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Comparison of life history and genetic properties of cowpea bruchid strains and their response to hypoxia

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ABSTRACT

The cowpea bruchid (*Callosobruchus maculatus*) is the most important storage pest of grain legumes and comprises geographically distinct strains. Storage under a modified atmosphere with decreased O_2 content represents an alternative to chemical fumigants for pest control of stored grains. In this study, we compared reproduction, development and survival, as well as genome size of bruchid strains from South India (SI), Burkina Faso (BF), Niger (CmNnC) and the United States (OH), reared on mung bean (*Vigna radiata*). Fecundity and egg-to-adult duration varied significantly among these strains. Notably, strain BF had the highest fecundity, and strain SI displayed the fastest development whereas strain OH was the slowest. Differences in adult lifespan among strains were only detected in unmated but not in the mated group. Genome size of SI females was significantly larger than that of OH females, and for all four strains, the female genomes were larger than those of their corresponding males. Furthermore, we studied effects of exposure to 1% O_2 + 99% N_2 on strains SI and BF. Mortality caused by hypoxia was influenced by not only developmental stage but also by insect strain. Eggs were most sensitive, particularly at the early stage, whereas the 3rd and 4th instar larvae were most tolerant and could survive up to 15 days of low O_2. Strain SI was slightly more resistant than BF in egg and larval stages. Proteolytic activity prior to, during and after hypoxia treatment revealed remarkable metabolic plasticity of cowpea bruchids in response to modified atmosphere.

1. Introduction

Terrestrial insects are dependent on atmospheric O_2 for generation of metabolic energy. Disinfestation of storage pests using a modified atmosphere with low O_2 (hypoxia) and/or high CO_2 (hypercapnia) content represents an alternative to fumigation with synthetic insecticides such as methyl bromide (Fields and White, 2002; Fleurat-Lessard, 1990). A hypoxic and/or hypercapnic environment can be achieved by hermetically sealing storage units so that the O_2 consumed by infesting insects in the storage units cannot be replaced, while respiration causes increased CO_2 concentration (Murdock et al., 2003; Sanon et al., 2011). Other approaches include directly purging the storage facility of air (O_2) using N_2 or CO_2, or by introducing gases generated outside the storage container from combustion of hydrocarbon fuels (Conyers and Bell, 2007). Depending on gas composition, exposure time, insect species and their developmental stages, effects of hypoxia vary (Donahaye et al., 1996; Hoback and Stanley, 2001; Soderstrom et al., 1990; Wang et al., 2001). Considerable research has been conducted on cosmopolitan pests such as *Tribolium castaneum*, *Tribolium confusum*, *Sitophilus zeamais*, *Sitophilus oryzae*, *Rhyzopertha dominica*, *Cryptolestes ferrugineus* and *Oryzaephilus surinamensis* (Carli et al., 2010; Chiappini et al., 2009; Finkelman et al., 2006; Lord, 2009; Riudavets et al., 2009). Generally, a reduction in the O_2 content to 3% or lower or an increase in CO_2 content to 60% or higher is effective for control of most storage pests (Navarro, 2006).

The cowpea bruchid, *Callosobruchus maculatus* (Coleoptera: Bruchidae) is the primary pest of stored legume seeds, especially the cowpea, *Vigna unguiculata* Walp. This bruchid thrives wherever its hosts are grown and stored, particularly in Africa where grain legumes are essential protein sources (Langyintuo et al., 2003). Infestations by the bruchids begin in the fields, but populations expand rapidly in storage. Adult females deposit their eggs on the seed surface, and hatched larvae burrow into and feed inside the seeds, where they complete four-instar larval and pupal

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development. Upon emergence, adults begin to mate and oviposit within a few hours, initiating another round of infestation. Complete infestation of cowpea can occur after 3–5 months of storage (Ajayi and Wintola, 2006). Damaged seeds can be completely hollowed out by feeding larvae, causing a severe loss of seed weight, nutrition, germination potential, and thereby the commercial value of the commodity (Boeke et al., 2004).

