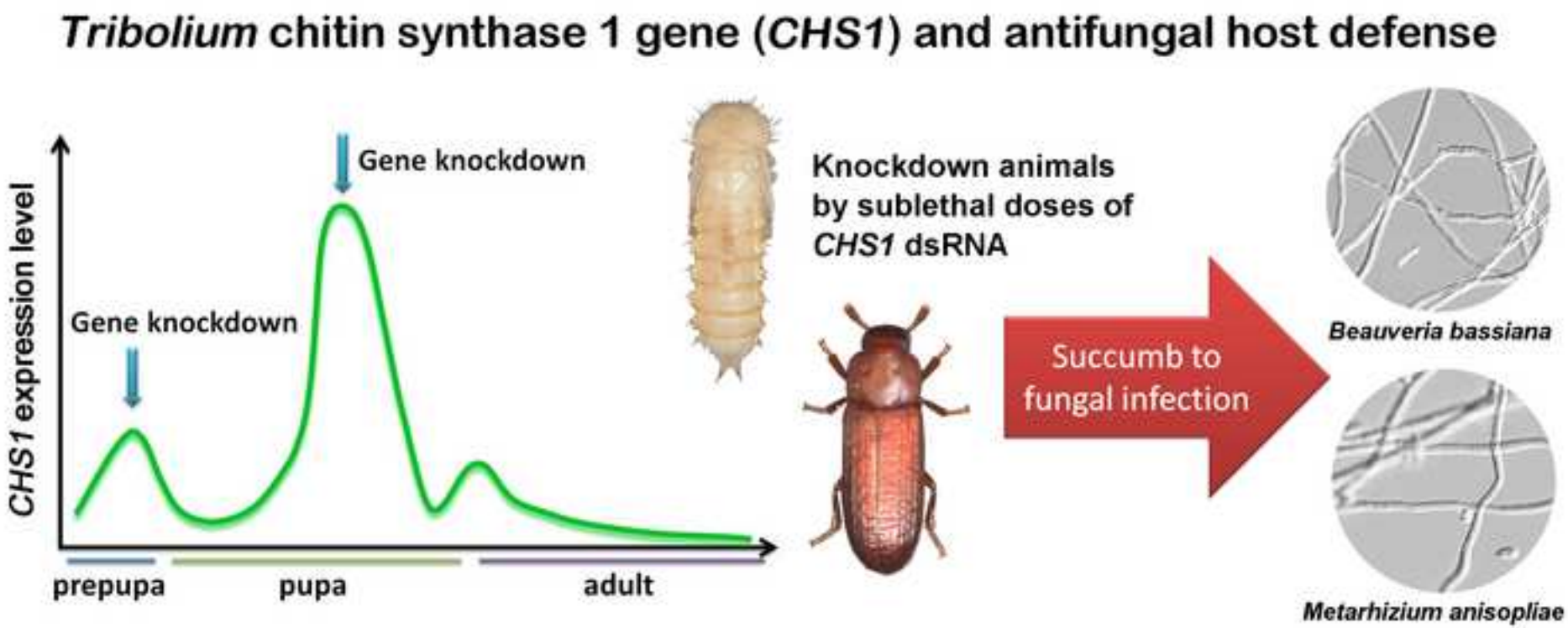
*Graphical Abstract



Highlights

·Both *Beauveria bassiana* and *Metarhizium anisopliae* can infect *Tribolium castaneum*.

·*M. anisopliae* exhibits higher infectivity to *T. castaneum* than *B. bassiana*.

·Adult beetles show higher refractoriness to fungal infection via the natural route.

·Intrahemocoelic inoculation of the two fungi is extremely virulent in *T. castaneum*.

·Knockdown of *chitin synthase 1* compromises significantly antifungal host defense.

Chitin synthase 1 gene is crucial to antifungal host defense of the model beetle, *Tribolium castaneum*.

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Abbreviations: AMP, antimicrobial peptide; PO, phenoloxidase; RNAi, RNA interference; CHS1, chitin synthase 1; qRT-PCR, real-time quantitative RT-PCR; RPL32, ribosomal protein L32; dsRNA, double strand RNA; malE, maltose binding protein E; GlcNAc, *N*-acetylglucosamine.

Abstract

The importance of the insect cuticle as a primary protective barrier against entomopathogens has long been noted. In the present study, we addressed this issue by utilizing an experimental infection system composed of the model beetle *T. castaneum* and two entomopathogenic fungal species, *Beauveria bassiana* and *Metarhizium anisopliae*. The pupae were relatively susceptible to these fungi by the natural route of infection, with some refractoriness developed with age, while the adults exhibited much higher refractoriness. Whereas *M. anisopliae* exhibited seemingly higher infectivity to the pupae compared to *B. bassiana* when the natural conidium infection was employed, direct inoculation of cultured hyphal body cells into the hemocoel was found highly and equally virulent in the pupae for the both fungal species. These results collectively suggest an important role of the cuticular integument in antifungal host defense, and we subsequently conducted the knockdown of *chitin synthase 1* gene (*CHS1*). We targeted the prepupal and mid-pupal peaks of the mRNA respectively by using injection of the dsRNA at very low dosages to avoid lethality. The resulting pupae looked normal, but the adults showed a mild phenotype with dimpled/wrinkled elytra. The *CHS1* gene knockdown compromised significantly host defense against the fungal infection via the natural route, except the configuration of knockdown pupae and *M. anisopliae*, suggesting an indispensable role of *CHS1*.

Key words: *Tribolium castaneum*, *Beauveria bassiana*, *Metarhizium anisopliae*, innate immunity, insect cuticle, chitin synthase.

1. Introduction

The insect immune system, which lacks antigen receptors with clonality, instead solely utilizes germline-encoded pattern recognition receptors for non-self recognition (Hultmark, 2003; Janeway and Medzhitov, 2002). The insect innate immune system involves well-suited cellular and humoral immune responses that function in coordination in host defense against several different pathogens (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007; Strand, 2008). Among these innate immune responses, the massive induction of antimicrobial peptide (AMP) genes represents one of the hallmarks of the insect immune system (Ferrandon et al., 2007). The intensive researches in this particular field using *Drosophila melanogaster* have identified two important immune signaling pathways, namely the Toll and the IMD pathways (Kleino and Silverman, 2014; Valanne et al., 2011), and have contributed greatly to our understanding of innate immunity of insect as well as vertebrates (Akira et al., 2006; Lemaitre and Hoffmann, 2007; Lemaitre et al., 1996). In addition, the melanization reaction catalyzed by pathogen- or wound-activated phenoloxidase (PO), which eventually results in melanin deposition around foreign bodies, is another critical constituent of insect immunity (Cerenius et al., 2010; Cerenius et al., 2008).

Those studies that have delineated the mechanistic aspects of the immune pathways or cascades often utilizes opportunistic pathogens of humans as model pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* or *Aspergillus fumigatus*, or purified pathogen-associated molecular patterns, and such elicitors are directly introduced into the insect hemocoel via injection or pricking. Meanwhile, our knowledge on the interplay between insects and natural entomopathogens are thus relatively limited. Fungi are the commonest entomopathogens, which as well offer an environment-friendly means of insect pest control

(Butt et al., 2016; Lu and St Leger, 2016). The majority of commercially available fungal biopesticides is based on species belonging to genera *Beauveria* or *Metarhizium*, and among them, *Beauveria bassiana* and *Metarhizium anisopliae* represent the fungal species widely used for practical applications as well as basic insect immunity studies. Entomopathogenic fungi exhibit a characteristic infection life cycle and can infect even the non-feeding stages of insect hosts such as pupae or ones during diapause, by penetrating directly the integument (Butt et al., 2016; Thomas and Read, 2007). The conidia adhere to the surface of host insects envelope (outer epicuticle) or waxy layer through hydrophobic interaction (Holder and Keyhani, 2005). The conidia germinate, and most species then differentiate infection structures, such as appressoria on the host cuticle. The hyphae penetrate inward the cuticle by using both chemical and physical forces, the former of which comprises hydrolytic cleavage of the cuticle constituents by proteases and chitinases (Kang et al., 1999; St Leger et al., 1994), while the latter involves the elevated hydrostatic pressure provided by lipid degradation (Wang and St Leger, 2007). Once the elongating hyphae reach the nutrition-rich host hemocoel, they transform into yeast-like unicellular hyphal bodies. The hyphal bodies colonize and propagate in the hemocoel while secreting toxic secondary metabolites (Amiri-Besheri et al., 2000; Kershaw et al., 1999; Xu et al., 2009). After the host death and the depletion of nutrient resource, the hyphal bodies transform into hyphae and penetrate outward the cuticle of the dead host. The hyphae cover the mycosed cadaver and then sporulate. While entomopathogenic fungi encounter both the cellular and humoral immune responses of host insects during the infection process, the critical importance of the cuticular integument as the first barrier has been noted repeatedly (Lu and St Leger, 2016; St Leger et al., 1994; St Leger et al., 1991).

