Hypoxia adaptation in termites: hypoxic conditions enhance survival and reproductive activity in royals

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Abstract

Termite royals (queen and king) exhibit extraordinary longevity without sacrificing reproductive performance, unlike most animals, in whom lifespan is generally negatively associated with reproduction. Therefore, the regulatory mechanisms underlying longevity have attracted much attention. Although the ageing process is influenced by environmental factors in many insects during their life cycle, it remains unclear whether any factors have an effect on the extended survival and high reproductive capacity of termite royals. Here, we show that hypoxia, possibly an important environmental factor in the nests, enhances survival and reproductive activity in incipient royals of the subterranean termite Reticulitermes speratus compared with those in control conditions. Quantitative real-time PCR analysis revealed that the expression levels of the vitellogenin gene in queens are maintained to a greater extent under hypoxic conditions than under control conditions. The expression levels of the antioxidant enzyme genes RsCAT1 and RsPHGPX are also significantly promoted by hypoxia in queens and kings respectively. These results suggest that hypoxic exposure can contribute in part to achieving high reproductive output by altering gene expression after founding of colonies in the royals. Our study provides novel insights into the effect of a nest environment on the reproductive characteristics in termite royals.

Keywords: ageing, antioxidant, hypoxia, reproduction, termite royals.

Introduction

The mechanisms underlying anti-ageing and longevity in termites have fascinated scientists for centuries. Owing to their extreme longevity and sustained reproduction capacity, termite royals, queen and king, are among the most promising subjects for ageing research (Keller and Jemielity, 2006). Because the royals avoid risky outer-field tasks, such as guarding the nest or foraging activities, their longevity has been explained by the evolutionary theory of ageing based on natural selection, in which the intrinsic lifespan is predicted to be directly related to the level of extrinsic mortality (Keller and Genoud, 1997). Consistent with this theory, queens and kings of the lower termite Zootermopsis nevadensis live for 7 years after colony foundation (Thorne et al., 2002). On the other hand, in most animals a trade-off between longevity and reproduction occurs (Partridge et al., 2005). However, termite royals can live for long periods (Keller and Genoud, 1997) and produce many offspring per day; for instance, 40 000 eggs are produced per day by the termite Macrotermes subhyalinus (Wyss-Huber and Lüscher, 1975). This is a remarkable number, suggesting the presence of an extraordinary anti-ageing mechanism.

Many organisms occupy unusual habitats where they benefit from reduced competition and gain physical shielding from predators. Environmental factors of habitats, such as oxygen (O₂), carbon dioxide (CO₂), temperature and nutrients, have pleiotropic and complex effects on organismal physiology, including the ageing process (Hoback and Stanley, 2001; Fanson et al., 2009; Hazell and Bale, 2011; LeBoeuf et al., 2016). Among these factors, low O₂ (hypoxia) is an especially strong inducer of many physiological processes, such as growth, development and metabolism (Harris, 2002; Cassavaugh and Lounsbury, 2011). Many insect species express adaptations that allow them to spend some proportion of their life...
cycles under hypoxic conditions, thus beneficially utilizing hypoxia (Hoback and Stanley, 2001). Indeed, the alfalfa leafcutting bee Megachile rotundata is naturally exposed to hypoxic conditions during the larval period, which promotes its survival during that period (Abdelrahman et al., 2014). These studies suggest that hypoxia has the potential to influence the ageing process as an environmental factor influencing insects living under hypoxic conditions.

The subterranean termite Reticulitermes speratus is one of the most well-researched termites, and its life cycle has been characterized in several studies (Matsuura et al., 2009; Kobayashi et al., 2013; Yashiro and Matsuura, 2014). Briefly, each colony of R. speratus produces numerous alates, which are the winged reproductive castes comprising the incipient royals, in the spring. The nests containing a high population density of individuals are separated from the external environment; here, the conditions of hypoxia may prevail due to poor gas exchange. After nuptial flights in April to May, a pair of female and male alates establishes a new colony in a new nest and produces offspring as queens and kings (Matsuura et al., 2002). This indicates that the royals must migrate from the hypoxic conditions of the nest to completely open-air conditions, followed by re-entry into their incipient nests separated from the external environmental conditions. The gas conditions of the incipient nest are also thought to be hypoxic. It is therefore hypothesized that the hypoxic environment contributes to the development of royal characteristics, such as extended survival and high reproductive activity.