Geographically different populations of cowpea bruchids, defined as strains, vary in numerous biological parameters. For instance, fecundity, developmental period, mortality and sex ratio differ significantly among bruchid strains Campinas, Yemen and IITA (Dick and Credland, 1984). The male's ejaculate size during mating and female's egg-spacing behavior are highly variable among populations collected worldwide (Messina and Mitchell, 1989; Savalli et al., 2000). Differences in larval respiration rate and seed consumption were also detected among strains (Guedes et al., 2003). Many of these variations are genetically-based (Bieri and Kawecki, 2003; Kawecki, 1995; Messina and Slade, 1997). Despite considerable investigation of bruchid strains, not all populations are well documented. For example, a strain originally collected from Columbus, Ohio (strain OH) is among the undocumented. Furthermore, genome size variation is thought to contribute to life history variation (Biemont, 2008; Ellis et al., 2014; Hessen et al., 2013), yet no information is available on genome size of bruchid strains so far. In addition, although hypoxia is known to affect growth and development of cowpea bruchids (Cheng et al., 2012, 2013; Mbata et al., 1996; Ofuya and Reichmuth, 1993; Storey, 1978), it is unclear whether hypoxia has differential impacts on various bruchid strains.

In this study, we compared biological (reproduction, development and mortality) and genetic (genome size) parameters of four different cowpea bruchid strains originating from Africa, Asia and America. We then investigated the hypoxic responses of two of these strains by comparing mortality at different developmental stages of two strains when exposed to 1% O_2 + 99% N_2, and measured their midgut proteolytic activities under hypoxia and normoxia.

2. Materials and methods

2.1. C. maculatus strains

The four cowpea bruchid strains used in the current study were collected originally from infested cowpeas in Niamey, Niger (CmNnC) and Ouagadougou, Burkina Faso (BF), and from infested mung beans in Tirunelveli, South India (SI) and Columbus, Ohio, the United States (OH), respectively. Prior to this study, strain SI had been maintained on mung bean seeds, whereas strains CmNnC, BF and OH had been maintained on cowpea seeds for over 10 generations. Comparisons of life history traits, genome sizes and hypoxic response among bruchid strains were performed on mung bean. To minimize possible previous host effects, new populations of strains CmNnC, BF and OH (previously maintained on cowpea) were grown for two generations on mung bean before the start of these experiments. All cultures of four strains were maintained in 500 mL wide-mouth glass bottles, and all experiments in this study were conducted in an environmental chamber with 27 °C and 60% R.H.

2.2. Reproduction, development and mortality of cowpea bruchid strains

For each bruchid strain, approximately 200, 1–4 day old adults were introduced into a wide-mouth glass bottle containing 400 mung beans for mating and oviposition. The adults were removed 2 h later to obtain an age-synchronized population. Seeds with a single egg on a mung bean were selected. Batches of 50 eggs (4–6 h old) were placed into 30 mL clear plastic cups with their lids and sides perforated for air exchange. The number of hatched eggs was recorded 8 days later, using color change as the indicator of hatching (Shazali et al., 2004). Since larvae were hidden inside the seeds, the number of successfully hatched eggs was used to determine the initial larval and pupal numbers in the seeds. Emerged adults from these seeds were recorded twice a day until no further emergence occurred. There were three replicates for each strain. Egg viability, adult emergence from hatched eggs, and egg-to-adult developmental duration were calculated.

To determine the longevity of mated adults and the fecundity of individual females of four strains, 25 mung beans were introduced into 30 mL clear perforated plastic cups. Three 0–4 h old virgin adults (one female and two males) of a single strain were released into each cup, and deaths of female and male adults were recorded daily. The total number of eggs laid by each female was also documented. This was repeated ten times for each strain. To measure longevity of unmated adults, 5 females or males of each strain, 0–4 h old, were released into a 30 mL perforated clear plastic cup for observation of adult death. This experiment was repeated six times for each bruchid strain.

