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RESEARCH ARTICLE

Effects of FABP knockdown on flight performance of the desert locust, *Schistocerca gregaria*

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ABSTRACT

During migratory flight, desert locusts rely on fatty acids as their predominant source of energy. Lipids mobilized in the fat body are transported to the flight muscles and enter the muscle cells as free fatty acids. It has been postulated that muscle fatty acid binding protein (FABP) is needed for the efficient translocation of fatty acids through the aqueous cytosol towards mitochondrial β-oxidation. To assess whether FABP is required for this process, dsRNA was injected into freshly emerged adult males to knock down the expression of FABP. Three weeks after injection, FABP and its mRNA were undetectable in flight muscle, indicating efficient silencing of FABP expression. At rest, control and treated animals exhibited no morphological or behavioral differences. In tethered flight experiments, both control and treated insects were able to fly continually in the initial, carbohydrate-fueled phase of flight, and in both groups, lipids were mobilized and released into the hemolymph. Flight periods exceeding 30 min, however, when fatty acids become the main energy source, were rarely possible for FABP-depleted animals, while control insects continued to fly for more than 2 h. These results demonstrate that FABP is an essential element of skeletal muscle energy metabolism *in vivo*.

KEY WORDS: RNAi, Fatty acid binding protein, Insect flight, Lipid transport

INTRODUCTION

For many centuries, locusts have inflicted severe damage to human populations in African and Asian countries. Every few years, when weather conditions are favorable, locusts that normally develop dispersed in their solitary stage accumulate in large numbers and undergo a phase transformation to their gregarious form (Pener and Simpson, 2009). As adults, gregarious locusts form gigantic swarms that can migrate in a coordinated manner for several hundred kilometers, touching down for feeding and eradicating much of the vegetation along their path. Migratory flight of locusts is among the most energy demanding of activities, and insects have developed an efficient mechanism to fuel this metabolic activity (Wegener, 1996). In the initial phase of flight, the readily available disaccharide trehalose serves as the main energy source for muscle contraction, but within 30–60 min, lipids become the predominant metabolic fuel (Mayer and Candy, 1969).

Lipids are stored as triglycerides in the fat body. Their mobilization is initiated by the release of adipokinetic hormone (AKH), which activates a signal transduction pathway that triggers the action of a lipase in the fat body. One fatty acid chain is cleaved from the triacylglycerol molecule, and the resulting diacylglycerol (DAG), which is the major transport form of lipids in insects, is released into the hemolymph (Van der Horst and Rodenburg, 2010).

Locusts use an effective transport system, often referred to as the 'lipophorin shuttle', to assure sustained delivery of DAG to the flight muscle (Van der Horst and Rodenburg, 2010). In resting insects, the predominant hemolymph lipoprotein is the high-density form of lipophorin (HDLp), a protein composed of the two apoproteins apoLp-I (~250 kDa) and apoLp-II (~80 kDa), as well as phospholipids, DAG and smaller amounts of other lipids, which together amount to around 20% of the mass of the lipophorin particle. Upon their release from the fat body, numerous DAG molecules associate with HDLp, and the lipid-enriched particle is stabilized by the binding of several molecules of a third apoprotein, apoLp-III (~18 kDa). The resulting low-density lipophorin (LDLp) has a density of ~1.02 g ml^{-1} and contains more than 40% lipid, mostly in the form of DAG. A lipoprotein lipase located at the flight muscle membrane hydrolyzes DAG; free fatty acids enter the flight muscle, while glycerol and apoLp-III are released into the hemolymph. Lipophorin returns to the high-density form HDLp, which remains in the hemolymph and can resume transporting DAG from the fat body to the flight muscle (Van der Horst and Rodenburg, 2010).

Although the transport of lipids through the hemolymph has been studied extensively, less is known about how fatty acids enter the flight muscle cells and translocate through the aqueous cytosol to the mitochondria, where beta-oxidation takes place. It is widely believed that fatty acid binding proteins (FABPs) play a role in intracellular transport of fatty acids, especially in muscle cells (Haunerland and Spener, 2004). FABPs belong to an ancient family of genes now called the intracellular lipid binding protein (iLBP) family, which originated more than a billion years ago (Schaap et al., 2002). The first gene duplication appears to have occurred approximately 900 mya, long before the vertebrate–invertebrate divergence, and hence all animals seem to have at least two distinct FABPs, reflecting the two major branches of the phylogenetic tree. Subsequent gene and genome duplications gave rise to the variety of FABP found today (Schaap et al., 2002). In mammals, more than 14 different members of the gene family have been identified, with distinct differences in tissue-specific expression patterns. In contrast, fewer paralogs have been characterized in insects, which appear to express only one or two isoforms on each of the two branches (Haunerland and Thakrar, 2009). In locusts, only one FABP has been characterized to date, but recent expressed sequence tag or genome sequencing projects suggest a potentially larger number of paralogs. FABP was first discovered in the flight muscle of adults of the desert locust, *Schistocerca gregaria* (Haunerland and Chisholm, 1990), and later in *Locusta migratoria* (Van der Horst, 1990; Maatman et al., 1994). FABP is the most abundant