To determine the influence of hypoxia on survival and reproductive activity after foundation in R. speratus royals, we assessed the survival and the number of eggs laid in alate mating pairs under laboratory conditions. Hypoxia significantly promoted survival and egg production in the royal pairs. We therefore investigated whether these phenotypic changes were caused by altered gene expression due to hypoxic exposure for 2 weeks. First, we compared the gene expression levels of vitellogenin – known as a yolk protein, contributing to the developing oocytes in queens – under hypoxic conditions and under control conditions, using quantitative real-time PCR (qPCR) analysis. In addition to the vitellogenin gene expression analysis, we also compared several antioxidant enzyme genes reported as potentially being associated with fertility and longevity in the termite (Tasaki, Kobayashi et al., 2017).

Results
Hypoxia increases egg production and survival in incipient termite royals

We monitored the number of eggs laid and survival in alate female–male mating pairs (FM) under 21% O₂ (control conditions) or 5% O₂ (hypoxic conditions) in a multi-gas incubator (n = 126; Fig. 1). The FM units kept under hypoxic conditions produced significantly more eggs per female than the pairs kept under control conditions (Fig. 2). Amazingly, survival tests demonstrated that the FM units showed a markedly higher survival rate under hypoxic conditions than under control conditions (Fig. 3).

The survival rates at 2 weeks were 68.25% and 77.78% for FM units under control conditions and hypoxic conditions respectively (Fig. 3). To test whether hypoxic exposure affected the expression level of the vitellogenin gene in termites, we used queens from FM units, and kings from FM units as a negative control; all FM units were exposed to control or hypoxic conditions for 2 weeks. As indicated by our qPCR analysis, vitellogenin gene expression levels in queens from FM units were maintained at 2 weeks under hypoxia but not under control conditions (Fig. 4).

Hypoxia upregulates several antioxidant enzyme genes in termite queens and kings

In addition to vitellogenin gene-expression analysis, we investigated whether the expression of antioxidant genes that make major contributions to the reductions in oxidative stress associated with fecundity and survival (Finkel and Holbrook, 2000; Agarwal et al., 2003) are modulated by...
Hypoxia increased the number of eggs laid in termite royals. Mean number (plus/minus SEM values) of eggs laid per queens per colony \( n = 44 \) (control female–male mating pairs FM) at 2 weeks \( (2 \text{ w}) \), \( n = 57 \) (hypoxia FM at 2 w), \( n = 49 \) (hypoxia FM at 3 w), \( n = 43 \) (hypoxia FM at 4 w), \( n = 33 \) (control FM at 4 weeks \( 4 \text{ w} \)) and \( n = 44 \) (hypoxia FM at 4 w). \( P \)-values were obtained using unpaired \( t \)-tests. Error bars indicate SEM.

**Figure 2.** Hypoxia increased the number of eggs laid in termite royals.

Hypoxic conditions promote survival in termite royal pairs. Kaplan–Meier survival curves for female–male mating pairs units under control \( n = 63 \) and under hypoxia \( n = 63 \). \( P \)-values were obtained using log-rank tests (Peto–Peto test, \( P < 0.001 \); Cochran–Mantel–Haenszel test, \( P < 0.001 \)) and Peto–Prentice–Wilcoxon test \( (P < 0.001) \).

**Figure 3.** Hypoxic conditions promote survival in termite royal pairs.

Hypoxia increased the number of eggs laid in termite royals. We sought to determine whether hypoxic exposure in termites for 2 weeks affected the expression level of catalase genes \( Rs\text{CAT}1 \) and \( Rs\text{CAT}2 \), peroxiredoxin genes \( Rs\text{PRX}1, Rs\text{PRX}4, Rs\text{PRX}5 \) and \( Rs\text{PRX}6 \) and glutathione peroxidase genes \( Rs\text{GPX} \) and \( Rs\text{PHGPX} \). Under hypoxic conditions, the expression levels of \( Rs\text{CAT}1 \) and \( Rs\text{PRX}1 \) were higher in queens at 2 weeks than at 1 day, but under control conditions no effect was observed (Fig. 5A, C). There was no significant difference in the expression levels of \( Rs\text{CAT}2, Rs\text{PRX}5, Rs\text{PRX}6, Rs\text{GPX} \) and \( Rs\text{PHGPX} \) in queens at 1 day and at 2 weeks (Fig. 5B, E–H). Moreover, we confirmed that the expression levels of \( Rs\text{PRX}5 \) and \( Rs\text{PHGPX} \) increased only in hypoxia-exposed kings, whereas these expression levels did not differ under control conditions (Fig. 5E, H). There was no significant difference in the expression levels of \( Rs\text{CAT}1, Rs\text{CAT}2, Rs\text{PRX}4, Rs\text{PRX}6 \) and \( Rs\text{GPX} \) in kings at 1 day and 2 weeks (Fig. 5A, B, D, F, G). In both control and hypoxic conditions, kings and queens showed higher expression levels of \( Rs\text{PRX}1 \) and \( Rs\text{PRX}4 \) at 2 weeks than at 1 day respectively (Fig. 5C, D).