2.3. Measurement of genome sizes of cowpea bruchid strains

Male and female adults from each of the four strains were collected and prepared for genome size estimates as described in Hare and Johnston (2011). Briefly, the head of a single male or female adult was placed into 1 mL ice-cold Galbraith buffer in a 2 mL Dounce tissue grinder along with the head of a single female *Drosophila virilis* which served as a genome size standard (1C = 328 Mb). The heads were ground and the resultant solution filtered through 40 μm nylon mesh and then stained in 25 μg/mL propidium iodide for 30 min. The relative fluorescence of diploid nuclei from the head of the bruchid and the standard were scored using a CyFlow flow cytometer (Partek America, Swedesboro, NJ). The 1C amount of DNA in each bruchid was calculated as the ratio of the mean 2C fluorescence of the bruchid and standard times the amount of DNA in the standard. A minimum of 9 adults for each sex of each strain were scored.

2.4. Hypoxic treatment of strains BF and SI

Due to their similar developmental time, strains BF and SI were subjected to 1% O_2 + 99% N_2 treatment to determine whether low O_2 differentially influenced mortality of different bruchid strains. The precise developmental sub-stages for both strains (Table 1) were pre-determined as previously described (Cheng et al., 2012), and prepared accordingly. Seeds, each carrying a single insect at a specified developmental stage including eggs of three stages (early, intermediate and black-headed), or larvae of four instars or pupae were selected.

Certified pre-mixed gas (1% O_2 + 99% N_2) was purchased in the form of pressurized cylinders from Brazos Valley Welding Supply (Bryan, TX). Batches of (respectively) 50 eggs of each stage, 30 larvae of each instar, 30 pupae and 20 adults (ten females and ten males, 4–6 h old) were separately placed into one-liter septum bottles (Industrial Glassware, Millville, NJ) and exposed to 1% O_2 + 99% N_2. Specifically, the septum bottles with infested mung bean seeds were connected to the outlet of the pressure gage of gas cylinders via plastic tubing. The gas was delivered to the bottles for 7 s at 70 kPa as measured on a manometer and controlled by a regulator. After treatment, the bottles were immediately sealed to prevent any diffusion of air. The level of O_2 was verified using a head-space analyzer (Mocon PAC CHECK®, Model 325, Minneapolis, MN). The

Table 1
Defining developmental substages of cowpea bruchid strains for hypoxia treatments.

Developmental stage	Substage	Developmental time for hypoxia treatment (h)[a]	
		BF	SI
Egg	Early	4–6	4–6
	Intermediate	50–52	50–52
	Black-headed	96–98	96–98
Larva	First instar	144–146	146–148
	Second instar	216–218	210–212
	Third instar	264–266	258–260
	Fourth instar	337–339	327–329
Pupa		482–484	458–460
Adult		4–6	4–6

[a] Substages of egg, larva and pupa were calculated from egg laying; and adult stage was from emergence.

exposure time was 12, 24, 48 or 72 h for eggs; 3, 6, 9, 12 or 15 days for larvae and pupae; and adults stayed in the treatment conditions until death. For exposure periods longer than 2 days, gases in septum bottles were replenished every 2 days to maintain relatively constant hypoxic conditions. Control insects were subjected to the same manipulations as their respective treatments but exposed to ambient air. For every developmental stage, each treatment was replicated three times. Egg hatch, adult emergence and death were recorded. For eggs, those whose eggshells remained transparent or appeared shriveled or wrinkled were assumed dead, whereas those that turned cream white were considered alive (Shazali et al., 2004). For larvae and pupae, individuals that failed to emerge were considered dead. Adults remaining motionless after being stimulated by turning the container several times were also presumed dead. Mortality from different exposure times for each developmental stage was calculated.

2.5. Hypoxic treatments for gut proteolytic analyses

To determine the hypoxic effects on strains SI and BF, we measured midgut digestive proteolytic activity of the 4th instar larvae due to relative ease of midgut dissection at this stage. Early 4th instar larvae of strains SI and BF were exposed to 1% O_2 + 99% N_2 for 24 and 72 h. A portion of the 72 h group were transferred to ambient air for another 48 h normoxic treatment. Larvae collected prior to hypoxic treatment served as the experimental controls. Upon completion of treatment at each time point, larvae were immediately removed from the seeds and their midguts were dissected. Collected midguts (5 per tube) were stored in 0.1 M sodium acetate buffer with 1 mM EDTA (pH 5.5) and kept in the −80 °C freezer. Midgut extract (0.5 guts per reaction) was used for proteolytic analysis as previously described (Cheng et al., 2012). All experiments were repeated three times.