We have been studying insect immunity in the past few years using the model beetle, *Tribolium castaneum*, which is amenable to RNA interference (RNAi)-mediated gene

knockdown (Tomoyasu et al., 2008). First, we investigated the immune signaling pathways of the model beetle by employing RNAi-based approaches combined with microinjection of opportunistic model microbial pathogens, such as *E. coli*, *Micrococcus luteus* and *Saccharomyces cerevisiae*, and have demonstrated that the Toll and the IMD pathways indeed exist in *T. castaneum*, but the specificity of microbial activation of the pathways is distinctive from that in *Drosophila* (Koyama et al., 2015; Yokoi et al., 2012a; Yokoi et al., 2012b). We established our *Tribolium* experimental system to study insect immunity in the course of these studies, and subsequently performed the functional analyses of *Tribolium* prophenoloxidase genes, with *B. bassiana* introduced in the experimental system as an entomopathogen (Yokoi et al., 2015). In the present study, we employed another entomopathogenic fungal species *M. anisopliae*, and investigated the role of the cuticular integument in host defense against these fungal species. We knocked down modestly to avoid lethality *T. castaneum* chitin synthase 1 gene (*CHS1*), which provides a crucial component of the cuticle, and examined the defense phenotypes of resulting animals.

2. Materials and Methods

2.1. Insect

T. castaneum was reared at 30 ^{o}C as described previously (Yokoi et al., 2012a; Yokoi et al., 2012b). When fungal infection was conducted, animals were transferred to 25 ^{o}C conditions just after the exposure to fungi unless otherwise stated.

2.2. Fungal species used

The *B. bassiana* strain used in this study was isolated by K. K. in Fukui prefecture, Japan (Yokoi et al., 2015). The *M. anisopliae* strain was also isolated by K. K. in Saitama prefecture, Japan (Kamiya et al., will be published elsewhere). The hyphal body cells and conidium suspensions of *B. bassiana* were prepared as described in our previous paper (Yokoi et al., 2015). Those of *M. anisopliae* were prepared in a similar fashion except that PDA was used as a solid medium for this species. See section 2.5 for more detail.

2.3. RNA extraction and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the whole body of *T. castaneum* prepupae, pupae and adults using TRIZOL reagent (Invitrogen) according to the manufacturer's instruction. After confirming that the A260/A280 and A260/A230 ratios of RNA preparations were at least above 1.7 and 2.0 respectively, these preparations were used as templates for 1st strand cDNA synthesis with a PrimeScript RT reagent Kit with gDNA Eraser (TAKARA), which digests contaminating genomic DNA prior to reverse transcription reaction. A primer pair for *ribosomal protein L32* (*RPL32*, GLEAN_06106) spans an exon-intron boundary while that for *CHS1* (AY291475) does not. Sequences of the primers are listed in Table 1. Transcript abundance of the target gene was determined using a SYBR Premix Ex Taq Perfect Real Time Kit Tli RNaseH Plus (TAKARA) and a Thermal Cycler Dice Real Time System (Model TP800, TAKARA). Threshold cycle number for target transcript in each sample was determined based on the second derivative of its primary amplification curve and normalized to that of *RPL32* in the same sample, giving a relative abundance of target transcript. For more details regarding cDNA synthesis and qRT-PCR conditions, refer to our previous papers (Koyama et al., 2015;

Yokoi et al., 2012b). RNA extraction followed by cDNA synthesis and qRT-PCR analysis was done using a pool of three animals, and these experiments were independently repeated three times.

2.4. RNAi

Gene knockdown via RNAi was performed as described previously (Yokoi et al., 2012a; Yokoi et al., 2012b). While we routinely treated animals with 100 ng of double strand RNA (dsRNA) in our previous studies, this dosage causes lethality in the case of *CHS1* dsRNA. Therefore, we injected 0.5 to 1.0 ng dsRNA depending on the developmental stages of animals in order to obtain a mild phenotype. T7 RNA polymerase promoter sequence-tagged primer pairs were used to prepare cDNA templates for dsRNA synthesis with a MEGAscript RNAi Kit (Ambion). Purified dsRNAs were determined spectrophotometrically, diluted in 10 mM Tris-HCl, pH 8.0 and injected into day 0 prepupae or day 1 pupae by using a Nanoject II (Drummond Scientific Company). Animals treated with dsRNA were used for determination of either RNAi efficiencies or defense phenotypes upon fungal infection. The primer pair sequences used for the preparation of *CHS1* dsRNA are shown along with those of a negative control *maltose binding protein E* (*malE*) in Table 1. In addition, appearance of pupae or adults resulting from the mild *CHS1* knockdown were observed and photographed under a microscope (Olympus, model SZX12) along with those of control knockdown animals.

2.5. Survival assay upon fungal infection

T. castaneum pupae or adults were subjected to *B. bassiana* and *M. anisopliae* infection, and afterward, the survival was recorded every 24 h. Two distinct ways of infection were employed, namely immersing animals in conidium suspension, which mimics the natural way of infection (hereafter referred to as the immersion method) or micro-injecting cultured hyphal body cells directly into the hemocoel (the injection method), for both fungal species. The infection procedures for *B. bassiana* were as described in our previous paper (Yokoi et al., 2015). Those for *M. anisopliae* were conducted basically in a similar way but with some minor modifications. *M. anisopliae* conidia were prepared from its fully-grown culture on PDA plates, instead of SDY agar plate for *B. bassiana*. The conidia were collected from dried plates and stored at 4 ^{o}C in the presence of desiccant. Since the *M. anisopliae* conidium stock exhibits a shorter shelf life under these conditions compared to *B. bassiana* one, we usually prepare it every 3 months. The stored conidia were suspended in 0.02% Tween-80 just prior to use, filtered through absorbent cotton, counted on a hemocytometer and diluted. To prepare *M. anisopliae* conidium suspension of 3.0 x 10^{8} cell/ml, 0.1% Tween-80 was used because of a more hydrophobic nature of its conidium surface. Animals were immersed in the conidium suspensions for 1 min, put on filter paper to remove excessive liquid, and place in 12-well culture plates. The plates were put in a moist container at 25 ^{o}C, and the survival of animals observed every 24 h. Life and death decisions of animals were performed by observing the responses when animals were stimulated by a thin and flexible plastic rod under a stereoscopic microscope (Olympus, model SZX12 and SZX16). Data were drawn in Kaplan-Meier plots, and *P*-values calculated by Gehan-Breslow-Wilcoxon test by utilizing a commercial software package (Ekuseru-Toukei 2010, Social Survey Research Information Co., Ltd.).

Hyphal body cells of the both fungi were prepared by inoculating a piece of hyphae into SDY liquid media from a SDY or PDA slant stored at 4 ^{o}C. After shaking culture at 25 ^{o}C

for two (for *B. bassiana*) or three days (for *M. anisopliae*), culture media were filtered through absorbent cotton, and the filtrate centrifuged. The hyphal body pellet was washed twice in PBS, counted on a hemocytometer and diluted in PBS. Animals were microinjected with hyphal body suspensions or vehicle PBS, and thereafter processed as described above for the immersion method.

3. Results

3.1. Pupal-adult development and refractoriness to B. bassiana infection

Indeed, natural populations of *T. castaneum* in Pakistan are reportedly infected with several fungal species including *B. bassiana* and *M. metarhizium* (Wakil et al., 2014). We have also demonstrated that *B. bassiana* can infect and kill *T. castaneum* in our previous study (Yokoi et al., 2015). While in that study we used day 3 pupae, here we wanted to know whether the refractoriness to the infection changes during the course of development. Thus, we conducted infection experiments with early and middle stage pupae as well as early adults by the immersion method using relatively high concentration of *B. bassiana* conidium suspension (Fig. 1). As shown here, while day 1 (on the next day of pupation) and day 2 pupae totally succumbed to *B. bassiana* infection by 96 h (Fig. 1A and Fig. 1B), day 3 pupae seemed to develop refractoriness to some extent (Fig. 1C). Moreover, day 1 adult beetles on the next day of emergence exhibited complete refractoriness to *B. bassiana* infection via the immersion method under the same conditions (Fig. 1D). These results suggest that the refractoriness of *T. castaneum* to the fungal infection varies by the developmental stages as well as by the age in days of the same developmental stage.

3.2. Survival of day 3 pupa after B. bassiana or M. anisopliae infection

Here, day 3 pupae were employed, and dose-response studies were conducted for both the immersion and injection methods. In these experiments, we used another entomopathogenic fungus *M. anisopliae* besides *B. bassiana*. The changes of survival rates were recorded for the respective infection methods, and the results are illustrated in Fig. 2 and Fig. 3. Fig. 2 shows survival curves of pupae infected with either *B. bassiana* (A) or *M. anisopliae* (B) by the immersion method. Under the conditions that we employed *M. anisopliae* exhibited apparently higher infectivity to *T. castaneum* pupae, with 61% survival by 168 h at the concentration of 1.0×10^3 conidia/ml (Fig. 2B). As regards *B. bassiana*, a similar but even higher survival rate (70%) was obtained with two orders of magnitude higher concentration of 1.0×10^5 conidia/ml (Fig. 2A).

The survival of day 3 pupae was also examined after the infection by injecting directly hyphal body cells of the both fungal species (Fig. 3). Since the injection method can skip germination and cuticle penetration steps, it kills animals faster than the natural infection via the immersion method. We used hyphal bodies in the range of 5 to 500 cells in these tests, and found that both *B. bassiana* and *M. anisopliae* were extremely virulent in *T. castaneum* when introduced directly in the hemocoel. Of note is that as small as five hyphal body cells were able to kill c.a. 80% of individuals by 72 h in both cases of *B. bassiana* and *M. anisopliae*, suggesting a crucial role of the initial barrier provided by the cuticular integument.