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cytosolic protein in mature adult locusts, amounting to almost 20% of all soluble proteins (Haunerland et al., 1992). Interestingly, the protein and its mRNA are completely absent in mesothorax muscles of nymphs. FABP expression starts right after adult ecdysis and continues strongly to increase for the following 7–8 days. *fabp* mRNA levels decline afterward, while the protein reaches its maximum 2 weeks after ecdysis (Haunerland et al., 1992). FABP is a very stable protein, and its level in the flight muscle remains constant for several weeks after reaching its maximum, requiring only a low amount of mRNA to replace degrading FABP (Zhang and Haunerland, 1998). The three-dimensional structure and the binding affinity of locust FABP are very similar to vertebrate FABP3 (Haunerland, 1994), which is expressed strongly in the heart and other lipid-dependent muscles.

In mammals, skeletal muscle contains relatively small amounts of FABP (1–2% of all cytosolic proteins), whereas in cardiac muscle, the most lipid-dependent tissue in mammals, FABP amounts to up to 5% (Kaikaus et al., 1990; Al-Hadi et al., 2009). The metabolic rates encountered in the flight muscles of a migratory shorebird (the western sandpiper, *Calidris mauri*) and of the desert locust are 2- and 3-fold higher than in the mammalian heart, and these muscles also possess twice or three times as much FABP, respectively (Guglielmo et al., 2002; Haunerland and Spener, 2003). There appears to be a clear relationship between the cellular FABP content and the rate of beta-oxidation encountered by a muscle, suggesting that FABP facilitates the uptake and transport of fatty acids for energy production in those muscles (Veerkamp and van Moerkerk, 1993). However, in spite of many attempts, conclusive proof that FABP is necessary for sustained muscle activity has not yet been obtained. Ablation of the muscle *fabp* gene in mice did not result in a distinct phenotype, and knockout mice remained active and viable, with the possible exception of somewhat reduced exercise tolerance and a slight reduction in fatty acid uptake rates (Binas and Erol, 2007). This, however, cannot be interpreted as an argument against an essential role of FABP, as compensatory overexpression of other members of the iLBP family with similar characteristics has been observed (Haunerland and Spener, 2004).

We believe that the desert locust may be an advantageous model that allows for the demonstration of the function of FABP in energy metabolism. If indeed FABP is required for fatty acid transport during vigorous muscle activity, a substantial reduction of this protein in the flight muscle should greatly limit migratory flight capacity. RNA interference (RNAi), which has been shown to be highly effective in locust species (Wynant et al., 2012), may be an efficient means to prevent the accumulation of *fabp* mRNA and its subsequent translation into the protein, especially because FABP expression in locusts commences only after adult ecdysis. In contrast to gene ablation, RNAi-mediated knockdown of proteins is less likely to result in compensatory expression of other proteins (Rossi et al., 2015), and insects possess fewer FABP paralogs than vertebrates that could compensate for the loss of muscle FABP. Taking advantage of the adult-specific nature of locust FABP and its well-defined expression pattern, the present study was carried out to investigate the consequences of a reduction in or elimination of FABP on the flight performance of the desert locust.

MATERIALS AND METHODS

Insect rearing

Desert locusts (*Schistocerca gregaria* Forsskål 1775) were reared under long-term gregarious conditions in the insectary of Simon Fraser University at 30°C and 16 h daily illumination. Insects were fed daily *ad libitum* on a diet of rye grass and wheat bran. For treatment and FABP measurements in adult locusts, freshly emerged male adults were removed and reared separately until used at the specified age. Individuals between 0 and 12 h after adult molting are referred to as day 0 adults, with each subsequent day representing an additional 24 h period.

Synthesis of dsRNA

The dsRNA constructs were prepared using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA), which is designed for the synthesis of dsRNA molecules of >200 bp. Using cDNA prepared from *S. gregaria* flight muscle as a template, the 400 bp target region of the FABP transcript (GenBank accession no. AH010557) was first amplified in a simple PCR reaction with forward primer sgFABP2F (5′-TGGTGAAGGAATTCGCAGGC-AT-3′) and reverse primer sgFABP401R (5′-TGGGCCTTGTAT-ATTCTCGTTGCC-3′) using RedTaq DNA polymerase (Sigma-Aldrich, Burlington, ON, Canada) (95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 57°C for 45 s and 72°C for 45 s, then 7 min at 72°C).

