

## RESEARCH ARTICLE

# Flea fitness is reduced by high fractional concentrations of CO<sub>2</sub> that simulate levels found in their hosts' burrows

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## ABSTRACT

Nidicolous ectoparasites such as fleas and gamasid mites that feed on small and medium-sized mammals spend much of their time in their hosts' burrows, which provide an environment for living, and often feeding, to their pre-imaginal and/or adult stages. Thus, these ectoparasites should be adapted to environmental conditions in burrows, including high fractional concentrations of CO<sub>2</sub> ( $F_{CO_2}$ ). We examined how a high  $F_{CO_2}$  (0.04) affected survival and reproductive success of a hematophagous ectoparasite of burrowing rodents using fleas *Xenopsylla ramesis* and Sundevall's jirds *Meriones crassus*. In the first experiment, fleas fed on hosts housed in high-CO<sub>2</sub> ( $F_{CO_2}=0.04$ ) or atmospheric-CO<sub>2</sub> ( $F_{CO_2}\approx 0.0004$ ) air, and were allowed to breed. In a second experiment, fleas were maintained in high CO<sub>2</sub> or CO<sub>2</sub>-free air with no hosts to determine how CO<sub>2</sub> levels affect survival and activity levels. We found that at high  $F_{CO_2}$  fleas laid fewer eggs, reducing reproductive success. In addition, at high  $F_{CO_2}$ , activity levels and survival of fleas were reduced. Our results indicate that fleas do not perform well in the  $F_{CO_2}$  used in this experiment. Previous research indicated that the type and intensity of the effects of CO<sub>2</sub> concentration on the fitness of an insect depend on the  $F_{CO_2}$  used, so we advise caution when generalizing inferences drawn to insects exposed to other  $F_{CO_2}$ . If, however,  $F_{CO_2}$  found in natural mammal burrows brings about reduced fitness in fleas in general, then burrowing hosts may benefit from reduced parasite infestation if burrow air  $F_{CO_2}$  is high.

**KEY WORDS:** Development, Carbon dioxide, Ectoparasite, Life history, Reproductive success, Survival

## INTRODUCTION

Parasites are ubiquitous in that almost every living organism is parasitized by other organisms. Some parasites live inside the host's body (endoparasites), while others live on the host's surface (ectoparasites). Many ectoparasites of terrestrial vertebrates are nidicolous arthropods that live on burrowing hosts. These ectoparasites spend much of their time in the host's burrow, which provides an environment for living, and often feeding, to pre-imaginal and/or adult stages (Holland, 1964). These ectoparasites usually alternate between spending time on and off the body of their host; thus, they must contend with the two types of environment: (i)

the environment on board the host, including its immune and behavioral responses; and (ii) the off-host environment, including the physical conditions inside the host's burrow.

Ectoparasites of small mammals benefit from some of the same advantages of living in burrows as their hosts. For example, the microclimate within the burrow is more stable than that outside the burrow (Kenagy, 1973; Reichman and Smith, 1990; Shenbrot et al., 2002). However, the air in some rodent burrows contains high fractional concentrations of CO<sub>2</sub> ( $F_{CO_2}$ ), and this is particularly true for burrows of fossorial rodents and those that have blocked entrances (Maclean, 1981b; Kuhnen, 1986; Burda et al., 2007). In contrast,  $F_{CO_2}$  in most burrows of semi-fossorial rodents and those that maintain open burrow entrances is close to the atmospheric value, such as in burrows of Merriam's kangaroo rat (*Dipodomys merriami*) and banner-tailed kangaroo rat (*Dipodomys spectabilis*) (Soholt, 1974; Kay and Whitford, 1978). Nonetheless, even in burrows with open entrances,  $F_{CO_2}$  can be higher than atmospheric concentrations. For example,  $F_{CO_2}$  in natural burrows of eastern chipmunks (*Tamias striatus*) ranged between 0.009 and 0.029, and  $F_{CO_2}$  increased with distance from the burrow entrance (Maclean, 1981b). In the same study, nest chambers in artificial burrows that were inhabited by eastern chipmunks had  $F_{CO_2}$  ranging from 0.012 to 0.064 (Maclean, 1981b). Similarly,  $F_{CO_2}$  as high as 0.022 – that is, 50-fold greater than atmospheric concentrations – occurred in nest chambers of permeable, artificial burrows inhabited by Sundevall's jirds (*Meriones crassus* Sundevall) (Brickner-Braun, 2014). Although  $F_{CO_2}$  in the tunnels of these artificial burrows is close to zero (Brickner-Braun et al., 2014), these observations highlight the variability of  $F_{CO_2}$  within the burrow and the need to consider microhabitat, such as nest versus tunnel, when investigating conditions experienced by individuals, especially because the nest is where  $F_{CO_2}$  is likely to be highest when occupied by a mother and offspring. In addition,  $F_{CO_2}$  in burrows is expected to be highest near the burrow inhabitant (Wilson and Kilgore, 1978; Burda et al., 2007). Thus, ectoparasites are likely to be exposed to  $F_{CO_2}$  higher than atmospheric  $F_{CO_2}$  when they dwell in the nest chamber, particularly when it is occupied by a host. Furthermore, ectoparasites may be disproportionately exposed to high  $F_{CO_2}$  relative to their mammalian hosts because when on their hosts they inhabit the layer between the host's skin and its hair surface, where  $F_{CO_2}$  may be further elevated because the air is poorly mixed with the surrounding burrow air.