3.3. Changes of transcript abundance of CHS1 during prepupal, pupal and adult development

The *CHS1* gene product represents a crucial component for cuticle formation and its integrity. Two distinct chitin synthase genes, namely *CHS1* and *CHS2* are encoded in the genome of *T. castaneum* (Arakane et al., 2004). Among them the product of *CHS1* is responsible for the chitin synthesis in the cuticle while that of *CHS2* is for peritrophic membrane synthesis in the midgut (Arakane et al., 2005). The results described in the above section implied an important role of healthy cuticle in antifungal host defense of this model beetle. Thus, we first examined the developmental changes of *CHS1* transcript by qRT-PCR (Fig. 4). The transcript had a peak on day 1 prepupae. This was followed by relatively low levels in early pupae, and then peaked again on day 3. The *CHS1* transcript consistently decreased after adult emergence, with a low level peak on day 0, and nearly disappeared by day 14. We postulated that the prepupal peak on day 1 was responsible for the formation of pupal cuticle, while the pupal peak on day 3 was for adult cuticle formation.

3.4. Knockdown of CHS1 and phenotypes of pupae and adults

We generally perform gene knockdown via RNAi by injecting 100 ng dsRNA in this model beetle irrespective of developmental stages. Since severe knockdown of vital genes, such as *CHS1* (Arakane et al., 2005), *IAP1* (*inhibitor of apoptosis protein 1*) and *actin* (our unpublished results) causes lethality in *T. castaneum*, here we used 0.5 and 1.0 ng dsRNA per prepupa and pupa, respectively to obtain mild phenotypes. To target day 1 prepupal peak or day 3 pupal peak of *CHS1* mRNA, day 0 prepupae or day 1 pupae were treated with above-mentioned doses of the dsRNA, and the knockdown efficiency was respectively estimated in day 1 prepupae or day 3 pupa (Fig. 5). The knockdown efficiency of *CHS1* in day 1 prepupae at 24 h after the injection of 0.5 ng dsRNA was found to be 51.5%, and that in

day 3 pupae at 48 h after 1.0 ng dsRNA injection was 42.3 %. Under these conditions, dsRNA-treated prepupae developed normally to pupae and adults. This held true for dsRNA-treated pupae as well, and the pupae metamorphosed to adults in a normal fashion. These adults, irrespective of the time point of dsRNA treatment, seemed to be vital and have a similar longevity to control ones (data now shown). We photographed the appearances of *CHS1* dsRNA-treated animals along with those of control, *malE* dsRNA-treated ones (Fig. 6). We were not able to recognize any morphological effect of *CHS1* knockdown under a stereoscopic microscope in day 3 pupae that had been injected with the dsRNA on day 0 of prepupal life. Meanwhile, the knockdown caused a clear phenotype in resulting adults. The elytra of knockdown adults exhibited dimpled or wrinkled appearance overall. This was recognizable in both dorsal and lateral views shown in Fig. 6, while the exoskeleton of the other portion appeared normal.

3. 5. Antifungal host defense in CHS1 knockdown pupae and adults

First, we investigated the effect of *CHS1* knockdown on host defense of pupae against *B. bassiana* and *M. anisopliae*. Day 3 knockdown pupae prepared as in Fig. 6 were subjected to fungal infection experiments by using the immersion method. The conidium concentration was set to 1.0 x 10^5 and 1.0 x 10^3/ml for *B. bassiana* and *M. anisopliae*, respectively. These doses correspond to ones resulting in 60-70% survival of naïve animals by 168 h post immersion as indicated in Fig. 2. The survival of the knockdown pupae were shown in Fig. 7 in conjunction with that of control. The *CHS1* knockdown clearly and significantly weakened the host defense against *B. bassiana* infection. While the control pupae were relatively resistant to *B. bassiana* infection with a survival rate of 83% at 168 h post conidial immersion,

the knockdown pupae succumbed to *B. bassiana* much faster. The survival rate dropped to 66% by 120 h and that at 168 h was 51%. Interestingly, the knockdown of *CHS1* did not influence the survival of pupae upon *M. anisopliae* infection. The knockdown pupae seemed to die faster than controls as shown in Fig. 7B, but the difference was not statistically significant.

Similar infection and survival experiments were conducted using knockdown adults. Day 1 pupae were injected with sublethal dose of *CHS1* dsRNA, and resulting adults were examined in terms of their defense phenotypes against *B. bassiana* and *M. anisopliae* by using the immersion method. As exemplified in Fig. 1D, the adult beetles are highly refractory to fungal infection. Therefore, we employed much higher conidial concentrations compared to the cases of pupae in these experiments, namely 1.0×10^9 and 3.0×10^8 conidium/ml for *B. bassiana* and *M. anisopliae*, respectively. Control adults were basically resistant to the two fungal species at respective dosages, while with 7% dead adults at 168 h post *M. anisopliae* infection (Fig. 8). The *CHS1* knockdown brought about conspicuous outcomes in the case of *B. bassiana* infection. The survival faction decreased to 77% by 96 h, and at 168 h post conidial immersion it dropped to 50% (Fig. 8A). Similar but somewhat moderate results were obtained by the *CHS1* knockdown upon *M. anisopliae* infection, with 74% survival at 168 h post conidial immersion (Fig. 8B).

4. Discussion

The cuticular integument provides insects a protective barrier against several life-threatening factors, such as a dry climate, predators, and microbial pathogens (Butt et al., 2016; Lu and St Leger, 2016; Moussian, 2010). The insect cuticle, which is secreted by the

epidermis and covers the entire body surface as well as the fore- and hindgut and trachea, comprises a few structurally distinct layers (Moussian, 2010). The outermost envelope is a lipid-rich hydrophobic layer facing the surrounding environment. The protein-rich epicuticle is assembled underneath the envelope. These two non-chitinous layers are lined by the innermost, much thicker procuticle that is composed mainly of chitin and cuticular proteins. Chitin is a β-1, 4-linked homopolymer of *N*-acetylglucosamine (GlcNAc) and widely found in fungi, nematodes and arthropods as a structural component that supports cells or body surfaces (Merzendorfer, 2006). Since the well-regulated chitin synthesis/degradation is essential to insect development and metamorphosis, the synthetic pathway of chitin offers an attractive target of the insect growth regulator class pesticide development (Merzendorfer, 2013). The key enzyme of the pathway is chitin synthase, an integral membrane protein that adds consecutively the sugar moiety of UDP-GlcNAc to the non-reducing end of elongating chitin chain. The microfibrils composed of chitin chains in the extracellular space provide a scaffold to assemble another major component, cuticular proteins, giving the composite procuticle layer via noncovalent or covalent bonds among the cuticle constituents with the help of oxidative enzymes. These oxidative processes, namely cuticle sclerotization and melanization, occur in all the cuticle layers, and the cuticular integument is thereby hardened, pigmented, and stabilized (Andersen, 2010; Merzendorfer, 2006; Moussian, 2010).

In the present study, we first investigated whether the susceptibility to entomopathogenic fungi changes depending on developmental stages, or ages of the same developmental stage of the model beetle, *T castaneum* when we employed the immersion method that mimics the natural route of *B. bassiana* infection. The results were that while the adult beetles were totally resistant to the fungal infection at the defined concentration of conidium, pupae were relatively susceptible, with increased refractoriness as the pupae

developed. Liu et al. (2014) have recently reported that the susceptibility of a scale insect species to entomopathogenic fungi belonging to genera *Lacanicillium* and *Fusarium* varies depending on the host insect's developmental stages, which differ in the procuticle thickness and deposition of waxy substances. We also considered that the increased refractoriness of aged pupae to *B. bassiana* might reflect the cuticle stabilization with age in days and that the remarkable resistance of adults to the infection might be attributed to their apparently thicker integument.

While the infection processes of entomopathogenic fungi are thought to be counteracted by host immunity-related factors in the integument as well as in the hemocoel, we focused on the former in this study. Thus, to evaluate the contribution of the cuticular integument to antifungal host defense, we employed two different ways of infection, the immersion method and the injection method. The former represents the natural way of fungal infection including the cuticle penetration, which is skipped in the latter method by directly inoculating cultured hyphal body cells into the hemocoel. For these tests, we used *M. anisopliae* in addition to *B. bassiana*. The immersion of day 3 pupae in *B. bassiana* conidium suspension resulted in around 30% mortality by 168 h post immersion at the concentration of 1.0 x 10^5 conidia/ml. *M. anisopliae* exhibited higher infectivity than *B. bassiana* under the conditions that we employed, and about 40% mortality was observed by 168 h at the conidium concentration of 1.0 x 10^3 cells/ml. We note here that conidial virulence of fungi can vary greatly depending on growth conditions, which has been demonstrated for both *B. bassiana* (Ortiz-Urquiza et al., 2016; Safavi et al., 2007) and *M. anisopliae* (Rangel et al., 2008; Shah et al., 2005). Thus, we consider that in this study we cannot do a direct comparison of conidial virulence to the host insect *T. castaneum* between *B. bassiana* and *M. anisopliae*, which were harvested from different growth media. On the other hand, when we employed the direct

injection of hyphal body cells, the both fungal species exhibited strikingly high virulence to the pupae, with only a few cells being sufficient to kill around 80% animals by 72 h post inoculation. These results collectively suggested the antifungal function of the cuticular barrier. Therefore, we then aimed to weaken the cuticle barrier by gene knockdown approaches. As mentioned above, the integrity of the cuticle is supported largely by the lattice made of chitin microfibrils and cross-linked cuticular proteins as well as by secreted enzymes responsible for the cuticle sclerotization/pigmentation. Among them we chose the cuticular chitin as a target and conducted the gene knockdown of *CHS1* that encodes the enzyme responsible for the cuticular chitin synthesis (Arakane et al., 2005).