The PCR product was purified by agarose gel electrophoresis and sequenced. For the synthesis of single-stranded sense and antisense RNA, a T7 promoter sequence (5′-GGATCCTAATACGACTCA-CTATAGG-3′) added to the 5′-end of the forward or reverse primer, respectively, and two separate PCR reactions with the respective unmodified reverse or forward primer were carried out to generate two separate single promoter templates (95°C for 3 min, followed by 10 cycles of 95°C for 30 s, 57°C for 45 s and 72°C for 45 s, then 35 cycles of 95°C for 30 s, 65°C for 45 s and 72°C for 45 s), and eventually kept for 7 min at 72°C. These templates were directly used in a single high-yield *in vitro* transcription reaction. Remaining ssRNA and DNA was removed by nuclease digestion, and the 400 bp dsRNA was further purified according to the manufacturer's instructions. Following quantification in a NanoDrop spectrophotometer (Thermo Fisher Scientific, Burlington, ON, Canada), dsRNA was diluted to 1 µg µl^{-1} in DEPC-treated water, analyzed on a 1% agarose gel and aliquoted for storage at −20°C.

For a non-targeting negative control, dsRNA corresponding to a 1155 bp region of the SWS1 opsin gene from the rainbow trout, *Oncorhynchus mykiss* (GenBank accession no. AF425074.1), was prepared as described above (Cheng and Novales Flamarique, 2007). Following amplification of the target with gene-specific primers (forward: 5′-AACCGCTGAACTACATCCT-3′, reverse: 5′-TAAC-ACAGAATGAAGGAGCA-3′), primers with the T7 promoter sequences (5′-GGATCCTAATACGAC-TCACTATAGG-3′) added to the 5′-end of the forward or reverse primers were used to produce the templates for the high-yield *in vitro* transcription reaction. In BLAST searches, the resulting opsin dsRNA shows no significant alignments >37 bp (3% of the query sequence) with expresses sequence tag or genomic databases from any locust species.

Treatment of insects

To knock down the FABP transcript, *fabp* dsRNA (4 µg in 4 µl H_2O) was injected with a 5 µl Hamilton syringe into the hemolymph of adult male locusts within 12 h of adult eclosion. Control male locusts were injected with an equivalent volume of DEPC-treated water. Four independent experiments, each with 3–5 control and 3–5 treated insects, were carried out. To exclude the possibility of off-target effects of the dsRNA injection, one additional experiment was carried out in which *fabp* dsRNA-treated insects were compared with insects treated with rainbow trout *opsin* dsRNA as control. *fabp* mRNA in the muscle was measured at specified

intervals, and insects were subjected to flight experiments 21 days after injection.

Total RNA preparation

Total RNA was extracted from the tissue homogenate with the Qiagen RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Toronto, ON, Canada). Freshly dissected tissue was ground up to a fine powder with a mortar and pestle under liquid nitrogen. Muscle powder (30 mg) was transferred into a 600 µl RLT buffer vial, homogenized with seven strokes through a 21 gauge needle in a 5 ml syringe, and centrifuged for 3 min at 12,000 ***g***. The supernatant was transferred to a new tube and mixed with the same volume of ethanol, and separated on an RNeasy spin column as described by the manufacturer. Total RNA concentration and purity were determined photometrically on a Nanodrop 2000C UV-Vis spectrophotometer (Thermo Fisher Scientific).

cDNA synthesis

Total RNA (1 µg) was transcribed into cDNA with a Bio-Rad iScript™ cDNA synthesis kit (catalog no. 170-8890) in an MJ Mini thermal cycler (Bio-Rad, Mississauga, ON, Canada; cDNA synthesis program: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, cooled down to 4°C). A parallel reaction was carried out without the addition of reverse transcriptase and used in subsequent PCR reactions as the negative control, to exclude the possibility of PCR products due to contamination with genomic DNA.

Quantitative real-time PCR (qPCR)

All reactions were performed in duplicate in 48-well plates on a MiniOpticon Real-Time PCR System (Bio-Rad). Each reaction contained 2 µl cDNA, 0.5 µl forward and reverse primer (10 µmol l^{-1}), 5 µl 2× SsoFast EvaGreen Supermix (Bio-Rad) and 2 µl water. For all qPCR reactions, the following thermal cycling profile was used: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. A melt curve analysis was performed to check for primer dimers. For all transcripts, only a single melting peak was found during the dissociation protocol. FABP transcript in the cDNA was quantified by real-time PCR with primers FABP127F (5′-GAGCTGGAGATCCTGGACGGT-3′) and FABP273R (5′-TCCGTCCTGAGTGATGGTGGA-3′), which yielded a 147 bp product with an experimentally determined amplification efficiency of 91.8%. *Elongation factor 1α* (*ef1α*, GenBank accession no. JG665860.1), which has been shown before to be a reliable reference gene in *S. gregaria* brain (Van Hiel et al., 2009), was used as reference gene in this study (forward primer EF1α271F: 5′-GATGCTCCAGGCCACAGAGA-3′; reverse primer SgEF1α336R: 5′-TGCACAGTCGGCCTGTGAT-3′; amplification efficiency 92.5%). In each sample, the expression of FABP was normalized against the reference gene by subtracting the cycle threshold (C_t) of FABP from the C_t of *ef1α* (i.e. ΔC_t). For each time point, the expression levels of *fabp* relative to its maximal expression were calculated assuming 100% amplification efficiency as $2^{-7-\Delta C^t}$, where −7 represents the mean ΔC_t value at day 7 in control animals. The differences between control and *fabp* dsRNA-treated animals were calculated using the $2^{-\Delta\Delta^{Ct}}$ method described by Livak and Schmittgen (2001), and the statistical significance was evaluated using paired-sample *t*-tests.