Several rodent species have physiological and behavioral traits that mitigate the effects of inspiring high  $F_{CO_2}$  (Boggs et al., 1984), including respiratory characteristics and blood properties that facilitate increased oxygen delivery to tissues (Foreman, 1954; Hall, 1966) and maintain blood acid–base balance. Given the long, shared evolutionary history of ectoparasites and their hosts (Holland, 1964; Waage, 1979; Balashov, 1984; Barker, 1994; Krasnov, 2008), it is likely that ectoparasites of burrowing rodents, such as fleas, encounter periods of increased  $F_{CO_2}$ . We therefore

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Received 25 March 2015; Accepted 7 September 2015

hypothesized that fleas have also evolved adaptations for living in environments with high  $F_{CO_2}$ , and thus predicted that high  $F_{CO_2}$  will not affect their fitness.

To test this hypothesis, we quantified the effect of an environment with a high  $F_{CO_2}$  (0.04) on fitness of fleas (*Xenopsylla ramesis* Rothschild) that were raised on their rodent hosts, Sundevall's jirds. Fleas (Siphonaptera) are nidicolous ectoparasites, which live mostly on small and medium-sized burrowing mammal species – only about 6% of flea species are parasites of birds (Krasnov, 2008). All adult fleas are obligate hematophages and most fleas alternate periods when they occur on their host and when they occur in its burrow or nest. Finally, in almost all flea species, with few exceptions, pre-imaginal development is off-host, so both adult and pre-imaginal fleas are strongly affected by environmental factors (Holland, 1964; Marshall, 1981; Krasnov et al., 2001a,b, 2002). For example, survival and development rates of fleas change with ambient temperature and relative humidity (Krasnov et al., 2001a,b, 2002).

We chose to use *X. ramesis* as a model for this research because we expected that *X. ramesis* typically experience environments with high  $F_{CO_2}$ . First, in the Negev desert, *X. ramesis* is the most abundant flea of Sundevall's jird, Tristram's jird (*Meriones tristrami*) and fat sand rats (*Psammomys obesus*) (Krasnov et al., 1997; Krasnov et al., 1998, 1999) and it spends most of its time in the burrow of the host (Krasnov et al., 2004). Both jird species occasionally plug the entrances to their burrows (Mendelsohn and Yom-Tov, 1999; Shenbrot et al., 2002); this happens particularly often in habitats where these species harbor *X. ramesis* (Krasnov et al., 1996; Shenbrot et al., 2002). In addition, *X. ramesis* has been recorded on a rodent species that often seals its burrow entrances, namely Wagner's gerbils (*Gerbillus dasyurus*) (Harrison, 1956). Furthermore, the tunnels of burrows of these rodents can be long (up to 10 m for Sundevall's jirds; Shenbrot et al., 2002) and deep (up to 80 cm for fat sand rats; Mendelsohn and Yom-Tov, 1999). In general, long burrows and burrows with plugged entrances have elevated  $F_{CO_2}$  (Kay and Whitford, 1978; Maclean, 1981b; Kuhnen, 1986; Burda et al., 2007), thus *X. ramesis* are likely to encounter high  $F_{CO_2}$  often. Second, the host–parasite system used in the present study serves as a model for other host–parasite pairs, where the host lives in sealed burrows that might have high  $F_{CO_2}$ . For example, the fleas *Xenopsylla conformis*, *Xenopsylla gerbilli*, *Xenopsylla hirtipes* and *Xenopsylla skrjabini* are closely related to *X. ramesis*. These species are common parasites of jerboas (Dipodidae), hamsters (*Cricetus*, *Mesocricetus*, *Cricetulus*) and ground squirrels (*Spermophilus*) that seal their burrows during hibernation, as well as fossorial mole voles (*Ellobius*) in Central Asia (Harrison, 1956; Popova, 1968; Krasnov, 2008) and, thus, might be exposed to high  $F_{CO_2}$ .

In our experiments, hosts were housed in an environment with either high or atmospheric  $F_{CO_2}$ . We predicted that if fleas are adapted to high  $F_{CO_2}$ , then their performance would not differ between environments with high and atmospheric  $F_{CO_2}$ . Specifically, we predicted that survival, egg production, the proportion of successfully hatched larvae, and reproductive success (number of imagoes produced) would be unchanged in fleas raised on hosts under high  $F_{CO_2}$ . We also explored differential sensitivity of male and female fleas to high  $F_{CO_2}$ . Earlier studies on *X. ramesis* showed that although males and females respond differently to relative humidity and temperature, these factors do not affect overall sex ratio of new imagoes (Krasnov et al., 2001a). Thus, we expected that the proportion of new female imagoes (i.e. the sex ratio) would not differ between the treatments.

In addition, we were interested in how living in a high- $F_{CO_2}$  environment affected behavior of fleas, specifically mobility.

The anti-parasite behavior of a host (i.e. grooming) is an important defense tool (Nelson et al., 1975; Hart, 1994), so reduced mobility may make fleas more susceptible to host defenses. To test the effects of high  $F_{CO_2}$  on activity, we housed fleas without a host in  $F_{CO_2}=0.04$ , or in  $CO_2$ -free conditions and quantified their mobility. As part of this experiment we also quantified how high  $CO_2$  directly affects survival of fleas.