The developmental expression profile of *CHS1* exhibited a large peak in day 3 pupae as well as a smaller one in day 1 prepupae. We speculated that the prepupal *CHS1* expression peak was prerequisite to the synthesis of the pupal cuticle while the mid-pupal, larger peak was responsible for the formation of the thicker adult cuticle, and targeted these peaks by dsRNA injection. As documented by a pioneering study (Arakane et al., 2005), *CHS1* knockdown with our regular dose of dsRNA (100 ng/animal) caused lethality through the defects in molting and metamorphosis. Therefore, we knocked down the gene only modestly using a much smaller amount of the dsRNA. The injection of 0.5 ng dsRNA in day 0 prepupae decreased the transcript level to one-half in day 1 prepupa, while 1 ng dsRNA administered to day 1 pupae resulted in about 40% reduction of the transcript level in day 3 pupae. The resulting day 3 pupae after prepupal dsRNA administration did not exhibit any recognizable differences in appearance compared to ones given control dsRNA. However, the defense phenotype to *B. bassiana* changed remarkably by this moderate *CHS1* knockdown, and the pupae succumbed much faster than controls to the *B. bassiana* infection by the immersion method. Interestingly, the knockdown did not compromise significantly the defense of the pupae against *M. anisopliae*.

This may be consistent with the results that *M. anisopliae* showed higher infectivity to naïve pupae than *B. bassiana* under the conditions we employed, and may suggest that the pupal cuticle of both knockdown and control pupae are similarly easy to be penetrated by *M. anisopliae*.

The moderate pupal knockdown of *CHS1* brought about a visible change in the adults. The elytra of knockdown adults showed a dimpled/wrinkled appearance. Interestingly, this phenotype associated with the elytra resembles ones obtained after the knockdown of two genes encoding elytra-enriched cuticular proteins, TcCPR27 and TcCRP18 (Arakane et al., 2012). These authors used more (200 ng/animal) dsRNA of respective cuticular protein genes, and the resulting adults have shorter, malformed elytra with deep wrinkles and die in one week after emergence. They discuss the possibility that these not-fully expanded elytra are generated through an excessive cross-linking among remaining cuticular proteins after the substantial reduction in the amount of major constituents, TcCPR27 or TcCRP18. Meanwhile, it is of interest to note that our moderate *CHS1* knockdown with much smaller amount of dsRNA resulted in a similar phenotypic change although the phenotype we obtained was less severe in terms of morphology and viability. We speculate that the stoichiometric changes among insect cuticle constituents could alter the physicochemical properties of resulting cuticular composite, in either a visible or invisible way depending on respective constituents and parts of the body, and that the elytra might be highly vulnerable to this kind of changes and tend to show recognizable phenotypes since they undergo more physical force while expanding just after emergence. The pupal knockdown of *CHS1* weakened significantly the host defense of the resulting adults against the natural infection of the both fungal species, indicating again the importance role of this gene in antifungal host defense.

The naïve or control knockdown adults showed much higher refractoriness to the fungal infection by the immersion method compared to the corresponding pupae. Indeed, most control adults survived after the exposure to 1.0 x 10^9 cells/ml of *B. bassiana* conidia or 3.0 x 10^8 cells/ml of *M. anisopliae* conidia. Apparently thicker adult cuticular integument may somewhat account for this point. In addition, the adults secrete benzoquinone-containing defense substances, which have been reported to impeded fungal germination and growth (Pedrini et al., 2015). Thus, the adult-specific defensive secretion may in part contribute to the elevated refractoriness found in the adults. The differences in susceptibility to fungal infection could also arise from stage-dependent modulation of host immune responses functioning in the hemocoel. Indeed, the infection of these fungi induces *T. castaneum* AMP genes to some extent (our unpublished observation by Kato et al.), and the magnitude and pattern of AMP gene induction by the injection of opportunistic model microbial pathogens do not differ greatly depending on the beetle's developmental stages (our unpublished observation by Kitamoto et al.). In *Drosophila*, some AMPs, such as Cecropins, Drosomycin and Metchnikowin are known to have fungicidal activity, but these AMPs do not always have effect against *M. anisopliae* and especially against *B. bassiana* (Hedengren et al., 1999). Moreover, these fungi are known to evade the immune responses in the host hemocoel by, for example, silencing the expression of protease genes that are needed for the host cuticle penetration but could trigger melanization reaction in the host hemocoel (Freimoser et al., 2005), or covering the hyphal body surface with collagenous proteins to evade the immune recognition by the host hemocytes (Wang and St Leger, 2006). Thus, we prefer the idea that the remarkable refractoriness of the adults to fungal infection depends largely on the adult-specific properties of the cuticle.

In the present study, we demonstrated the indispensable role of *CHS1* in antifungal host defense of both the pupae and adults of *T. castaneum*. At present we do not know exactly

how the modest *CHS1* knockdown influences the characteristics of the cuticle. It might be plausible to consider that subtle stoichiometric changes among major components could alter the physicochemical and immunological properties of the resulting cuticle to a greater extent, through the changes in the lattice structure itself, as well as through those in the settlement of immune-related components, such as PO and related factors (Asano and Ashida, 2001; Yokoi et al., 2015) in this extracellular matrices. More minute analyses on the properties and roles of the insect cuticle from a viewpoint of antifungal host defense will further advance our understanding of the function of barrier epithelia in innate immunity.

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Figure captions

Fig. 1. Developmental changes of refractoriness to *B. bassiana* infection during pupal-adult transition. Ten each of early to mid-stage pupae (day 1 (A), day 2 (B) and day 3 (C)) or day 1 adults (D) were immersed in a suspension of 1.0 x 10^8 *B. bassiana* conidia/ml for 1 min. These pupae or adults were reared at 30 ^{o}C until just before infection experiments. Ten each animals treated with a vehicle, 0.02 % Tween-80, served as controls. Animals were transferred to 25 ^{o}C and high humidity conditions, and the survival recorded thereafter. Results are shown in Kaplan-Meier plots.

Fig. 2. Survival of pupae upon *B. bassiana* and *M. anisopliae* infection through immersion in conidium suspension. Animals reared at 30 ^{o}C were transferred to 25 ^{o}C conditions at pupation, and day 3 pupae subjected to *B. bassiana* (A) or *M. anisopliae* (B) infection through the immersion method. The concentrations of conidia (cell numbers/ml) used for respective infections are as indicated. Animals treated with a vehicle 0.02 % tween-80 served as controls. Animals were then observed every 24 h, and the survival recorded. Ten to fifteen animals were used for experiments with respective conidium concentrations each time, and the experiments repeated independently three times. Survival rates were calculated based on the sum of the three independent experiments. Normal pupal development typically takes eight and a half days at 25 ^{o}C. When animals were undergone infection, adult emergence was observed at 96, 120 and 144 h post infection under 25 ^{o}C conditions. Thus, the survival rates are calculated based on the sum of both surviving pupae and adults.

Fig. 3. Survival of pupae upon *B. bassiana* or *M. anisopliae* infection through hyphal body injection. Animals reared at 30 ^{o}C were transferred to 25 ^{o}C at pupation, and day 3 pupae

subjected to *B. bassiana* (A) or *M. anisopliae* (B) infection through the injection method. The numbers of hyphal body (HB) cells injected are as indicated. Control animals were injected with a vehicle PBS only. Animals were thereafter observed every 24 h, and the survival recorded. The other details are the same as in Fig. 2.

Fig. 4. Developmental changes of *CHS1* transcript from prepupal to adult stages. The *CHS1* transcript levels in prepuae (Pp), pupae (P) and adults (A) were determined by qRT-PCR, and the values normalized to those of *RPL32*. Numerals following developmental symbols, such as 1 in 'Pp1', indicate age in days of respective stages. Total RNA extraction, 1st strand cDNA synthesis and the following qRT-PCR analyses were performed using a pool of three animals each time, and independently repeated three times. Animals were reared at 30 ^{o}C throughout. Each bar represents mean ± S. D of three independent experiments.

Fig. 5. Knockdown efficacy of *CHS1* via dsRNA injection. Day 0 purepupae or day 1 pupae underwent *CHS1* dsRNA injection at a dosage of 0.5 ng or 1.0 ng per animal, respectively. Control animals were treated with *malE* dsRNA at the same dosage. Animals were kept at 30 ^{o}C, and 24 hours (for prepupae) or 48 h (for pupae) later, total RNA was extracted from the whole body of day 1 prepupae (A) or day 3 pupae (B), and the transcript levels determined. Experiments were independently repeated three times with a pool of three animals each time. Bars represent means ± S. D of three independent experiments. *, significantly different from controls ($P < 0.05$ by Student's *t*-test).