2.6. Data analyses

All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL). Mean mortality of eggs, larvae, pupae and adults resulting from low O_2 treatment was corrected using control mortality by Abbott's formula (1925). Data on Abbott-corrected mortality, egg viability and emergence percentage from hatched eggs after arcsine square root transformation, development time, fecundity, longevity and genome sizes of cowpea bruchid strains, as well as midgut enzymatic activity were analyzed using a one-way ANOVA. Tukey's multiple range test was used for pairwise comparison of the difference between treatments for mean separation ($P < 0.05$). Data were also subjected to two-way ANOVA to determine the effects of mating status and sex on longevity. Difference in genome sizes between females and males of each strain was analyzed using independent samples Student's t test ($P < 0.05$).

3. Results

3.1. Biological features of four cowpea bruchid strains

No significant difference was detected among strains in egg viability ($F = 2.01$, df = 3, $P = 0.192$), nor in adult emergence percentage from hatched eggs ($F = 2.00$, df = 3, $P = 0.193$) (Table 2). However, egg-to-adult developmental time ($F = 634.95$, df = 3, $P < 0.001$) and mean total number of eggs laid per female ($F = 17.43$, df = 3, $P < 0.001$) varied significantly. Strain OH exhibited the slowest growth, reflected by its longest developmental time; in contrast, strain SI developed the fastest. Strain BF, on the other hand, had significantly higher fecundity than all other strains (Table 2).

Insect strains varied in the longevity of unmated adults (female $F = 12.98$, df = 3, $P < 0.001$; male $F = 17.88$, df = 3, $P < 0.001$), but not of mated adults (female $F = 2.38$, df = 3, $P = 0.088$, male $F = 2.24$, df = 3, $P = 0.091$). Both unmated female and male adults of strains OH and CmNnC lived longer than strains SI and BF, but there were no differences in the longevity between strains OH and CmNnC or between strains SI and BF (Table 3). Unmated adults lived longer than mated ones ($F = 128.26$, df = 1, $P < 0.001$), and female adults lived longer than males ($F = 82.67$, df = 1, $P < 0.002$). Significant mating × sex interaction ($F = 55.34$, df = 1, $P < 0.001$) indicated that mating has a more deleterious effect on females than on males.

3.2. Genome sizes of four cowpea bruchid strains

For all four strains, the genomes of females were larger than in their corresponding males (SI df = 22, $t = 2.95$, $P < 0.001$; BF df = 18, $t = 2.10$, $P < 0.001$; CmNnC df = 18, $t = 2.10$, $P < 0.001$; OH df = 21, $t = 2.08$, $P < 0.001$). The male genome sizes fell within a very narrow range, and did not vary significantly among strains ($F = 0.91$, df = 3, $P = 0.45$). Female genome sizes did vary among strains ($F = 3.41$, df = 3, $P = 0.026$). Strain SI had the largest genome, and strain OH the smallest, with strain BF and CmNnC intermediate in size, closer to the size of the strain SI female genome but not significantly different from either strain SI or OH (Table 4). Interestingly, there appeared to be a positive correlation between the genome size and body size; strain SI possessed the largest body size and the largest genome size. Likewise, the smallest body size of strain OH was correlated with the smallest genome size.

3.3. Hypoxic effect on strains SI and BF

Egg development was divided into three distinct stages: early, intermediate and black-headed. Early eggs (4–6 h old) were most susceptible to low O_2 as over 90% lost viability following 12 h exposure. In contrast, the intermediate staged eggs (50–52 h old) were most tolerant and less than 10% failed to hatch following a

Table 2
Comparison of four biological parameters of cowpea bruchid strains.