## MATERIALS AND METHODS

### Study species

We used fleas, *X. ramesis*, from our laboratory colony started in 1999 and collected from wild Sundevall's jirds, fat sand rats or Wagner's gerbils. Details on breeding and maintenance of fleas can be found elsewhere (Krasnov et al., 2002, 2003b; Khokhlova et al., 2009, 2010, 2012). In brief, fleas were maintained on rodents that were kept individually in plastic cages with a wire mesh floor over a pan with a mixture of sand and dried bovine blood (larval nutrient medium) at 25°C air temperature with a photoperiod of 12 h:12 h (light:dark). Every 2 weeks, all substrate and bedding material from the rodent's cage (including nest box and pan) were collected into plastic boxes with perforated lids and transferred to an incubator (FOC225E, Velp Scientifica, Milano, Italy; 25°C air temperature and 75% relative humidity) where the fleas developed. All fleas used in experiments were selected randomly from colonies.

We used Sundevall's jirds from a laboratory colony started in 1997–1999 from progenitors that were captured in the Negev desert (southern Israel). Details on rodent maintenance in colonies can be found elsewhere (Krasnov et al., 2002, 2003a; Khokhlova et al., 2009, 2012; Liberman et al., 2011). In short, rodents were maintained in plastic cages (35×25 cm and 15 cm high) at 25°C ambient temperature and 12 h:12 h (light:dark) light regime. The cages were supplied with wood shavings as bedding, paper towels as nesting material, and a pinecone for chewing. Millet seeds and alfalfa (*Medicago* sp.) were offered daily *ad libitum*. Commercial rodent chow was offered once a week. No water was available to the rodents as the alfalfa supplied enough water for their needs. This study was conducted under permits from Ben-Gurion University Committee for the Ethical Care and Use of Animals in Experiments (IL-36-9-2007).

### Experiment 1: flea fitness on hosts

#### Experimental design

To test for the effects of high  $F_{CO_2}$  on flea fitness, we used 18 female jirds and assigned nine of them to the high- $CO_2$  treatment and the remaining nine to the atmospheric- $CO_2$  treatment. To minimize maternal and genetic effects, we used pairs of sisters from nine litters for our experiment and assigned sisters to different treatments. We conducted the experiment in three sequential groups of hosts; each block included three rodents assigned to each treatment. To minimize the effect of age, all jirds were ~7 months old (144.8±1.5 days, mean±s.d.) when the experiment started.

Experimental cages were 9 l airtight plastic containers (20×26×17 cm, Lock&Lock, Hana Cobi, Korea). To create a high- $CO_2$  environment, we used two mass flow controllers (MFC-2, Sable Systems, Las Vegas, NV, USA) to pump pure  $CO_2$  and room air at rates that resulted in a final mixed  $F_{CO_2}$  of 0.035. This main airflow was then split into six channels with a series of Y-junctions. The flow of air through each line was ~300 ml min<sup>-1</sup> and was controlled with a needle valve. To maximize mixing of air within the experimental cages, the air inlet and outlet were spaced as far apart from each other as possible (i.e. the air inlet was in the lower right-hand corner of one side, and the air outlet was in the upper left-hand corner of the opposite wall of the cage). At the flow rate used, air in the experimental cage turned over approximately twice an hour. The average  $CO_2$  production of an adult female Sundevall's jird (~128 g) is 1.58 ml  $CO_2$  min<sup>-1</sup> (Brickner-Braun, 2014; I. Brickner-Braun, personal communication), so using equation 11.8 from Lighton (2008), the equilibrium  $F_{CO_2}$  in the high- $CO_2$  treatment cages was calculated to be 0.0402 (~0.04). The cages for jirds assigned to the atmospheric- $CO_2$  treatment were designed similarly, except that they received room air instead of mixed air. Based on the average metabolic rate of a jird and the flow rate of air for the experimental cages, we calculated that these cages would equilibrate at a  $F_{CO_2}$  of 0.005. We verified  $F_{CO_2}$  in the

experimental cages by measuring the excurrent air with a CO<sub>2</sub> analyzer (Foxbox, Sable Systems; data not shown). This was higher than atmospheric-CO<sub>2</sub> concentrations, but this level of CO<sub>2</sub> is biologically irrelevant for hosts (Boggs et al., 1984). Cages were fitted with wire mesh floors (5×5 mm) and the bottom of the cage was covered with a 3 mm layer of sand. This cage arrangement provided the fleas (see below) with access to sand so they could lay their eggs and also protected fleas from being killed easily by the host. These experimental cages also contained paper towels for nesting material, and the jirds were provided with millet seed *ad libitum* and alfalfa leaves.

After 3 days in the experimental cages to allow jirds to acclimate to air treatments, we added 100 female and 50 male fleas to each cage to mimic the number of fleas that reside in the burrow of an individual Sundevall's jird in the wild (Goüy de Bellocq et al., 2006). Fleas were combed from rodents and collected from the sand, counted and sexed, and replaced with a new batch of 150 fleas every 3–6 days until the end of the experiment (schedule details in Table 1). We used seven batches of fleas that were put on hosts consecutively, so that each host was exposed to seven batches of fleas in sequence. We did not quantify flea fitness for batch 2 because fleas were not on hosts long enough (<3 days) to lay the necessary eggs (Krasnov et al., 2004) (see below for details of fitness variables measured); thus, we used data from six batches of fleas. We do not have data for fleas on two hosts in the high-CO<sub>2</sub> treatment from the first group. In addition, for the first batch of fleas of the third group, we obtained no data for two of the hosts from the high-CO<sub>2</sub> treatment.