Protein extraction and analysis

Locust mesothorax muscles were dissected under phosphate buffered saline (PBS). The tissue was cleaned of adhering fat body and washed in saline, yielding approximately 30 mg of muscle tissue per locust. The tissue was rapidly frozen under liquid nitrogen and lyophilized for later usage. For protein analysis, 50 µg of the lyophilizate was homogenized with 125 µl PBS and centrifuged at 12,000 ***g*** to remove insoluble materials, and the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), following the protocol by Laemmli (1970). A 4 µl aliquot of the supernatant was boiled for 2 min with 6 µl Laemmli sample buffer (Bio-Rad), loaded on a stacking gel [5% total monomer (T), 20% crosslinker (C)] and separated on a 15% T, 5% C stacking gel. The Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific) was used to identify the size of the proteins. Gels were run for 1 h at 180 V (200 mA) in 1× Tank buffer. Gels were stained overnight with EZblue Gel Staining Reagent (Sigma-Aldrich).

Flight experiments

Sustained flight was induced by mounting locusts at 30°C in front of two large fans. The prothorax of the insect was fixed by means of a low-temperature glue stick to the tip of a wooden stick, which was attached to a stand in such a way that the mounted insects were suspended in the air and faced the fan directly. The wind speed was measured with an anemometer and adjusted to 4 m s^{-1}. Locusts generally engaged in flight as soon as the fans were turned on but sometimes needed to be gently nudged to initiate sustained flight. Insects that were not able to continuously fly for at least 5 min were discarded. Flight performance was measured at ∼15 min intervals and scored on a numerical scale, where 5 indicated continuous, uninterrupted flight, and 0 no flight activity even after repeated stimulation (up to three touches with a pencil tip). The values of 4, 3, 2 and 1 were assigned if stimulation led to continuing flight for at least 5 min, 1–5 min, 10–60 s and <10 s, respectively.

For the statistical analysis of the flight experiments, Fisher's exact test analysis was carried out with the statistical software R (https://www.r-project.org/) for each time point, comparing the flight scores for insects injected with *fabp* dsRNA, H_2O (control 1) and *opsin* dsRNA (control 2). To assess whether treatment affects the long-duration flight ability, the various measurements starting at 30 min were considered as multiple dependent measurements, and the Bonferroni correction was applied (corrected critical value 0.007).

Lipid analysis

Following the completed flight experiment, hemolymph was collected from both control and RNAi-treated insects. For hemolymph collection, one hind leg was cut off, and the hemolymph was flushed out into a pre-cooled Eppendorf tube by injection of PBS, pH 7.4, into the body cavity, as previously described by Chino et al. (1987). The tubes were centrifuged at 4°C for 10 min at 3500 ***g*** to remove the hemocytes. Lipids were extracted according to Bligh and Dyer (1959). Lipids were separated by two-step thin layer chromatography on a normal phase silica gel plate. The plate was developed with hexane:ether:acetic acid (60:40:1), followed by the second solvent, chloroform:methanol:water (65:40:5), which was allowed to move 4 cm up the plate. Lipids were visualized by exposure to iodine vapor, and the intensities of the spots for phosphatidylcholine (PC) and 1,3-diacylglycerol determined densitometrically with ImageJ software (Schneider et al., 2012).

RESULTS

FABP knockdown

Earlier work carried out with northern blots (Haunerland et al., 1992, 1993) established the expression pattern for locust muscle FABP, and provided a semi-quantitative estimation of the mRNA

Journal of Experimental Biology (2019) 222, jeb203455. doi:10.1242/jeb.203455

Table 1. Expression of *fabp* mRNA in control and dsRNA-treated locusts

	Control		RNAi treatment			
Day	ΔC_t±s.d.	% of max.	ΔC_t±s.d.	% of max.	$\Delta\Delta C_t$	$\Delta\Delta C_t$ significance
0	4.0±5.0	0				
3	−6.2±0.8[a]	58	1.4±0.2	0.3	7.6	P=0.0001, t_4=45.7
7	−7.0±0.3[b]	100	2.2±0.3	0.2	9.2	P=0.00001, t_5=17.2
10	−6.6±0.1[c]	74	1.7±0.6	0.2	8.2	P=0.00002, t_4=22.9
21	−1.5±0.2[d]	3	3.6±3.1	0.1	5.3	P=0.042, t_5=2.7
Opsin control						
21	−1.7±0.3[e]	3	3.6±3.1	0.1	5.0	P=0.038, t_5=2.8