Insect strain	Egg hatch (%)	Emergence from hatched eggs (%)	Egg-to-adult duration (days)	Fecundity
SI	82.7 ± 3.5 a	97.5 ± 1.5 a	23.2 ± 0.1 d	55.2 ± 2.5 b
BF	82.0 ± 7.0 a	98.5 ± 1.5 a	24.5 ± 0.1 c	81.0 ± 3.2 a
CmNnC	78.5 ± 3.7 a	98.4 ± 0.8 a	26.2 ± 0.1 b	63.8 ± 1.7 b
OH	67.3 ± 4.8 a	90.7 ± 4.9 a	31.6 ± 0.2 a	60.4 ± 3.4 b

Values shown as mean ± SE. Means followed by the same letter in a column were not significantly different by Tukey's multiple range test ($P < 0.05$).

Table 3
Mean longevity (days ± SE) of unmated and mated adults of cowpea bruchid strains.

Insect strain	Unmated adult		Mated adult	
	♀	♂	♀	♂
SI	12.0 ± 0.5 b	7.9 ± 0.3 b	7.9 ± 0.2 a	7.3 ± 0.2 a
BF	12.0 ± 0.7 b	7.8 ± 0.3 b	8.2 ± 0.2 a	7.6 ± 0.2 a
CmNnC	15.7 ± 0.7 a	10.3 ± 0.3 a	8.6 ± 0.2 a	8.1 ± 0.3 a
OH	16.0 ± 0.6 a	9.5 ± 0.2 a	8.0 ± 0.2 a	7.8 ± 0.2 a

Means followed by the same letter in a column were not significantly different by Tukey's multiple range test ($P < 0.05$).

24 h exposure (Fig. 1). Eggs of strain BF were more susceptible to hypoxia than strain SI, particularly after 24 h (for black-headed eggs) or 48 h (for intermediate eggs) exposure to 1% O_2 + 99% N_2 (Fig. 1).

Hypoxia tolerance increased in larvae and pupae compared to eggs. During larval development, 3rd and 4th instar larvae were more resistant to low O_2 than the 1st and 2nd instars (Fig. 2). Strain BF larvae were more susceptible than SI larvae, especially the 1st and 2nd instars subjected to 6-day hypoxia treatment (Fig. 2). As larvae continued to develop, the difference between the strains diminished. No difference in mortality was observed between the two strains in hypoxic pupae or adults (Figs. 2 and 3). Although more tolerant than any larval stage when exposed for 3 days, pupae became more vulnerable than late stage larvae when exposed to 1% O_2 + 99% N_2 for 9 days or longer. A similar trend was also detected in adults (Fig. 3).

3.4. Effect of hypoxia on digestive proteolytic activity in strains BF and SI

Midgut digestive protease activity was used as an indicator to determine the effect of hypoxia on metabolic status of strains BF and SI. Under low O_2, proteolysis was drastically suppressed in both strains; but when O_2 supply resumed, activity was restored, and even over-compensated (Fig. 4), implying an enormous flexibility in metabolic adjustment exists in cowpea bruchids.

4. Discussion

Life-history and genetic characteristics may vary among different strains of cowpea bruchids derived from different geographical regions. Other environmental challenges may further impact insect fitness traits. In the current study, insect fecundity, egg viability, egg-to-adult developmental time, adult longevity and genome size of four cowpea bruchid strains were evaluated. Such information will help us develop methods to control this important pest.

The life history parameters revealed high fecundity in strain BF and rapid growth in strain SI. Equally notable is the drastically slow development in strain OH. The measured difference in genome size

Table 4
Estimated genome sizes (mean ± SE) of four strains of cowpea bruchids.

Insect strain	♀		♂	
	Tested number	Genome size (Mb)	Tested number	Genome size (Mb)
SI	12	1239.6 ± 2.6 a	12	1204.1 ± 2.6 a *
BF	10	1235.0 ± 2.6 ab	10	1206.8 ± 3.3 a *
CmNnC	11	1235.9 ± 3.0 ab	9	1205.0 ± 1.9 a *
OH	12	1226.9 ± 3.5 b	11	1200.9 ± 2.2 a *

Means followed by the same letter in a column were not significantly different by Tukey's multiple range test ($P < 0.05$). Asterisks indicate significant differences between female and male in the same strain by independent samples' Student's t test ($P < 0.05$).