**Figure 4.** Effects of hypoxia on the vitellogenin gene expression in termite queens. Vitellogenin messenger RNA expression. The relative messenger RNA levels were measured by quantitative real-time PCR. One queen from the female–male mating pairs units: \( n = 3 \) (under control \( 21\% \text{ O}_2 \)) and \( n = 6 \) (under control and hypoxic conditions at 2 weeks). \( P \)-values were obtained using unpaired \( t \)-tests. Error bars indicate SEM.

**Discussion**

Our results revealed that hypoxia enhances survival and reproductive activity in long-lived termite royals. While \( \text{CO}_2 \) is a known factor affecting the reproductive activity in eusocial honeybees (Mackensen, 1947; Harris et al., 1996) and bumblebees (Amsalem and Grozinger, 2017), our findings revealed a novel effect of \( \text{O}_2 \) on the reproductive activity in eusocial insects. Intriguingly, the naked mole rat, a eusocial subterranean rodent, exhibits great longevity, with a maximum lifespan exceeding 30 years (Buffenstein, 2008), and it can epigenetically alter the expression levels of many genes associated with energy metabolism and redox control under hypoxic conditions (Kim et al., 2011). Parasitic nematodes and the ocean quahog \( Arctica islandica \) that live in hypoxic conditions – for instance, inside a host’s body and in muddy bottom sediments respectively – shift to anaerobic metabolism rather than oxidative stress-generating aerobic metabolism, resulting in slowing of ageing (Gems, 2000; Strahl, Brey et al., 2011). These findings imply that in adapting to hypoxic habitats the development of anaerobic energy-producing systems that avoid oxidative stress may allow termite royals to attain an extraordinary lifespan and sustained fecundity.
Figure 5. Effects of hypoxia on several antioxidant gene expressions in termite queens and kings. Catalase (CAT), peroxiredoxin (Prx) and glutathione peroxidase (GPx) messenger RNA expressions. The relative messenger RNA levels were measured by quantitative real-time PCR. One queen or king from the female–male mating pairs units: n = 3 [control (21% oxygen) and hypoxic (5% oxygen) conditions at 1 day] and n = 3 (control and hypoxic conditions at 2 weeks). P-values were obtained using unpaired t-tests. Error bars indicate SEM. [Colour figure can be viewed at wileyonlinelibrary.com]
Moreover, our findings demonstrated that hypoxic conditions maintained vitellogenin gene expression in queens (Fig. 4). In a previous study, vitellogenin gene expression levels and ovarian development were reported to change concomitantly in *R. speratus* (Maekawa et al., 2010). This implies that ovarian development in the queens is enhanced by hypoxia. In addition, vitellogenin is known to act as an antioxidant that promotes survival in honeybee queens (Seehuus et al., 2006; Corona et al., 2007). Antioxidant systems are thought to be associated with termite survival and also fecundity (Tasaki, Kobayashi et al., 2017; Tasaki, Sakurai et al., 2017). We found that, under hypoxic conditions, queens exhibited more than five times higher expression levels of *RsCAT1* at 2 weeks than at 1 day (Fig. 5A). As demonstrated in a previous study, a high level of *RsCAT1* expression is a major characteristic of mature queens and may play an important role in the antioxidant system (Tasaki, Kobayashi et al., 2017). On the other hand, kings exhibited more than twice the expression levels of *RSPHPX* at 2 weeks than at 1 day under hypoxic conditions (Fig. 5H). The selenoprotein PHGPx is known to be an essential antioxidant enzyme for sperm maturation (Ursini et al., 1999; Imai et al., 2001). These findings suggest that the expression of antioxidant systems in termite royals is triggered by hypoxic exposure during/after colony foundation and that these systems may be essential for achieving longevity without sacrificing high reproductive activity in termites.

The survival rate of termite royals under control conditions rapidly decreased compared with that under hypoxic conditions (Fig. 3), implying that atmospheric *O₂* is stressful for termite royals. *O₂* has been demonstrated to be a toxic molecule that must be supplied in carefully controlled concentrations due to the cause of oxidative stress (Fridovich, 1977). In *A. islandica*, adaptation to hypoxic conditions increases mitochondrial reactive oxygen species formation, which later induces oxidative stress under normoxia, due to a hypoxia adaptive metabolism (Strahl, Dringen et al., 2011). Consistent with the high sensitivity to atmospheric *O₂* conditions in termites (Tasaki, Sakurai et al., 2017), these findings suggest the possibility that termites have adapted for survival in hypoxic conditions. In addition, we observed that survival decreased to 25% after 20 weeks, even under hypoxic conditions (Fig. 3), because the experimental environment comprised extremely low nutrient conditions (only cellulose and water without nitrogen and micronutrients) that induced stress compared with those in natural nests. It will therefore be necessary to establish a more appropriate model. We also used termites kept under only estimated hypoxic conditions in the laboratory for all experiments in the present study. Thus, to address these limitations, it will be necessary to further measure the actual *O₂* and other gas concentrations (e.g., CO₂, nitrogen, hydrogen and methane) in natural *R. speratus* nests to understand their effects on the reproductive activity and physiology of termites. Apart from these limitations, this study highlights a novel aspect of behaviour and physiology of long-lived termite royals.