[a]Day 3/day 0: t_4=3.7, P=0.02; [b]day 7/day 3: t_4=1.6, P=0.18; [c]day 10/day 7: t_4=2.79, P=0.045; [d]day 21/day10: t_5=8.1, P=0.0005; [e]opsin day 21/H_2O day 21: t_5=0.72, P=0.51.

fabp mRNA was quantified by reverse transcription-qPCR prior to (day 0) and 3, 7, 10 and 21 days after injection of 10 µl H_2O (control), *opsin* dsRNA (opsin control, day 21) or *fabp* dsRNA (RNAi treatment). ΔC_t values are the mean of 3–4 independent replicates of C_t of the reference gene *ef1α* subtracted from the C_t of *fabp*. For the control group, the differences are statistically significant between adjacent time points except between days 3 and 7 (two-sample *t*-test results shown in footnotes a–d). Expression levels were calculated as described in the Materials and Methods, and expressed as percentage of maximal *fabp* mRNA levels observed in control animals at day 7 (% of maximum). For each day, the statistical significance of the difference in the ΔC_t values of control and treated animals ($\Delta\Delta C_t$) was analyzed in a two-sample *t*-test.

levels throughout adult maturation. This trend was verified by real-time PCR with a primer pair specific for FABP (Table 1). When normalized to the expression of the housekeeping gene EF1α and expressed relative to the maximal level of mRNA seen at day 7, the results from the real-time PCR match the data obtained previously (Haunerland et al., 1992) from the northern blots after normalization against β-actin (Fig. 1). *fabp* mRNA is near the detection limit within the first 12 h after ecdysis, and rapidly increases between days 1 and 7 to its maximal value, before declining over the next 2 weeks to the very low level needed to maintain the FABP protein concentration in the muscle. There are only small variations between individual insects, except during the steep increase of *fabp* expression around day 3, where slight variations in age can result in large differences of *fabp* mRNA. When 4 µg *fabp* dsRNA was injected within the first 12 h after ecdysis, *fabp* mRNA did not increase and remained near the detection limit for at least 21 days (Table 1). There were very low rates of mortality associated with the injection: less than 10% of all injected locusts died prior to being analyzed. As similar mortality was seen for both dsRNA-injected and H_2O-injected animals; the mortality is likely due to injury by the injection process, rather than the injected compound. Subsequent analysis of the protein expression pattern by SDS-PAGE revealed the absence of the strong band at 15 kDa that represents FABP (Fig. 2), confirming that *fabp* dsRNA indeed results in a nearly complete knockdown of FABP expression. In contrast, injection of *opsin* dsRNA had no apparent effect on FABP expression, as similar levels of *fabp* mRNA and protein were detected at day 21 as in the H_2O control (Table 1, Fig. 2).

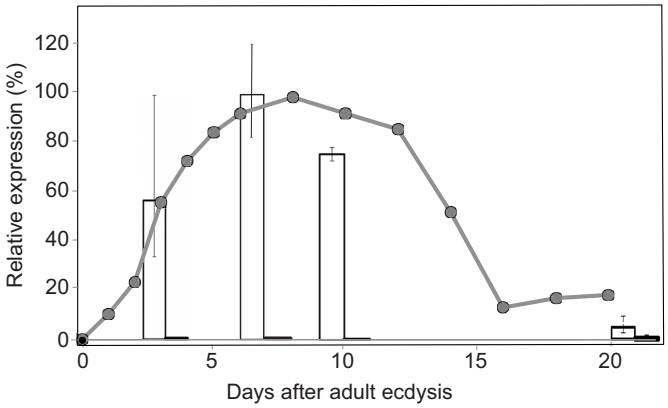

Fig. 1. Fatty acid binding protein (FABP) expression in adult locusts. *fabp* mRNA was quantified by reverse transcription-qPCR, as described in the Materials and Methods, and the expression levels calculated relative to the expression at day 7 after adult eclosion in the control group (see Table 1). Black circle: relative *fabp* mRNA level at day 0. White bars: relative *fabp* mRNA level in control animals 3, 7, 10 or 21 days after injection of 10 µl H_2O. Black bars: relative *fabp* mRNA level in animals before 3, 7, 10 or 21 days after injection of 10 µl *fabp* dsRNA. The error bars show the expression levels calculated from the standard deviation of the ΔC_t values. The previously published temporal expression pattern determined by northern blots (Haunerland et al., 1992) is shown in gray circles.