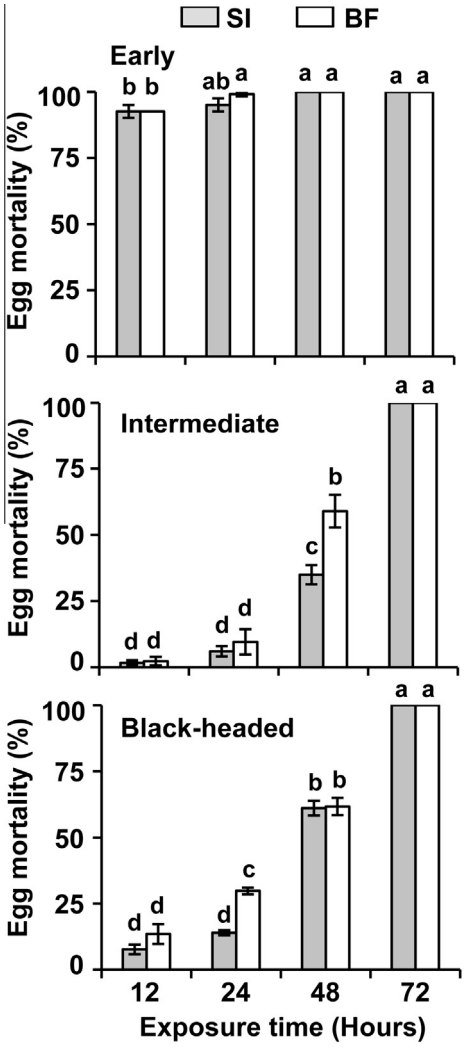

Fig. 1. Egg mortality of strains SI and BF exposed to 1% O_2 + 99% N_2. Bars represent the mean ± SE. One-way ANOVA results for early, intermediate and blackhead stages of eggs were $F = 9.39$, df = 7, $P < 0.001$; $F = 128.13$, df = 7, $P < 0.001$ and $F = 303.10$, df = 7, $P < 0.001$, respectively. Values followed by the different small letters were significantly different by Tukey's multiple range test ($P < 0.05$).

among strains supports the genetic basis of these trait differences. However, it is unclear whether the apparent superiority of BF and SI under controlled laboratory conditions is also a reflection of their performance in a natural setting. Likewise, it is unknown whether the seemingly inferior strain OH represents a population evolved to best adapt to some other undefined environment.

Apparently, mating negatively affected adult longevity. The cost of reproduction has been extensively studied in many insects (Kotiaho and Simmons, 2003; Messina and Fry, 2003; Onagbola et al., 2007; Sagarra et al., 2002). The trade-off between mating and longevity has also been reported for the cowpea bruchid (Paukku and Kotiaho, 2005). A commonly accepted explanation for the negative impact was that mating may divert limited resources to activities associated with reproduction such as courtship, copulation, egg production and sperm donation, thereby reducing resources available for somatic maintenance (Clutton-Brock and Langley, 1997; Kotiaho and Simmons, 2003; Onagbola et al., 2007; Paukku and Kotiaho, 2005). However, multiple mating in cowpea bruchids allowed to mate once with a single virgin male every two days has been shown to increase female longevity (Fox, 1993). It was speculated that during copulation, cowpea bruchid females may have received nutrients from male ejaculate