Female fleas were counted then placed in glass Petri dishes (100 mm diameter). We put ~10 females in each Petri dish (mean±s.d. of 9.3±1.9 female fleas per dish) and we created as many replicate Petri dishes for each host as allowed by the number of fleas collected from that host, up to six dishes (mean±s.d. of 4.1±1.2 dishes). Petri dishes were then placed in an incubator at 25°C and ~90% relative humidity; CO<sub>2</sub> treatments were not maintained during incubation. After 2 days, the female fleas were removed from the Petri dish and we calculated the mean number of eggs laid per female. The Petri dishes were put back into the incubator and the hatched larvae were counted and discarded 4, 5, 6, 7, 8 and 9 days after females were initially put in the Petri dish. We calculated the proportion of larvae hatched per day per female and the total proportion of eggs that developed into larvae.

When fleas for each batch were collected, the sand from the bottom of the cage was also collected, placed in a plastic box with a perforated lid, and incubated at 25°C and ~90% relative humidity until adult fleas completed emergence (see below). CO<sub>2</sub> treatments were not maintained during offspring development. Sand from each batch and from each host was incubated in separate boxes for 23 days and then the adult fleas that had emerged were counted. This was continued daily until no more fleas had hatched for 7 days.

### Statistics

The variables we quantified from experiment 1 were daily survival of adults for the duration of time they were on the host, average number of eggs laid per female, and hatching success of eggs. We calculated mean reproductive success, i.e. the mean number of new imago produced per mother flea, from data collected on imagoes emerging from the sand. The number of mother

fleas used in this calculation was calculated as the mean number of females added to the experimental cage at the beginning of the flea batch (e.g. batch 1, batch 3, etc.) and the number of females collected from the cage at the end of the same batch. We also calculated the proportion of female imagoes. Average development time from egg to emergence, number of days to first emergence, and number of days to last emergence were calculated to quantify flea development. Note that the data used in these calculations do not include the data from the eggs laid in Petri dishes and that these data were quantified for each batch of fleas separately.

We used general linear mixed effects models to determine the differences between fleas on hosts living in high and atmospheric CO<sub>2</sub>. Random effects were batch, host group, host mother ID and host ID. CO<sub>2</sub> treatment (high CO<sub>2</sub> or atmospheric CO<sub>2</sub>) was the only fixed effect. We determined whether CO<sub>2</sub> treatment was included in the best fit model using likelihood ratio tests (LRT) as recommended for general linear mixed effects models (Zuur et al., 2009). We used the following transformations to make the residuals normal: number of days to last emergence was log transformed, number of days to first emergence was transformed by raising to the power 0.2, mean development time was square root transformed, and the proportion of eggs hatched was transformed using an angular transformed. Statistics were performed in the program R v2.15.1 (R Development Core Team, 2011) using package lme4.

### Experiment 2: flea survival and movement without hosts

To determine how  $F_{CO_2}$  directly affected survival and activity of fleas, we put fleas in high-CO<sub>2</sub> ( $F_{CO_2}=0.04$ ) and CO<sub>2</sub>-free environments without a host. We used 10 replicates for each treatment, and for each replicate we put 10 fleas in a glass jar (20 ml) with a substrate of sand. The jars were covered with cloth that allowed gas exchange but prevented fleas from escaping. The jars for each treatment were then put inside a 9 l plastic experimental cage (described above) and these cages were put inside a temperature-controlled cabinet (Refritherm-5, Struers, Denmark) set at 25°C. To maintain the desired  $F_{CO_2}$ , experimental cages were attached to air pumps. For the CO<sub>2</sub>-free air treatment, incurrent air was pumped into the system through a purge gas generator (Pure Gas, Broomfield, CO, USA, model no. PCDA-1-12-m-32-C) to remove CO<sub>2</sub> and water vapor, both to less than 1 ppm. For the high-CO<sub>2</sub> treatment, we mixed dry, CO<sub>2</sub>-free air with bottled CO<sub>2</sub> with a two-way gas mixing pump (Wösthoff DIGAMIX 5KA27, Bochum, Germany) set to a  $F_{CO_2}$  of 0.04. For both treatments the flow rate of incurrent air was set to 300 ml min<sup>-1</sup>.

On days 5 and 6 we counted the surviving fleas. On day 5 we returned the fleas to their jars, and returned the jars to their experimental treatments. On day 6 we also quantified activity by pouring the sand and fleas into the bottom of a Petri dish (100 mm diameter) elevated 6 cm. Fleas were allowed to jump out of the dish for 5 min and then the top was put on the Petri dish to prevent the remaining fleas from escaping. We then counted the number of fleas that had jumped out of the dish and used it as a proxy for mobility.

### Statistics

We tested for differences in survival using a general linear mixed model. Survival was measured as the cumulative proportion of fleas surviving on days 5 and 6 of the experiment. We transformed both survival and activity with an angular transformation to normalize the distribution of residuals. CO<sub>2</sub> treatment (high  $F_{CO_2}$  or CO<sub>2</sub> free), day (5 or 6 days in treatment), and interaction between CO<sub>2</sub> treatment and day were potential fixed effects. Replicate (i.e. jar) was the only random effect. We determined whether CO<sub>2</sub> treatment was included in the best fit model using LRT as recommended for general linear mixed effects models (Zuur et al., 2009). We tested for differences in mobility for fleas in high-CO<sub>2</sub> and CO<sub>2</sub>-free treatments using a *t*-test. Statistics were performed in program R v2.15.1 (R Development Core Team, 2011). General linear mixed models were run using package lme4.