Flight experiments

The *fabp* dsRNA-treated insects and control locusts that had been injected with H_2O or *opsin* dsRNA were mounted in a suspended position on the flight stand, and flight was induced by a headwind of 4 m s^{-1} (Fig. 3). Insects engaged in uninterrupted flight within 5 min were observed at 15 min intervals and scored for their flight performance. As shown in Fig. 4, there was no significant difference between control and treated insects for the first 15 min. Within the following 115 min, however, *fabp* dsRNA-treated animals showed a distinct decline, as noticeable in more frequent rest periods and shorter flight durations. The results were similar for both control treatments (Fig. 4, legend): at 15 min, the flight ability score of *fabp*

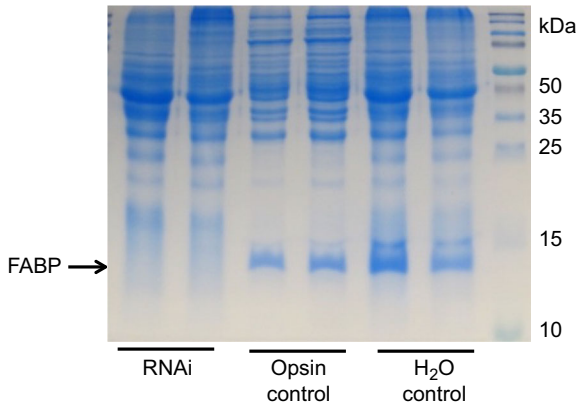

Fig. 2. SDS-PAGE of soluble muscle proteins. Newly emerged adult locusts were injected with *fabp* dsRNA, *opsin* dsRNA (control) or water (control), and muscle proteins were analyzed 21 days later by SDS-PAGE, as described in the Materials and Methods. Two representative samples for each treatment are shown.

Journal of Experimental Biology (2019) 222, jeb203455. doi:10.1242/jeb.203455

Fig. 3. Tethered flight of locusts. Control and dsRNA-treated adult males 21 days after adult eclosion were mounted in a suspended position for induced flying, as described in the Materials and Methods.

RNAi-subjected insects was statistically not different from those of both H_2O and *opsin* dsRNA-injected control insects ($P>0.05$). For each subsequent time point, *fabp* RNAi-treated animals scored significantly lower than either control ($P<0.05$). At 45 min and each time point after 60 min, *P*-values were lower than 0.007, the critical value when applying the Bonferroni correction for multiple dependent comparisons, confirming that treatment with *fabp* dsRNA prevents long-duration flight.

Control animals usually remained in their migratory flight position for more than 2 h, as shown in Fig. 4; any insect that stopped flying could be easily stimulated to continue its flight. Attempts to stimulate resting FABP-knockdown locusts to continue their flight were rarely successful, and if so, flight did not continue for more than a few minutes. Sometimes these insects bent their forelegs, abdomen or wings, as shown in Fig. 5, until their tarsi had contact with some part of the locust body.

Lipid content analysis

Hemolymph samples of control and dsRNA-treated locusts collected 3 h after the initiation of flight were analyzed for their lipid content and compared with animals that had not been allowed to fly. While DAG was present only in small amounts in the hemolymph of resting locusts, it became the most prominent lipid after flight in both control and FABP-deficient animals (Fig. 6). Through densitometric analysis of the iodine-stained thin layer chromatograms, we estimated an almost 5-fold increase in DAG upon flight in both the control and dsRNA-treated animals, confirming that the mobilization of lipids and the resulting availability of fuel in the hemolymph is not affected by dsRNA treatment.

DISCUSSION

RNAi is used increasingly as an effective tool for functional studies of individual proteins, as this technique allows gene-specific degradation of mRNA at any time point in the life of the organism. Although its mechanism is well understood, the effectiveness of this technique varies widely, owing to variances in delivery, uptake and stability of interfering RNA molecules. In insects, this technique has been used with mixed results, as the effectiveness of the RNAi depends on various factors, such as the concentration of dsRNA, the target species, the corresponding transcript and the delivery procedure (reviewed by Huvenne and Smagghe, 2010; Terenius et al., 2011; Gu and Knipple, 2013; Scott et al., 2013). However, studies in the laboratory of Vanden Broeck and others (Wynant et al., 2012; Santos et al., 2014) have demonstrated that RNAi works extraordinarily well in locust species. A single injection of small amounts of dsRNA generally causes a strong systemic response that persists for 10 days or more