**Experimental procedures**

**Sample**

This study uses insect species and so did not require approval from an animal ethics committee. To obtain *R. speratus* alates (winged adults) during the swarming season from April to May, we collected 10 colonies (colonies A–J) from the experimental forest of Yamaguchi University, which is part of Mt Himeyama in Yamaguchi, western Japan, with approval. After they had shed their wings (de-alated), female and male alates were randomly selected from each colony and placed in 35 mm Petri dishes containing wet cellulose paper. Finally, 126 mating pairs were prepared, which comprised one female and one male randomly selected from different colonies. Briefly, de-alated termites were assigned to the following foundation units of FMs. FM units were constructed using the following combinations, with F and M indicating the female and the male respectively, and the subscript indicating the colony: F₂M₁, F₂M₂, F₂M₃, F₂M₄, F₂M₅, F₂M₆, F₂M₇, F₂M₈, F₂M₉, F₁M₁, F₁M₂, F₁M₃, and F₁M₄. The Petri dishes were kept under 21% or 5% *O₂* at 27 °C in a multi-gas incubator (9000EX, Waken B Tech Co. Ltd, Kyoto, Japan). One study measured gas concentrations in the mound nests of the higher termite *Macrotermes michaeleni* (Turner, 2001); however, the actual *O₂* concentration in natural nests of the subterranean termite *R. speratus* is unknown because of the methodological difficulties associated with its measurement. Therefore, as a first attempt to produce a model, we chose hypoxic conditions (5% *O₂*) based on previous hypoxia studies in *Drosophila melanogaster* (Lavistallanos et al., 2002; Zhou et al., 2007; Van Voorhis, 2009) for all experiments in this study. Using a stereoscopic microscope, we counted the number of live individuals and eggs laid every week, and the old Petri dishes were then replaced with new ones containing wet cellulose paper. To mitigate potential confounding effects of parental care for eggs and larvae, we removed all eggs weekly.

**Measuring gene expression levels**

Using next-generation RNA sequencing, the whole transcriptome of *R. speratus* was examined as described previously (Mitaka et al., 2016). We used the messenger RNA sequence of the vitellogenin gene *Vg1* and several antioxidant enzyme genes described in previous studies (Mitaka et al., 2016; Tasaki, Kobayashi et al., 2017). Since beta-actin (*RsACT*) has been evaluated as the most reliable reference gene in *Reticulitermes* species, it was selected as the reference gene (Isitani and Maekawa, 2017). Additionally, the expression levels of *RsCAT1* were compared between normoxia and hypoxia using a numbers of 126 mating pairs.
We designed primer pairs for each gene using PRIMER3 (version 1.1.4; Table S1) (Rozen and Skaletsky, 2000). Using ISOGEN® reagent (Nippon Gene, Tokyo, Japan), total RNA was extracted from the whole bodies of individuals that had been frozen in liquid nitrogen and stored at −80 °C until needed. To prevent RNA degradation, we never thawed insect samples before extraction. We synthesized cDNA immediately from the extracted RNA using a PrimeScript™ RT reagent kit (Takara Bio, Kyoto, Japan), total RNA was extracted from the whole bodies of individuals that had been frozen in liquid nitrogen and stored at −80 °C until needed. To prevent RNA degradation, we never thawed insect samples before extraction. We performed all of the procedures according to the manufacturers’ protocols. We calculated relative expression levels using the standard ΔΔCt method. We performed three biological replicates for queens and kings of R. speratus.

Statistical analysis
We used the R software package (version 3.2.2) for most of the statistical analyses and performed unpaired t-tests with the different data sets (Holm, 1979), presenting all of the data in graphs as the mean plus/minus SEM. For survival tests using the Kaplan–Meier method, we performed statistical analyses with the Peto–Peto and Cox–Prentice–Wilcoxon test in EXCEL (Microsoft, Redmond, WA, USA). All of the relevant P-values are shown in the figures, but where not shown the values were P > 0.05.

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**Supporting Information**
Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Table S1. Primer sequences.