## RESULTS

### Experiment 1: flea fitness on hosts

In high  $F_{CO_2}$ , 25.7±2.3 (estimated mean±s.e.) fleas died per day, whereas only 20.3±2.3 fleas died per day in atmospheric CO<sub>2</sub>. Thus, 27% more fleas died per day when on hosts in high  $F_{CO_2}$  than in

**Table 1. Timeline for experiment 1**

Flea batch	Experimental day		Days on host
	Placed on host	Removed from host	
1	3	6–9	3–6
2	6–9	10	1–4
3	10	15	5
4	15	18	3
5	18	23	5
6	23	28	5
7	28	32	4

For details, see Materials and methods.

atmospheric CO<sub>2</sub> (LRT between model with CO<sub>2</sub> treatment as a fixed effect and null model, LR<sub>1</sub>=21.6,  $P<0.001$ ). On average, females in high  $F_{CO_2}$  produced 0.3 less of an egg per day than those in atmospheric CO<sub>2</sub> (LR<sub>1</sub>=6.7,  $P=0.009$ ; Fig. 1). The hatching success of eggs was the same regardless of CO<sub>2</sub> treatment (LR<sub>1</sub>=1.4,  $P=0.24$ ; Fig. 1).

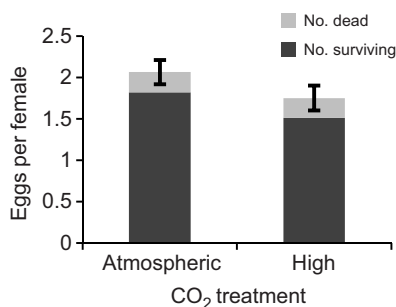
Mean reproductive success was 25% lower for fleas in high  $F_{CO_2}$  than in atmospheric CO<sub>2</sub> (LR<sub>1</sub>=7.0,  $P<0.001$ ; Fig. 2). The proportion of newly emerged imagoes that were female did not differ between treatments (LR<sub>1</sub>=1.36,  $P=0.24$ ; Fig. 2). Time of development until the first new imago emerged was, on average, 1.9 days longer in high CO<sub>2</sub> than in atmospheric CO<sub>2</sub> (LR<sub>1</sub>=850.1,  $P<0.001$ ; Table 2) and mean development time of the new generation of fleas was 2 days shorter in high  $F_{CO_2}$  (LR<sub>1</sub>=10.2,  $P<0.001$ ; Table 2) than in atmospheric CO<sub>2</sub>. However, the time of development assessed as the time from the first egg laid to the last adult flea to emerge did not differ between treatments (LR<sub>1</sub>=0.007,  $P=0.93$ ; Table 2).

### Experiment 2: flea survival and movement without hosts

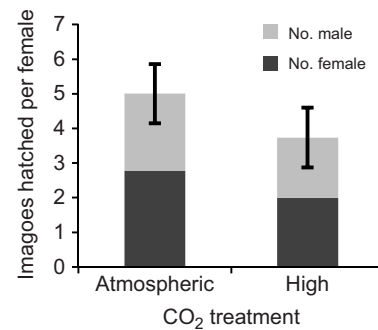
Cumulative survival of fleas was 25.4% lower in the high- $F_{CO_2}$  treatment than in the CO<sub>2</sub>-free treatment (LRT between model with just treatment and day plus treatment, LR<sub>1</sub>=4.7,  $P=0.03$ ; Fig. 3A) and decreased as the experiment progressed (LRT between model with just day and day plus treatment, LR<sub>1</sub>=29.9,  $P<0.001$ ; Fig. 3B). We found no interaction between number of days since the beginning of the experiment and CO<sub>2</sub> treatment on cumulative survival (LRT between model with interaction and additive model, LR<sub>1</sub>=0.49,  $P=0.48$ ). Fleas in the high- $F_{CO_2}$  treatment were also less active than those in the CO<sub>2</sub>-free treatment ( $t_{1,17}=-5.53$ ,  $P<0.001$ ; Fig. 4).

### DISCUSSION

The results of our first experiment indicate that the fleas we studied are not physiologically equipped to persist in environments with high  $F_{CO_2}$  because fleas in such conditions have reduced fitness relative to those in low  $F_{CO_2}$ ; both survival and reproductive output were reduced when  $F_{CO_2}$  was 0.04. The second experiment demonstrated that flea survival was reduced when fleas were exposed to high  $F_{CO_2}$ , as opposed to being killed by hosts, which is consistent with studies on other insects (Nicolas and Sillans, 1989). High  $F_{CO_2}$  may directly affect survival of fleas by altering gas exchange rates. Elevated levels of CO<sub>2</sub> cause insects to increase



**Fig. 1. Egg laying and hatching success at different  $F_{CO_2}$ .** Females in high  $F_{CO_2}$  produced 0.3 less of an egg per day than those in atmospheric  $F_{CO_2}$ , but the proportion of eggs that hatched did not differ between the treatments (atmospheric CO<sub>2</sub> 88.2±0.01%, high CO<sub>2</sub> 86.3±0.01%). Estimated means (±s.e.) were obtained from linear mixed models. The proportion of eggs that hatched was angular transformed for statistics, but the graph shows the back-transformed estimate. Error bars indicate s.e. for the number of eggs produced per female.