Fig. 6. Appearances of animals treated with sublethal doses of *CHS1* dsRNA. Prepupae were treated with 0.5 ng of the dsRNA on day 0, and the appearances of resulting day 3 pupae

are shown. Similarly, pupae were injected with 1.0 ng of *CHS1* dsRNA on day 1, and the resulting adults photographed on day 1. Animals were reared at 30 ^{o}C throughout. Ten each animals were used for respective knockdown experiments, and typical appearances are shown.

Fig. 7. Moderate *CHS1* knockdown and defense phenotype of pupae against fungal infection. Ten to fifteen day 0 prepupae were injected with either *CHS1* or *malE* dsRNA. Animals were reared at 30 ^{o}C until pupation, and then transferred to 25 ^{o}C. Resulting day 3 pupae were subjected to fungal infection by the immersion method, and the survival recorded every 24 h. Experiments were repeated independently three times, and the survival rate calculated based on the sum of the three experiments. Results of *B. bassiana* (A) or *M. anisopliae* (B) infection are shown in Kaplan-Meier plots. Statistically different survival curves are marked with asterisks along with *P*-values.

Fig. 8. Moderate *CHS1* knockdown and defense phenotype of adults against fungal infection. Day 1 pupae were injected with either *CHS1* or *malE* dsRNA. Animals were reared at 30 ^{o}C, and emerged adults were subjected to fungal infection by the immersion method on day 1. Then, animals were transferred to 25 ^{o}C conditions, and the survival of animals monitored every 24 h. The other details are the same as in Fig. 7.

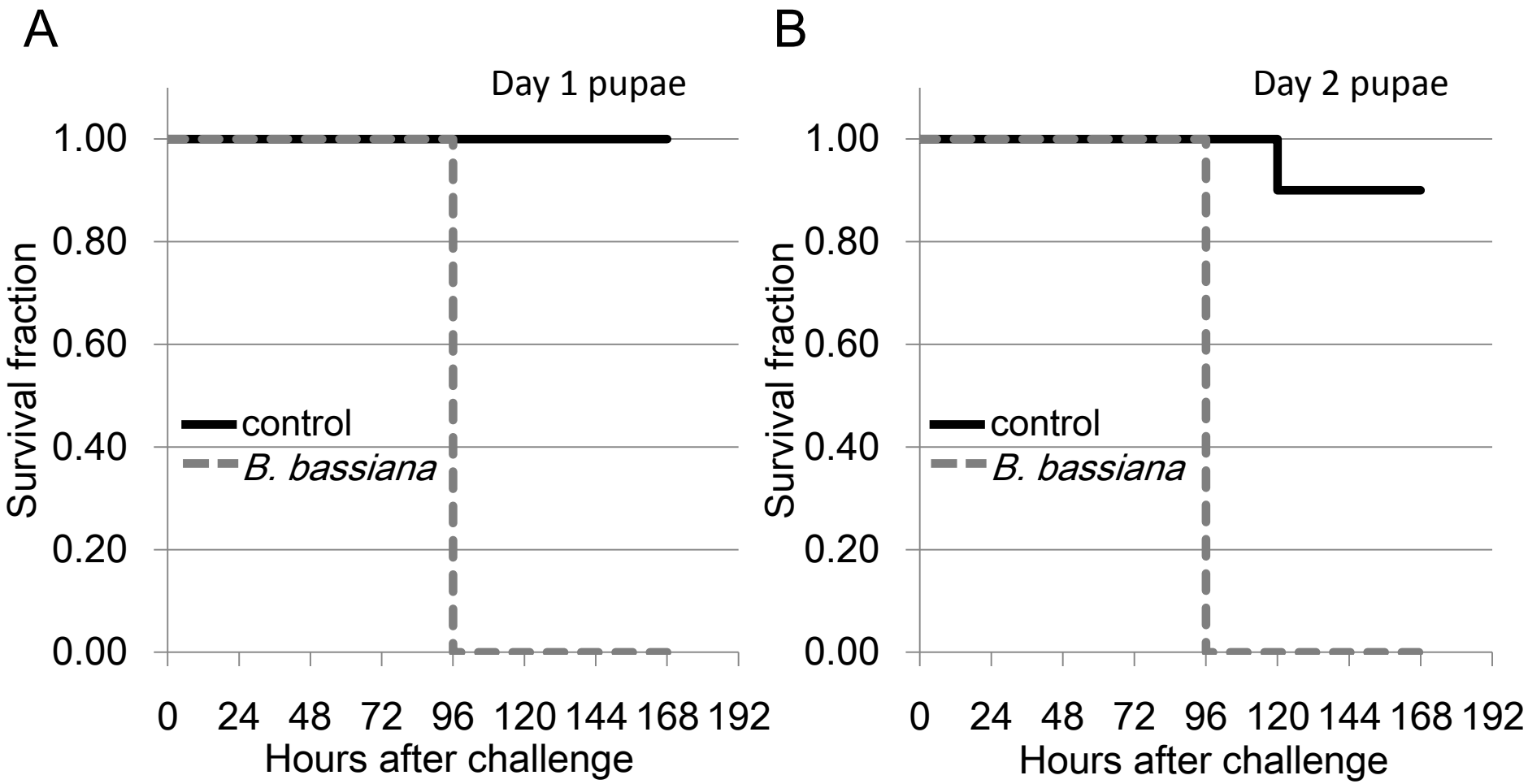
Tables

Table 1. Sequences of primers used in this study

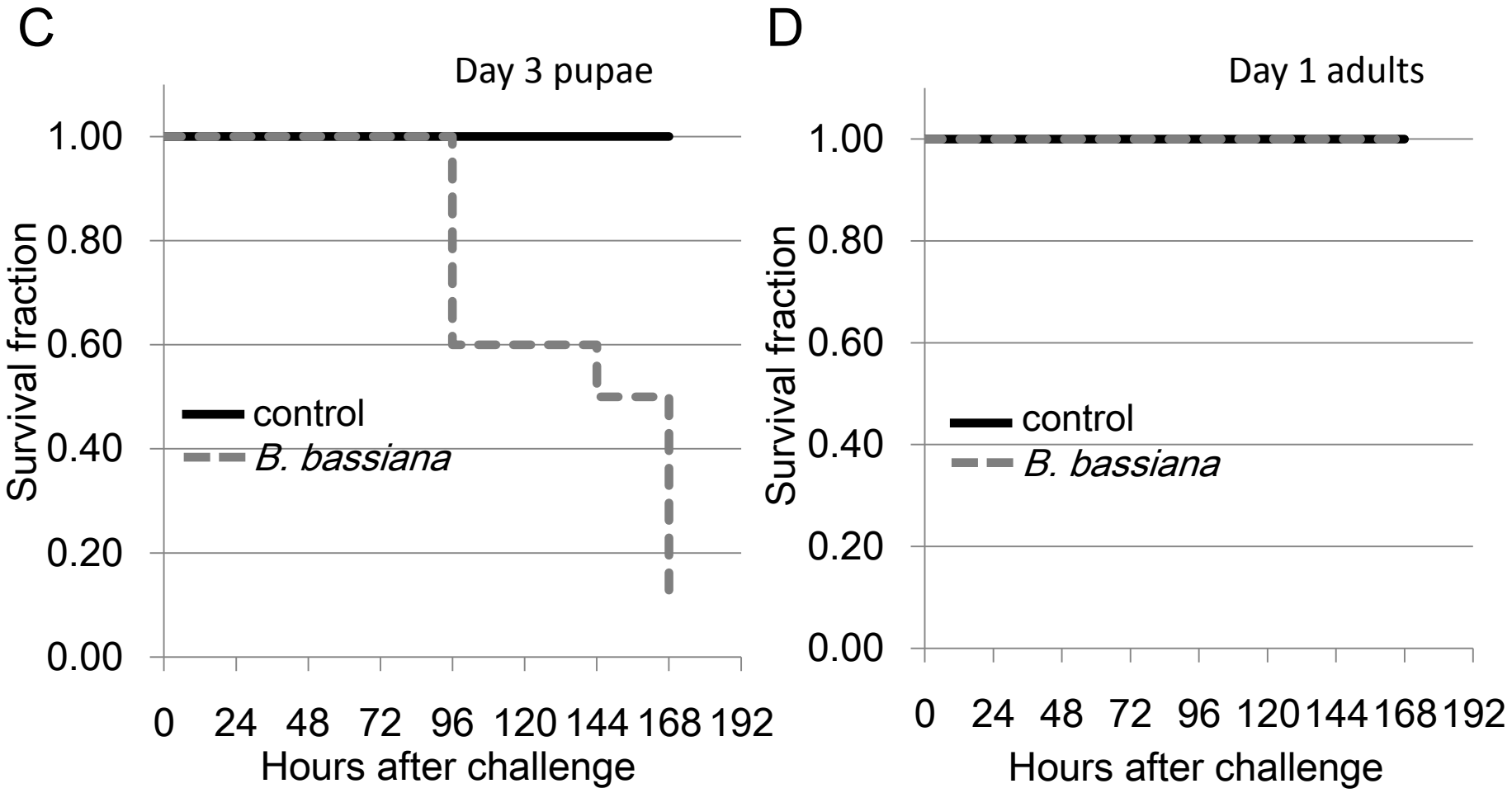
For qRT-PCR		
Gene	Forward (5' to 3')	Reverse (5' to 3')
CHS1	GCGTTTGGCTCGGACTGAGAA	CTCATCCCCTTTCTCCTTCTG
RPL32	ACCGTTATGGCAAACTCAAACG	TGTGCTTCGTTTTGGCATTGGA
For dsRNA synthesis		
Gene	Forward (5' to 3')	Reverse (5' to 3')
CHS1	*TAATACGACTCACTATAGGG* -TCGGCTGGTGGGAGAACTAC	*TAATACGACTCACTATAGGG* -GCCCTGGATCATGATCTTGCA
malE	*TAATACGACTCACTATAGGG* -ATTGCTGCTGACGGGGGTTAT	*TAATACGACTCACTATAGGG* -ATGTTCGGCATGATTTCACCTTT

T7 RNA polymerase promoter sequences are in italic.