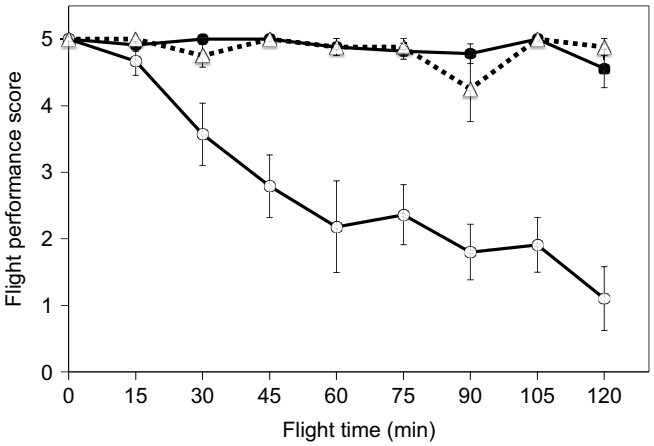

Fig. 4. Flight performance of adult locusts. Newly emerged adult locusts were injected with *fabp* dsRNA (open circles), *opsin* dsRNA (control 1; triangles) or water (control 2; closed circles), and subjected to tethered flight 21 days later, as described in the Materials and Methods. Flight performance was measured at ∼15 min intervals and scored on a numerical scale, as follows: (5) continuous, uninterrupted flight; (4 to 1) gentle stimulation was required to restart flight of a duration of (4) >5 min; (3) 1–5 min; (2) 10–60 s; and (1) <10 s; (0) no flight. Each data point is the mean±s.e.m. of the scores of 8–11 (controls) or 10–14 (*fabp* dsRNA) individual insects. Fisher's exact test analysis was carried out for each time point, comparing the flight scores for insects injected with *fabp* dsRNA, H_2O (control 1) and *opsin* dsRNA (control 2) (15 min, n=10: H_2O P=0.72, opsin P=1; 30 min, n=14: H_2O P=0.043, opsin P=0.028; 45 min, n=14: H_2O P=0.0009; opsin P=0.005; 60 min, n=11: H_2O P=0.030, opsin P=0.027; all subsequent time points, n=10–11, P<0.007).

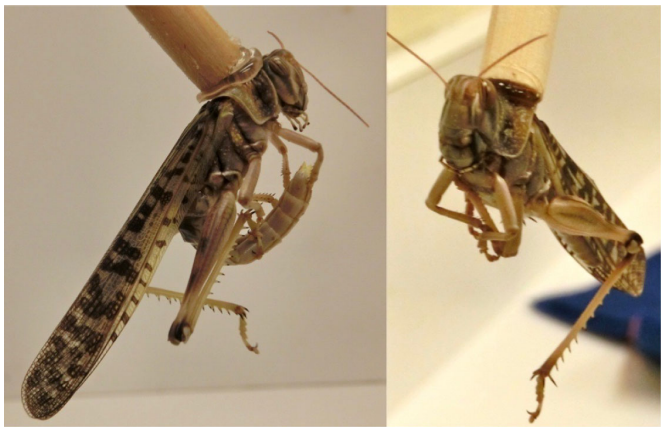

Fig. 5. Behavioral changes of exhausted FABP knockdown locusts. Insects treated with *fabp* dsRNA no longer capable of continuing flight sometimes folded their appendages around the body, with the tarsi touching the thorax or abdomen.

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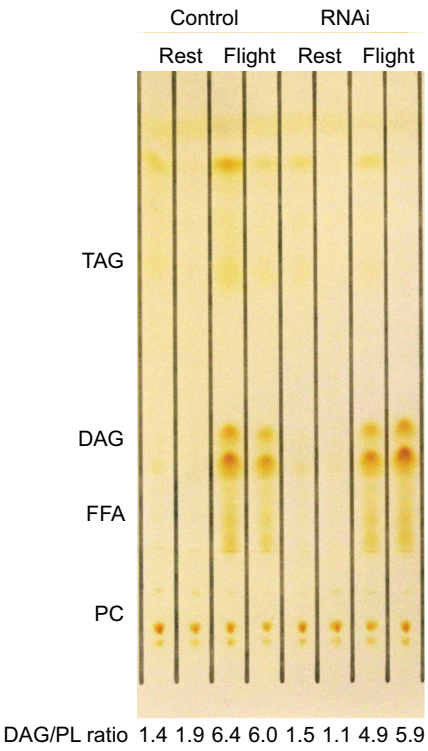

Fig. 6. Thin layer chromatography separation of hemolymph lipids. Total lipids were extracted from the hemolymph of locusts at rest and immediately after the flight experiments. Subsequent densitometric analysis of the individual lanes revealed an almost 5-fold increase in DAG upon flight (average DAG/PL ratio: control, rest 1.55, flight 7.6; RNAi, rest 1.35, flight 6.45). PC, phosphatidylcholine; FFA, free fatty acid; DAG, diacylglycerol; TAG: triacylglycerol; HC, hydrocarbon.

(Wynant et al., 2012; Santos et al., 2014), in a variety of tissues including muscle, fat body, midgut and the central nervous system. Nevertheless, it was not clear at the onset of our study whether it was possible to obtain a similar reduction of FABP, which is the most prominent soluble protein in the flight muscle of mature adult locusts. As muscle FABP expression is specific for adult flight muscle, we injected dsRNA within 12 h of adult ecdysis, when *fabp* mRNA was minimal. A single injection of 4 µg dsRNA very effectively prevented the accumulation of *fabp* mRNA and of FABP itself, as evident from the absence of the characteristic protein band in SDS gels. There were no apparent anatomical or behavioral differences during maturation and adult development between the dsRNA-treated and control animals. However, FABP and its mRNA were almost undetectable in treated animals, even 3 weeks after adult ecdysis, when muscle FABP levels normally reach their maximum and insects are fully capable of migratory flight. This confirms that locusts are excellent organisms for RNAi studies targeted at prominently expressed proteins.