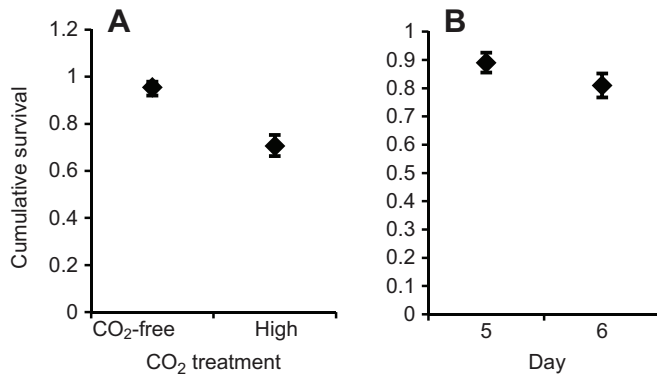
**Fig. 2. Reproductive success at different  $F_{CO_2}$ .** The mean number of imagoes emerged per female was 33% greater for fleas on hosts in atmospheric  $F_{CO_2}$  than high  $F_{CO_2}$ . The proportion of hatched imagoes that were female did not differ between CO<sub>2</sub> treatments (atmospheric 55.5±0.02%, high 53.4±0.02%). Means (±s.e.) were estimated from linear mixed models. The proportion of hatched female imagoes was angular transformed for statistics, but back-transformed estimates are presented. Error bars indicate the s.e. for the mean number of imagoes emerged per female.

respiration and spiracles to dilate, which in turn increases desiccation and may cause mortality (Bursell, 1974; Nicolas and Sillans, 1989). Specifically, spiracles remain open when CO<sub>2</sub> tensions are above a threshold and the spiracle is very sensitive to CO<sub>2</sub> (Burkett and Schneiderman, 1967; Förster and Hetz, 2010). In the flea *X. cheopis*, opening of the spiracles is controlled by want of oxygen, but the duration that spiracles remain open is determined by how long it takes CO<sub>2</sub> to diffuse out of the spiracle (Wigglesworth, 1935). Diffusion time increases in environments with higher  $F_{CO_2}$ , and *X. cheopis* kept their spiracles open constantly when respiring air with a  $F_{CO_2}$  of 0.02 (Wigglesworth, 1935). In addition, water loss increases when spiracles are open (Mellanby, 1935). Thus, fleas in our study that were respiring air with a  $F_{CO_2}$  of 0.04 likely kept their spiracles open continuously, leading to a higher rate of evaporative water loss. Indeed, high levels of CO<sub>2</sub> caused tsetse flies (*Glossina morsitans*) to keep their spiracles open and the rate of water loss increased as the percentage CO<sub>2</sub> in the air increased (Bursell, 1957). Experimentally reduced relative humidity negatively affects the survival of many insects, including *X. ramesis*, presumably because desiccation rates increase at low relative humidity (e.g. Krasnov et al., 2001a; Hulasare et al., 2005). Indeed, critical threshold experiments indicate that *X. ramesis* lose water to the environment when relative humidity drops below some point between 60% and 65%, and they lose water at a rate of 1.4–2.4% mass h<sup>-1</sup> when exposed to dry air, depending on the life history stage (Fielden et al., 2002). Small insects, such as fleas, would be particularly susceptible to water loss caused by high  $F_{CO_2}$  because resistance to desiccation decreases with decreasing body size (Hood and Tschinkel, 1990; Lighton et al., 1994; Lehmann et al., 2000), although some insects are capable of shifting patterns of gas exchange in low relative humidity to minimize water loss (Duncan et al., 2002).

**Table 2. Estimated mean (±s.e.) number of days to first hatch of imagoes, last hatch of imagoes, and mean generation time for fleas in atmospheric- and high-CO<sub>2</sub> treatments**

Hatch response variable (days)	CO <sub>2</sub> treatment		P-value
	Atmospheric	High	
First hatch	25.7±0.60	27.6±0.66	<0.001
Last hatch	63.6±7.9	63.2±7.9	0.933
Mean hatch	40.3±1.8	38.3±1.8	0.001

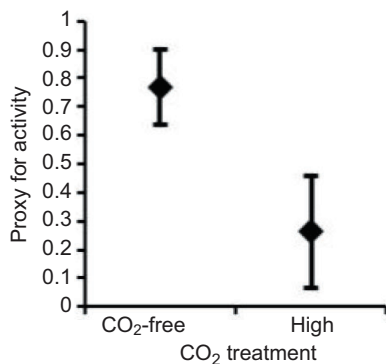
For details, see Materials and methods. P-values are from  $\chi^2$  tests to determine whether treatment effect was significant.



**Fig. 3. Flea survival at different  $F_{CO_2}$  and on different days of the experiment.** (A) Survival, calculated as the cumulative proportion of fleas alive since the beginning of the experiment, was lower in the high- $F_{CO_2}$  treatment than in the CO<sub>2</sub>-free treatment. (B) Survival was also lower on day 6 than on day 5 of the experiment for both CO<sub>2</sub> treatments. The cumulative proportion of fleas alive was square-root transformed for statistics, but the graph depicts back-transformed estimates of means ( $\pm$ s.e.).