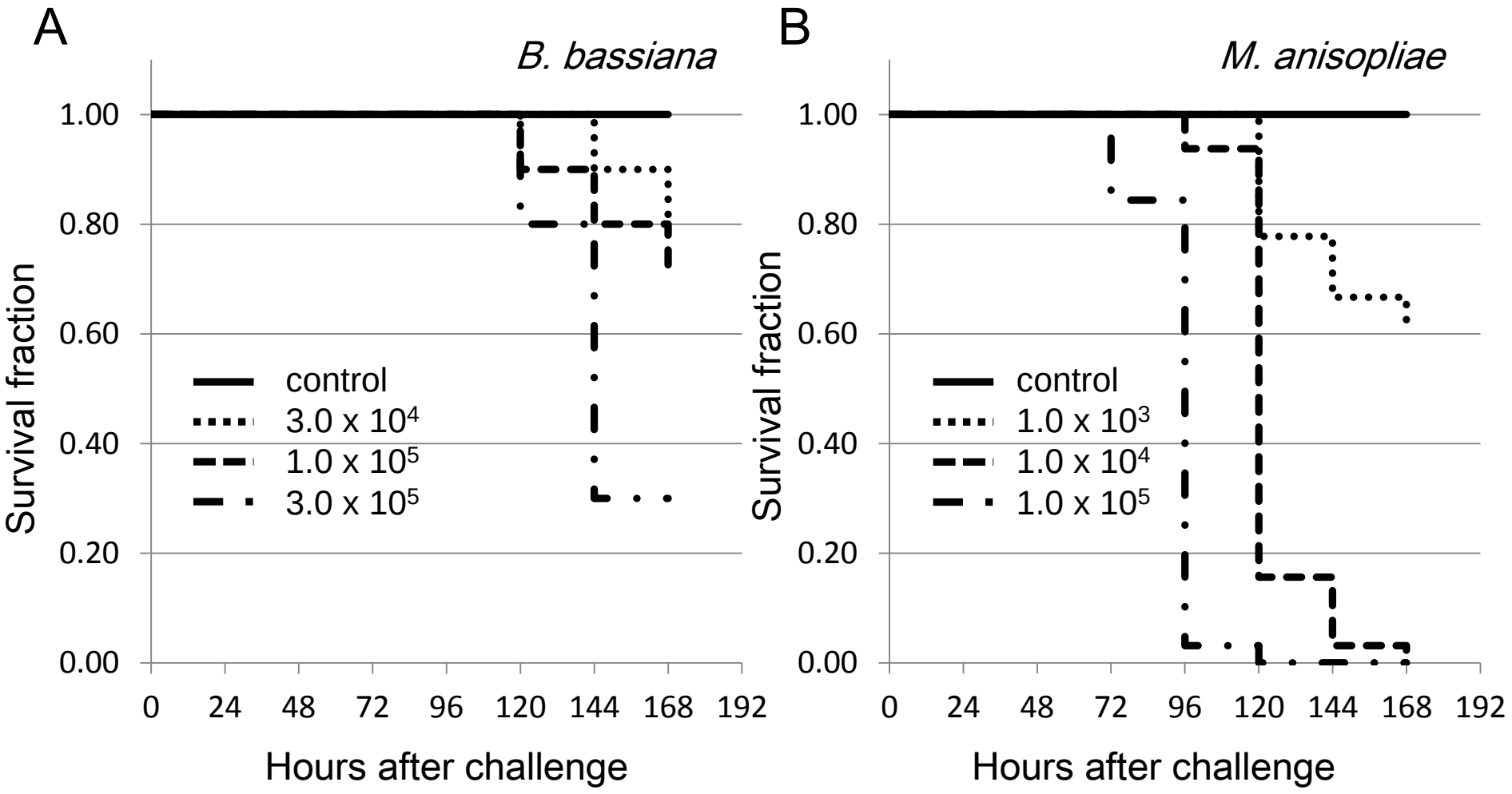
Figure



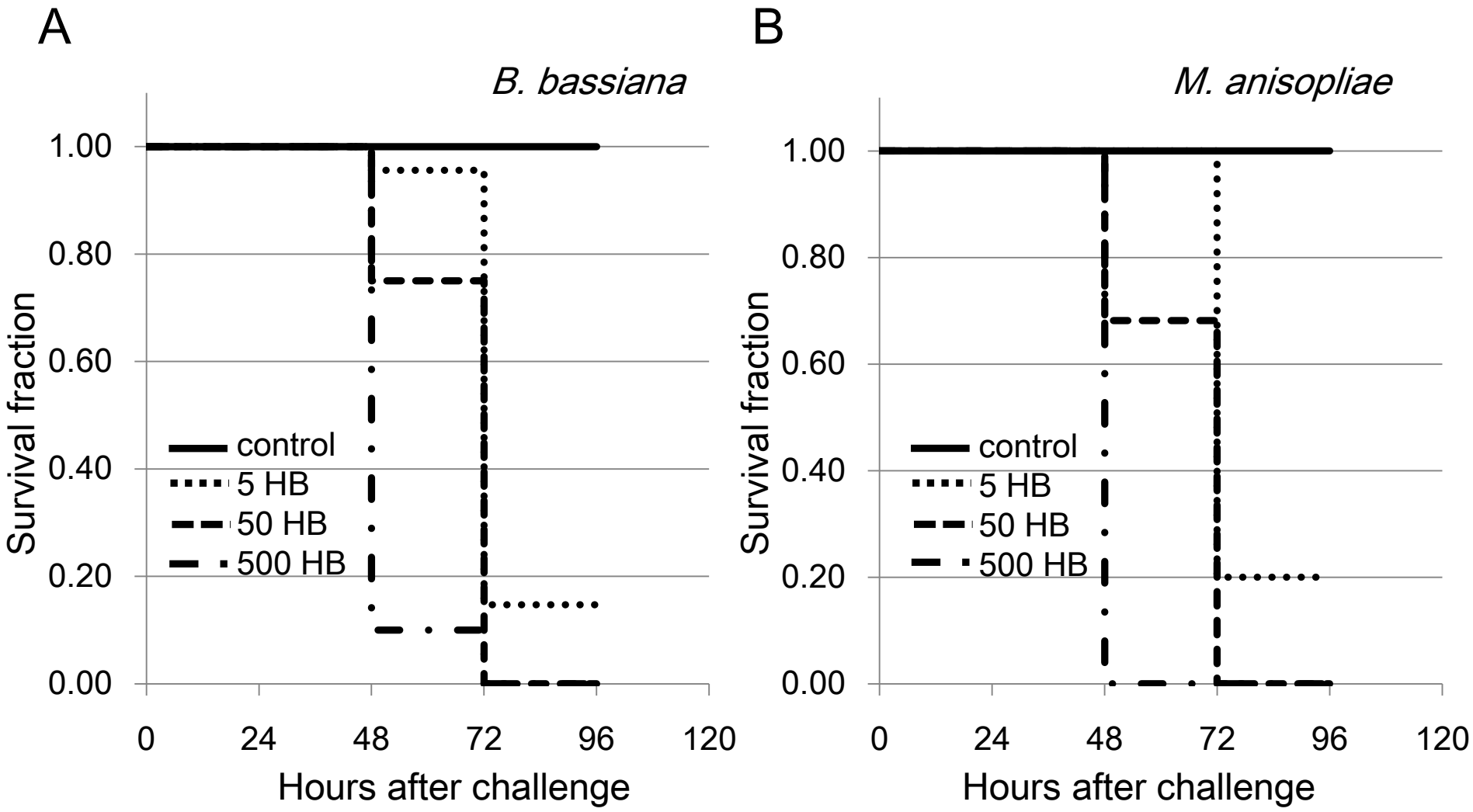
Hayakawa et al., Fig. 1.



Hayakawa et al., Fig. 1 (continued).



Hayakawa et al., Fig. 2.