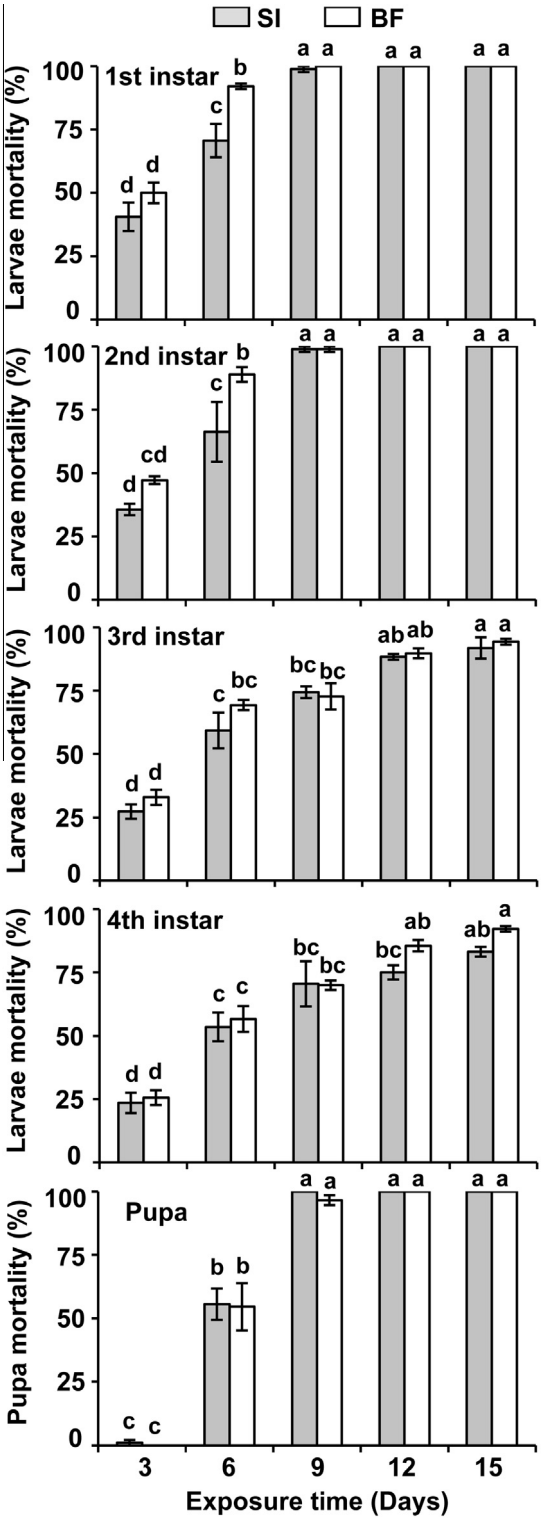

Fig. 2. Larval and pupal mortality of strains SI and BF exposed to 1% O_2 + 99% N_2. Bars represent the mean ± SE. One-way ANOVA results for larvae and pupae were df = 9, $F = 87.19$, $P < 0.001$ (1st instar); df = 9, $F = 52.67$, $P < 0.001$ (2nd instar); df = 9, $F = 27.03$, $P < 0.001$ (3rd instar); df = 9, $F = 31.64$, $P < 0.001$ (4th instar); and df = 9, $F = 176.02$, $P < 0.001$ (pupae), respectively. Values followed by different small letters were significantly different by Tukey's multiple range test ($P < 0.05$).

composed of nutritious liquid and more abundant in virgin males than non-virgin (Savalli and Fox, 1999; Paukku and Kotiaho, 2005). It is likely that the net cost-benefit outcome determines whether mating will compromise longevity. Following this idea, because mated females in our experimental setting were confined with males throughout their lives, the female adults were constantly harassed by their male partners, while receiving reduced ejaculate-derived nutrients, resulting in shorter longevity compared to unmated insects. Furthermore, adult lifespan was longer for females than for males, possibly due to more food consumption by the female larvae (Credland and Dick, 1987). Interestingly, insect strain had a significant effect on longevity of unmated but not mated adults, suggesting different resource acquisition during larval stages. This strain-associated impact, however, was masked by mating activity.

Significant differences in genome size were detected in female adults between strains SI and OH, and between male and female within all strains. It is postulated that increased genome size causes increased cell size and thus body size both between and among species (Hessen et al., 2013). Positive association of genome size and body size has been reported in invertebrates and other plants and animals (Finston et al., 1995; Rasch and Wyngaard, 2006; Hessen et al., 2013). Our experimental data provided further supportive evidence. Differences in genome size may result from duplications, deletions and inversions in coding and noncoding portions of the genome, copy number variations, as well as other differences between strains presumably necessary for adaptation to specific environments. Modification of genomic architecture could in turn affect insect life history (Finston et al., 1995; Hessen et al., 2013; Ellis et al., 2014; Huang et al., 2014). Our data showing the rapid development of strain SI and slow development of strain OH, however, did not support the general assumption of developmental rate being negatively associated with genome size. Given that the SI and OH strains were collected in India and USA respectively, adaptation to specific local trophic environments of different cowpea bruchid strains likely accounts for the apparent deviation. It has been shown that genome size contributes to life history traits in an environmentally dependent manner (Ellis et al., 2014). For strains BF and CmNnC that have comparable genome sizes, considering that they were originally collected from two African locations that are geographically close, exchanges of genetic material could occur and similar growth and development patterns are expected to be shared by the two strains.