In addition, our results indicate that high  $F_{CO_2}$  indirectly reduces flea survival because fleas in high  $F_{CO_2}$  were less active, which might increase the probability of being dislodged or killed by a host. This finding is consistent with the common practice of using sublethal doses of CO<sub>2</sub> to anesthetize insects in the laboratory. Mechanistically, flea activity may be reduced because CO<sub>2</sub> may block synaptic transmission at the skeletal neuromuscular junction, as was found in *Drosophila melanogaster* (Badre et al., 2005).

Reproductive success was also reduced in high  $F_{CO_2}$ , and this reduction was the result of fleas laying fewer eggs in the environment with a high  $F_{CO_2}$  as opposed to that environment rendering eggs unviable. Furthermore, the proportion of new female imagoes did not differ between treatments, suggesting that sensitivity to high CO<sub>2</sub> was not sex dependent. Our results partially concur with those from experiments with *D. melanogaster* where it was found that flies laid fewer eggs and the fraction of eggs that hatched decreased when in a  $F_{CO_2}$  of 0.13; the decrease was even greater when  $F_{CO_2}$  was 0.20 (Helenius et al., 2009). In addition, exposure of insects to sublethal doses of CO<sub>2</sub> (often  $F_{CO_2}=1$ ) for short durations for anesthetic purposes resulted in insects laying fewer eggs and in reduced hatching success of those eggs (Press



**Fig. 4. Flea activity at different  $F_{CO_2}$  levels.** Fleas from the high- $F_{CO_2}$  treatment were less mobile than those from the CO<sub>2</sub>-free treatment. We measured activity as the proportion of fleas that jumped out of the Petri dish within 5 min of being removed from the CO<sub>2</sub> treatment. Thus, a higher proportion that jumped from the dish equated with more mobile fleas. Statistics were run on angular-transformed data, but the graph shows raw means ( $\pm$ s.d.).

et al., 1973; Mbata et al., 1998). Differences regarding the viability of eggs between the present study and other studies might result from differences in experimental  $F_{CO_2}$  and duration of exposure. Sub-lethal doses of CO<sub>2</sub> reduce production by preventing maturation of oocytes in female red flour beetles (*Tribolium castaneum*) (Press et al., 1973). In addition, breathing air with a high  $F_{CO_2}$  could affect reproductive performance by reducing feeding and drinking, as was found in house cricket (*Acheta domestica*) larva (Woodring et al., 1978), which would reduce the resources available for egg production. However, we did not investigate the mechanism by which breathing air with a high  $F_{CO_2}$  reduced reproductive success, so we cannot draw a general conclusion at this point.

High  $F_{CO_2}$  could also indirectly affect flea fitness through changes in the chemistry of the host blood. For example, a host inspiring air with a high  $F_{CO_2}$  might develop hypercapnia with high levels of CO<sub>2</sub> and bicarbonate in the blood (Henderson and Haggard, 1918), and inspiring high  $F_{CO_2}$  also changes gene expression of hosts, including genes associated with immune function (Cummins et al., 2010; Taylor and Cummins, 2011). These and other changes in host physiology might affect parasites by changing the nutritional value of the blood, similar to what happens to phytophagous insects when high  $F_{CO_2}$  indirectly affects insect fitness by altering the nutritional quality of host plants (Fajer et al., 1989; Coviella and Trumble, 1999), but this idea would have to be tested directly.

The duration of development time to emergence of imagoes increased in the high- $F_{CO_2}$  treatment, which concurs with results found in other studies of invertebrates in which high  $F_{CO_2}$  was reported to increase development time (Nicolas and Sillans, 1989; Bouletreau-Merle and Sillans, 1996). The duration of development encompasses the effects of changes during the egg, larval or pupal stages of flea development. However, fleas for which development time was calculated were only exposed to different  $F_{CO_2}$  treatments as eggs, indicating that exposure during egg development within the mother or the egg stage underlies differences in the duration of development. Further evidence to support this idea is the lack of a difference between CO<sub>2</sub> treatments for the duration of time until the last imago emerged (Table 2). All eggs from each batch of fleas were collected from the host cages at the same time, so eggs laid later would have been exposed to their CO<sub>2</sub> treatment for less time, resulting in less time for plastic traits associated with development to have been altered.

The result concerning the duration of development time to emergence of imagoes indicates that conditions during early development alter traits throughout an individual flea's lifetime. The change in duration of development could have occurred through at least three mechanisms. Most simply, eggs in high  $F_{CO_2}$  could take longer to hatch, delaying the whole development cycle. Alternatively, female fleas exposed to high  $F_{CO_2}$  might alter offspring development time via maternal effects such as investment in resources provided to the eggs, resulting in slower development overall. Finally, exposure of eggs to high  $F_{CO_2}$  could push the individual into a 'slow-development' phenotype through changes in gene expression, resulting in an increased overall duration of development – duration of development being the result of developmental canalization (Cohen et al., 2012). We are inclined to believe that the first explanation is correct because it is the simplest. However, CO<sub>2</sub> exposure can alter expression of transcription factors (Cummins et al., 2010; Taylor and Cummins, 2011) and can alter biochemical pathways including NADPH production (Friedlander et al., 1984). Even short-term exposure to

high  $F_{CO_2}$  can cause long-term disruption of neuroendocrine function in house crickets (Woodring et al., 1978). Thus, any of these proposed explanations might be correct and experimental work is needed to distinguish among them.