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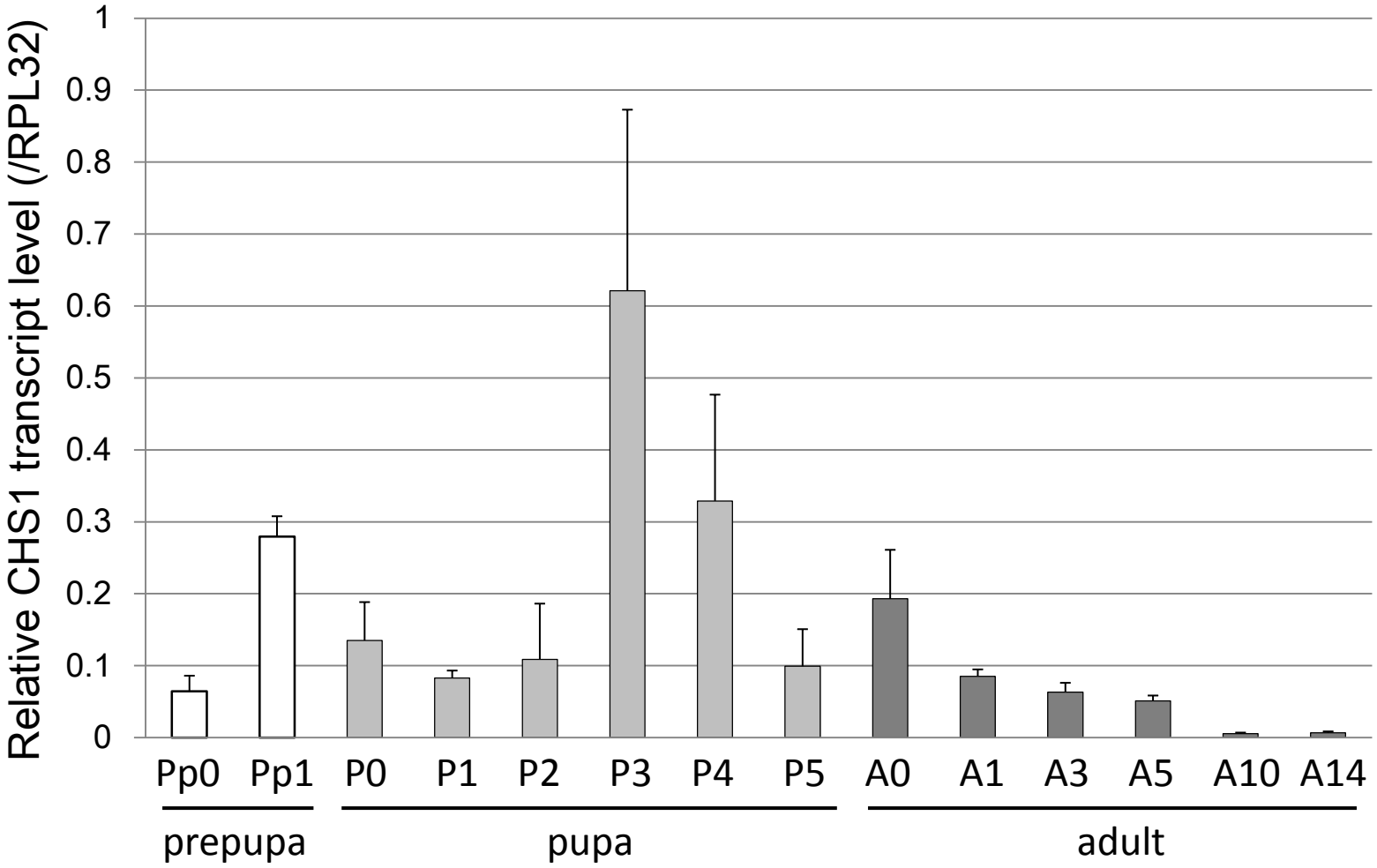
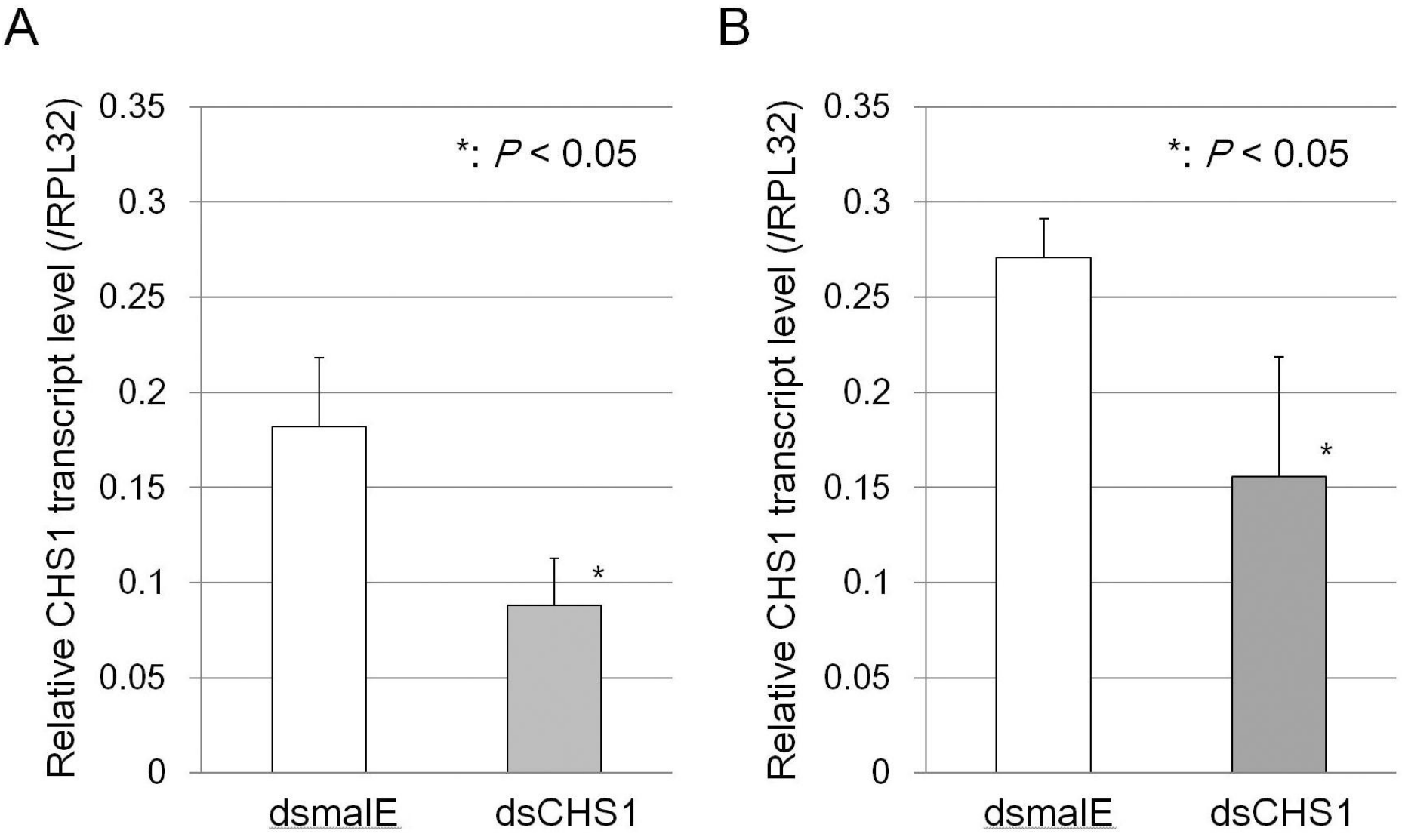
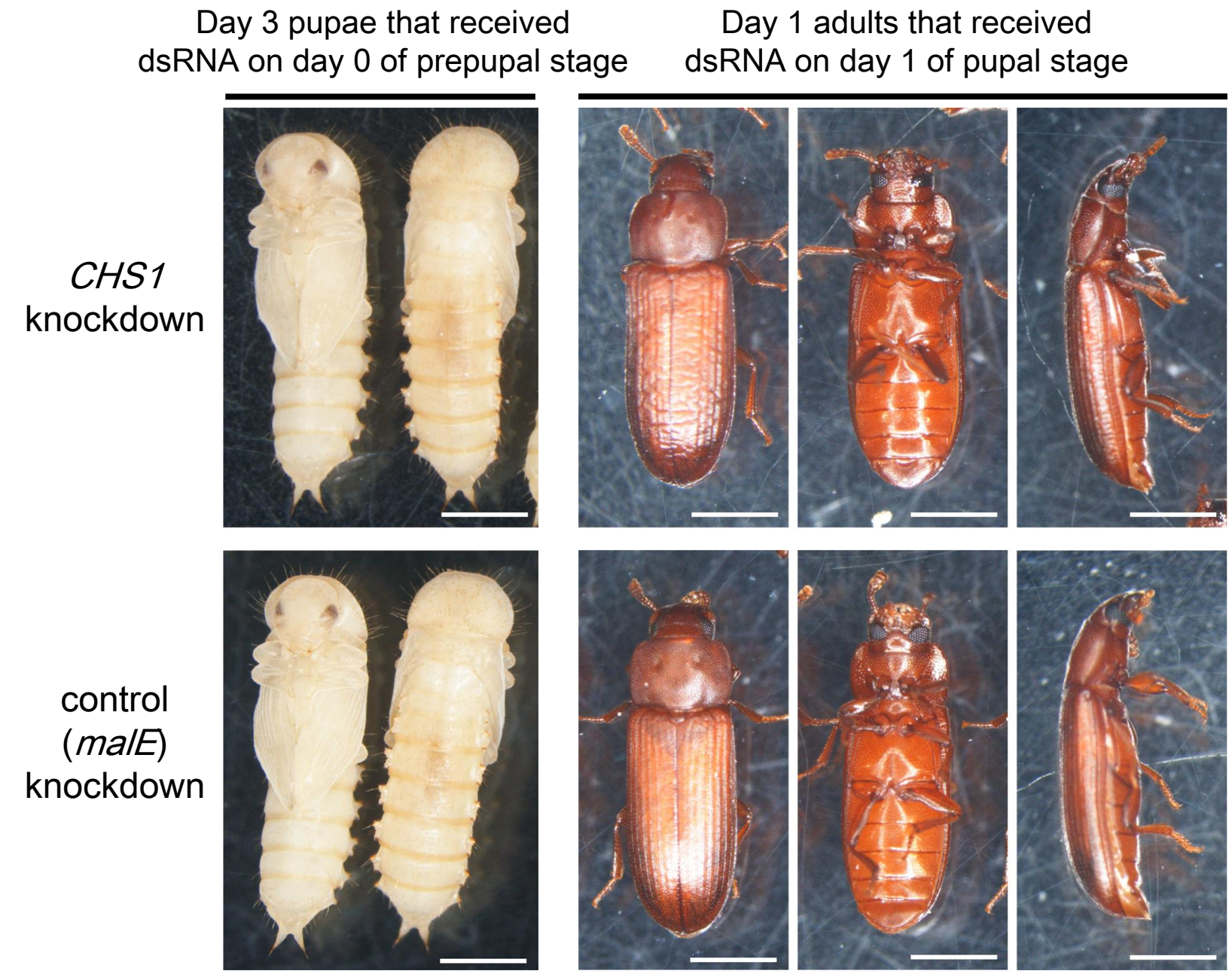