Because FABP was essentially absent in the flight muscle, treated animals were ideal for testing the physiological significance of FABP. While it has generally been assumed that muscle FABP fulfills an important role in the uptake and transportation of fatty acids, unambiguous proof for this function has never been obtained for any animal species. Our study clearly demonstrates that the absence of FABP in the flight muscle of locusts prevents sustained flight activity, while it can be easily induced in control animals in a tethered flight setup. Locust flight is initiated when their legs are lifted off the ground, and wind-sensitive hairs on the head detect air movement (Weis-Fogh and Jensen, 1956). Flight is maintained as long as there is sufficient wind resistance (>2 m s^{-1}) and the wings continue to oscillate in the wind. All insects used in this study initiated flight once mounted on the flight stand, and, after occasional stops in the first few minutes, engaged in uninterrupted flight for approximately 30 min. There was no apparent difference between control and treated animals, which could be expected as the initial phase of locust flight is fueled by trehalose and easily mobilized carbohydrate stores. In the following hour, however, after carbohydrate resources had been depleted, and lipids gradually became the sole fuel, the flight performance of insects lacking FABP rapidly declined. Once these insects stopped moving their wings they could initially be stimulated to continue flying for short durations, but eventually, it was apparent that they struggled to keep moving their wings. While there were individual differences in length of time until uninterrupted flight ceased, none of the insects lacking FABP was capable of flying continuously after 90 min, while all control animals continued to fly for 2 h and beyond.

Treated insects eventually kept hanging motionless in the flight stand, and occasionally we observed unusual movements, such as bending a foreleg so that it would touch the mouth, folding one of the inner wings so that it would rest under the body of the insect, or bringing the abdomen in contact with the tarsi of the forelegs or hindlegs (Fig. 5). We presume that these motions were aimed at providing tactile contact at the tarsi, in order to suppress the flight reflex that otherwise would force the insects to continue moving their wings (Weis-Fogh and Jensen, 1956), even though no substrate was available to fuel the muscle activity.

The present study clearly demonstrates that FABP plays an essential role in lipid uptake and transport in the flight muscle of locusts, and that its absence prevents long-distance flight activity. FABP may act as a sink for fatty acids in the cytosol, thus maintaining a concentration gradient across the plasma membrane, as well as a transport vehicle to allow for the rapid transport of the hydrophobic fatty acid molecules through the aqueous cytosol. Our findings add to earlier circumstantial evidence from vertebrate and invertebrate studies that suggest a role of FABP in sustained muscle activity, including the correlation between FABP content and lipid-fueled metabolic rates and the observations that FABP expression can be induced by increased lipid supply (e.g. Qu et al., 2007). Endurance training in rodents, humans, birds and insects leads to increased FABP expression in skeletal muscle (Haunerland, 1994; Clavel et al., 2002), as do pathological conditions with elevated plasma levels (diabetes, obesity) (Maeda et al., 2003; Choi et al., 2009; Atshaves et al., 2010). We have demonstrated before that endurance flight in fully mature locusts, in spite of the already very high levels of FABP, upregulates FABP expression in the flight muscle (Chen and Haunerland, 1994), likely in response to the increased lipid supply encountered during flight. Indeed, a similar upregulation of FABP expression was seen in resting locusts when lipid release and LDLp accumulation in the hemolymph was induced by injecting the lipid-mobilizing hormone adipokinetic hormone (AKH), or by simply injecting externally provided LDLp (Chen and Haunerland, 1994). In the present study, our experiments indicate that AKH signaling and lipid release is unaffected by RNAi and the resulting lack of FABP, and that there were no apparent differences in availability of lipids in the hemolymph. Following the initial, carbohydrate-fueled phase of flight, lipids were mobilized to induce a similar rise in the DAG content of the hemolymph in both treated and control animals. Therefore, the flight-induced mobilization of lipids in the fat body and the lipophorin shuttle in

the hemolymph appear fully functional in FABP-deficient animals as well.

The present study provides compelling evidence for an essential role of FABP in skeletal muscle energy metabolism *in vivo*, possibly owing to the advantageous locust model system with the stage-specific, very high levels of FABP, their total dependency on lipid as an energy source for sustained flight, and the extraordinarily strong response to dsRNA treatment. The structural and functional similarities between vertebrate and invertebrate muscle and their FABP structure and regulation suggests that FABP fulfills similar functions in vertebrates as well.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.H.H.; Methodology: S.R., D.Q., N.H.H.; Formal analysis: N.H.H.; Investigation: S.R., D.Q., A.S.A., N.H.H.; Writing - original draft: A.S.A., N.H.H.; Writing - review & editing: J.R.-H., N.H.H.; Supervision: J.R.-H., N.H.H.; Project administration: J.R.-H.; Funding acquisition: N.H.H.

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