The effect of a high  $F_{CO_2}$  on parasite fitness likely depends on species, microclimatic conditions (e.g. relative humidity, temperature,  $F_{CO_2}$ ) and life stage. For example, *D. melanogaster* had increased development time and reduced viability when continuously exposed to a  $F_{CO_2}$  of 0.2 (Bouletreau-Merle and Sillans, 1996), but not when exposed to a  $F_{CO_2}$  of 0.018 (MacAlpine et al., 2011). Similarly, a  $F_{CO_2}$  of 0.05 improved fecundity in *D. melanogaster* (Bouletreau-Merle and Sillans, 1996), but a  $F_{CO_2}$  of 0.018 did not alter fecundity (MacAlpine et al., 2011). Furthermore, the duration of exposure to  $CO_2$  that causes mortality depends on temperature and relative humidity (Nicolas and Sillans, 1989). Thus, caution should be exercised when generalizing the results of this study.

Furthermore, caution should also be exercised when drawing conclusions about the ecological implications of our results because we investigated the role of a single  $F_{CO_2}$  value, 0.04, on flea fitness. Mean  $F_{CO_2}$  in burrows of semi-fossorial rodents typically ranges between 0.005 and 0.064 (Maclean, 1981a,b; Kuhnen, 1986; Roper et al., 2001), although values as high as 0.108 have been recorded in an artificial burrow with sealed entrances (golden hamster, *Mesocricetus auratus*; Kuhnen, 1986). Furthermore,  $F_{CO_2}$  ranged from 0.002 to 0.08 in nest chambers of artificial burrows designed to match the geometric properties of natural burrows of Sundevall's jirds (Brickner-Braun, 2014). Thus, the  $F_{CO_2}$  we chose was within the range of  $F_{CO_2}$  found in burrows and is in the middle of the range found in artificial nest chambers of jirds. The  $F_{CO_2}$  used, however, is the upper end of the typical range and is potentially outside the range found in natural burrows of jirds, which has never been measured. More experimental work is needed to determine how more moderate  $F_{CO_2}$  affects fitness of fleas and to better understand the ecological context of these results. If the effects of inspiring high  $F_{CO_2}$  on the fitness of fleas are generalizable, the results of our experiment suggest an interesting hypothesis about the benefits for hosts of high  $F_{CO_2}$  in their burrows.

Studies of burrow-dwelling animals, including rodents, report that the architecture of the animals' burrow may reduce the build-up of  $CO_2$  in some cases (Vogel et al., 1973; Kleineidam and Roces, 2000; Brickner-Braun et al., 2014). Indeed, this behavior is expected if there are costs associated with high  $F_{CO_2}$ , because burrows are the product of natural selection acting on the builder (Turner, 2000). Some animals, however, live in burrows with high  $F_{CO_2}$ , particularly those with sealed entrances (Maclean, 1981b; Kuhnen, 1986; Mendelsohn and Yom-Tov, 1999; Shenbrot et al., 2002; Burda et al., 2007). This leads to the question, do rodents build burrows designed so they do not to completely eliminate the elevated  $F_{CO_2}$ ? Our results provide a potential answer: if our results are generalizable and high  $F_{CO_2}$  reduces parasite fitness and thus reduces the fitness costs of parasite infection on the host more than elevated  $F_{CO_2}$  reduces the host's fitness, then selection should favor hosts that build burrows with slightly elevated  $F_{CO_2}$ . This explanation does not exclude other explanations about how the balance of other costs – e.g. digging costs (Vleck, 1979) – and benefits results in selection on burrow design. Rather, reduction of parasite load may be just one of the many factors contributing to the complex costs and benefits equation of burrow design.

For this explanation, involving reducing parasite pressure, to be true, the  $F_{CO_2}$  found in natural burrows must bring about reduced

fitness of the flea that feeds on the host, but simultaneously minimize the costs to the host. Previously, it was reported that rodents that inhabit burrows are physiologically adapted to living in them, and those physiological attributes allow rodents to tolerate high inspired  $F_{CO_2}$  (Boggs et al., 1984; Ar, 1987). Those studies suggest that rodents can tolerate high  $F_{CO_2}$  with little negative effect on their physiology and therefore high  $F_{CO_2}$  might not affect fitness. The results of the present study indicate that exposure to a  $F_{CO_2}$  of 0.04 reduces flea survival and reproductive success. Combined, these observations support the notion that high  $F_{CO_2}$  in burrows may benefit some hosts by reducing the fitness of ectoparasites.

#### Acknowledgements

This is publication no. 882 of the Mitrani Department of Desert Ecology. We thank Inbal Brickner-Braun for helpful advice and discussion during the planning of this experiment, and thank two anonymous reviewers for their constructive feedback.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

C.J.D. conceived the idea for the experiment; all authors helped design the experiment; C.J.D. and I.S.K. executed the experiment; C.J.D. and B.R.K. interpreted the experiment and drafted the manuscript; and all authors revised the manuscript.

#### Funding

This study was partly supported by the Israel Science Foundation (grant numbers 26/12 to B.R.K. and I.S.K. and 136/10 to B.P. and Pedro Berliner). C.J.D. was the recipient of the Blaustein Postdoctoral Fellowship of the Blaustein Center for Scientific Cooperation, and was also funded by the Kreitman School of Advanced Graduate Studies and the Swiss Institute for Dryland Environmental and Energy Research.

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