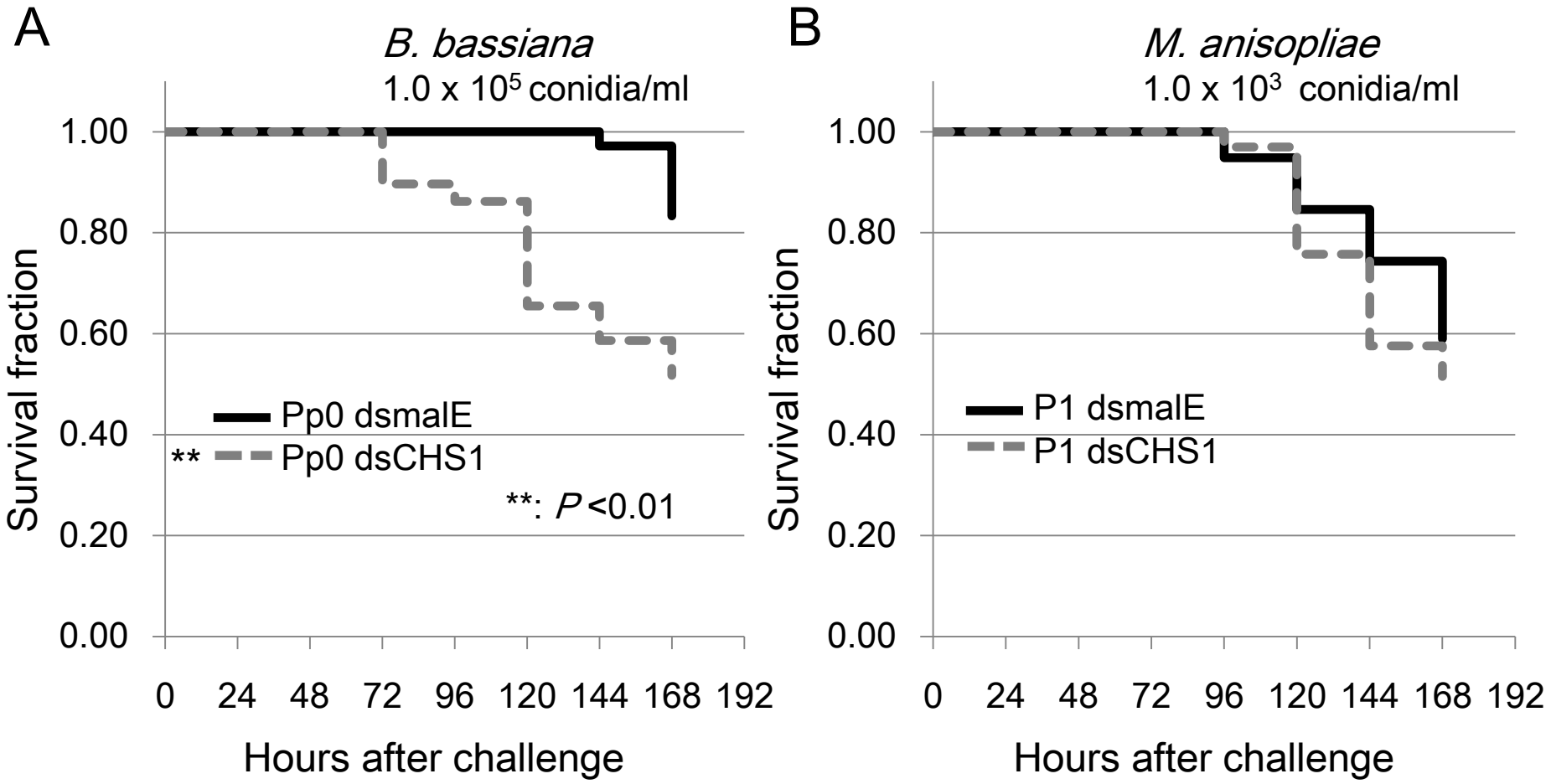
Hayakawa et al., Fig. 4.



Hayakawa et al., Fig. 5.



Hayakawa et al., Fig. 6.



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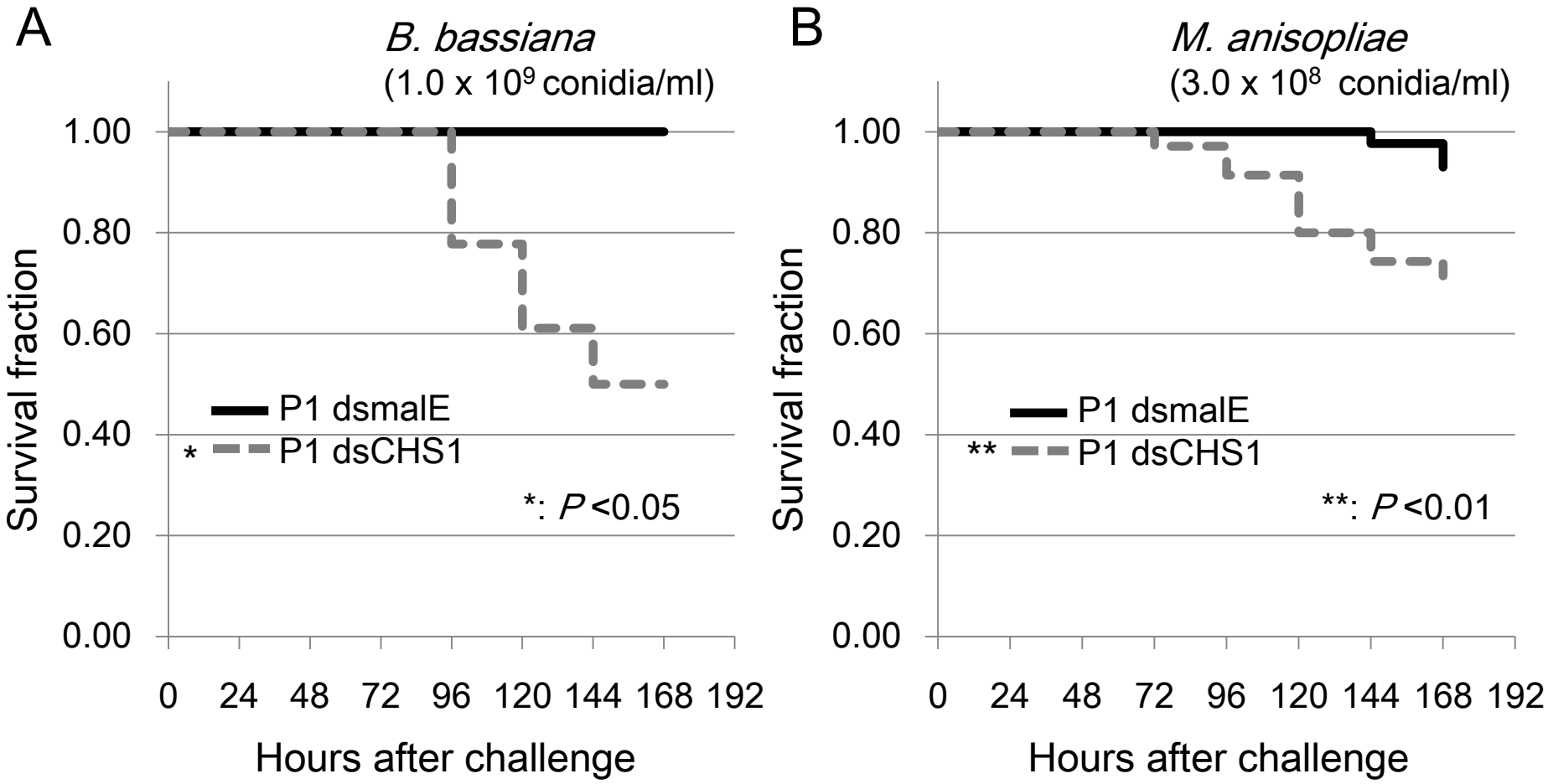

Hayakawa et al., Fig. 8.