Modified atmosphere has become a widely-adopted technique for control of storage pests. Its potential use as an alternative to synthetic fumigants has also gradually been recognized. As a result, it is important to determine factors influencing its efficacy. We have shown here that bruchid strains can be affected differently by low O_2 in their earlier developmental stages. The differential responses between strains BF and SI could be due to host factors; Strain SI used in this study has been reared on mung beans since

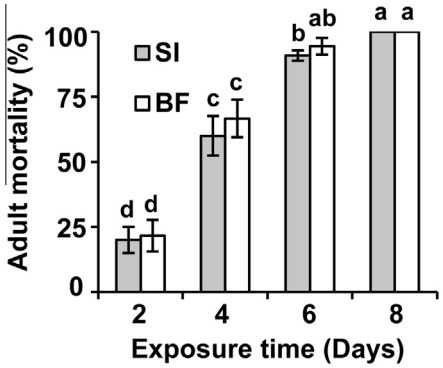

Fig. 3. Adult mortality of strains SI and BF exposed to 1% O_2 + 99% N_2. Bars represent the mean ± SE. One-way ANOVA was used for data analyses (df = 7, $F = 47.92$, $P < 0.001$). Values followed by different small letters were significantly different by Tukey's multiple range test ($P < 0.05$).

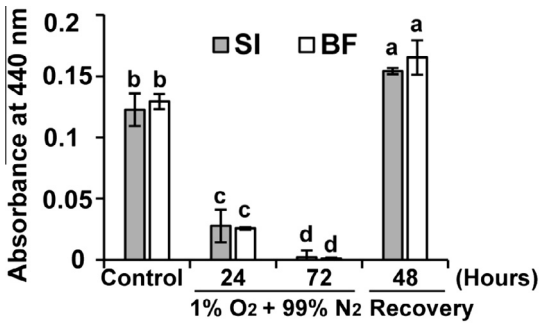

Fig. 4. Proteolytic activity of strains SI and BF in response to 24 and 72 h hypoxic (1% O_2 + 99% N_2) and subsequent 48 h normoxic treatments. Midguts of treated cowpea bruchids were collected as described in Section 2, and total midgut proteolytic activity was determined by azocasein hydrolysis. Data were analyzed using one-way ANOVA (df = 7, $F = 191.03$, $P < 0.001$). Values followed by different letters were significantly different by Tukey's multiple range test ($P < 0.05$).

collection, and thus was more adapted to nutrient and defensive compounds in this legume than was the BF strain. For the latter, the host transfer (cowpea to mung bean for two generations) may represent a further stress imposed on the insect in addition to hypoxia. On the other hand, it is interesting to speculate that the larger (despite statistically insignificant) genome in strain SI implies higher energy reserves, and this may have given strain SI an advantage over BF, enabling them to cope better with O_2 deprivation.

This environmentally friendly storage technique to control storage pests has been challenged by insects' development of tolerance to low O_2 atmospheres. We have previously demonstrated that cowpea bruchid strain CmNnC is highly tolerant to hypoxia particularly at the late larval stage (Cheng et al., 2012). Midgut digestive protease activity indicates that the bruchids endure hypoxia by suppressing metabolism thus decreasing the demand for ATP. Once normoxia resumes, they are able to recover their metabolic activity to prior levels. In the current study, we observed such tolerance in cowpea bruchid strains SI and BF.

The readiness to alter metabolic activity according to environmental variation is presumably most closely associated with insects' hypoxic habitat, although these geographic strains showed small but significant differential tolerance to hypoxia. Lack of O_2 has been shown to cause higher mortality in the pesticide-resistant *T. castaneum* strain than in regular laboratory susceptible strains (Jay and Pearman, 1971), suggesting that modified atmospheres may have higher anti-insect efficacy when used in combination with other defense mechanisms.

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