatment haemolymph volume of insect 3 is most likely a result of underestimating its initial volume rather than a result of water uptake from the air stream. Insect 4 showed virtually no difference between its actual pre- and post-treatment haemolymph volumes. There is no evidence, therefore, for believing that the air stream itself can cause decreases in haemolymph volume.

Prolonged incubation of the [^{14}C]inulin by a resting insect for 4·5 h had little apparent effect on the haemolymph volume. The calculated volume at the end of the incubation period in a 79·7 mg insect was 21·1 μl, a 1·0 μl increase from the actual initial volume (20·1 μl), and 1·5 μl higher than the estimated initial volume (19·6 μl). If the difference is real it would probably indicate a slight degree of sequestration of label from the haemolymph.

DISCUSSION

Under the present experimental conditions, flight activity in male *R. prolixus* can result in a reduction in haemolymph volume by up to half the initial volume. The diuresis observed in many insects appeared rather copious at times, and it is possible that the decrease in haemolymph volume may be accounted for by the amount of urine which is voided during flight. The quantitative relationship between these two phenomena is currently being investigated.

To our knowledge this is the first instance that flight has been demonstrated to cause either excretion or a decrease in haemolymph volume in any insect. Certainly it is the first time that a natural diuresis in *R. prolixus* has been shown to be related to something other than a blood meal.

Most of the decline in haemolymph volume which occurs during flight may be a result of increased Malpighian tubule activity. Such flight-related activity occurs in locusts (Goldsworthy, 1976) and, presumably, is important in disposing of the end products of flight metabolism. Unlike locusts, however, *R. prolixus* is apparently unable to reabsorb the bulk of the tubular water when it reaches the hind gut.

It has been suggested that haemolymph can act to buffer the tissues against acute dehydration (Shaw & Stobbart, 1972), but since the air stream alone did not have any desiccating effect, the decline in haemolymph volume during flight was more likely related to enhanced urine production than to a shift of water from the haemolymph space into the tissues because of tissue dehydration.

The extent to which evaporation cooling was a factor in reducing haemolymph volume in *R. prolixus* during flight is not presently known. This is not thought to be of great significance in the present experiment however, since the insects were exposed to a saturated air stream which would inhibit water loss through such a process. In general, little of the metabolic heat produced in flying insects is lost by evaporative cooling (Church, 1960*a*, *b*; Cockbain, 1961; Heinrich, 1970, 1971*a*–*c*). Makings (1968), however, has suggested that enhanced transpiration through Slifer's patches in flying Acrididae may be an important cooling mechanism.

The percentage water in the haemolymph (91·0%) of male *R. prolixus* is comparable to other insects (e.g. see Altman & Dittmer, 1961). The fact that it did not decrease as a result of the reduction in haemolymph volume indicates that solute was being removed at a proportional rate. Otherwise, a 39–47% decrease in haemolymph volume, as a result of water loss only, would have expected to reduce the relative amount of water to 83–82%. The haemolymph of one insect which had flown (30 min) contained 83% water (Fig. 2), and it is possible that concentrating effects were present but largely obscured by individual variability.

Although *R. prolixus* does fly in the wild (Havilland, 1931), the extent to which it uses flight as a means of locomotion is uncertain. The decline in haemolymph volume may present problems to the flying insect. It may be that a decline is a contributing factor in limiting the duration of flight. Perhaps a critical level of circulating haemolymph is reached which prevents a sufficient amount of essential metabolites from reaching the flight muscles or adequate removal of waste products from them. Or, if *R. prolixus* relies on haemolymph conduction as a heat dissipating mechanism, perhaps the insect becomes overheated at this critical level.

Whether reduction in haemolymph volume in flying *R. prolixus* serves, within limits, any useful purpose is difficult to say. Possibly this is a mechanism by which internal water concentration is maintained in the face of substrate depletion. Perhaps the elimination of water from the haemolymph compartment, as substrate is removed and metabolized, prevents the haemolymph from becoming too dilute and ensures that an adequate concentration gradient is maintained for continuous uptake of substrate by flight muscle.

The fact that the haemolymph volumes in air-stream and resting controls (Tables 2 and 3) which were injected with isotope prior to treatment remained virtually unchanged over the test period indicates that very little sequestration of the [^{14}C]inulin label from the haemolymph occurred. Loughton & Tobe (1969) found that uptake of [^{14}C]inulin in locust tissue and faeces, and metabolism of the label, were negligible during the first 30 min after injection but appreciable after 72 h. Fat body, in particular, contained relatively high amounts, whereas Malpighian tubules and faeces were among the lowest.

The possibility exists that uptake and metabolism of label in *R. prolixus* increased during flight as a result of generally enhanced metabolic activity. If this is the case, the apparent decrease in haemolymph volume must therefore be regarded as a conservative estimate.

The authors gratefully acknowledge Ms Rosemary Hewson for her skillful execution of the graphs and editing of the manuscript, Dr J. E. Paloheimo for helpful sug-

gestions concerning the statistical tests, Dr S. S. Tobe for use of the scintillation counter and review of the manuscript, Dr F. M. Barrett for technical assistance and review of the manuscript, and Dr J. J. B. Smith for critical review and very helpful suggestions. This work was supported by a grant to W.G.F. from the National Research Council of Canada.

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