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Source: Australian Journal of Zoology, 69(4) : 158-165

Published By: CSIRO Publishing

URL: <https://doi.org/10.1071/ZO21052>

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Exoskeleton ageing and its relation to longevity and fecundity in female Australian leaf insects (*Phyllium monteithi*)

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Handling Editor:

Paul Cooper

Received: 10 December 2021

Accepted: 6 April 2022

Published: 13 May 2022

Cite this:

Bonduriansky R and Creak C (2021)
Australian Journal of Zoology, **69**(4), 158–165.
doi:[10.1071/ZO21052](https://doi.org/10.1071/ZO21052)

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ABSTRACT

Senescence is a decline in reproduction and survival rate with advancing age resulting from deterioration of somatic tissues and systems throughout the body. Age-related somatic changes (somatic ageing) have been studied extensively in vertebrates but are less well known in other animals, including insects. Since adult insects have very limited ability to repair their exoskeleton, somatic ageing could involve deterioration and discolouration of the cuticle. We investigated age-related changes in wing pigmentation and abdominal cuticle necrosis in females of the Australian leaf insect *Phyllium monteithi*. Adult females varied markedly in the extent and pattern of pigmentation on their bodies, and we found that pigment spots on the forewings increased in size with age in most individuals. As females aged, most individuals also exhibited increasing levels of abdominal cuticle necrosis, resulting in the loss of abdominal cuticle along the margin of the abdomen. Neither the extent of pigmentation nor cuticle loss were clearly associated with reduced fecundity or longevity in the protected laboratory environment, but it remains unknown whether these age-related changes have functional implications in the wild. Our results show that the *P. monteithi* exoskeleton undergoes complex changes with age, with potential implications for functional traits and fitness.

Keywords: cuticle, exoskeleton, fecundity, longevity, necrosis, Phasmatodea, *Phyllium monteithi*, senescence.

Introduction

Senescence (ageing) is a decline with age of diverse aspects of organismal performance, resulting in reduced reproductive rate, survival probability and residual reproductive value (Partridge and Barton 1996). Despite many years of research on senescence, variation in the rate and pattern of senescence across species, and between individuals within species, remains incompletely understood. Although senescence has been documented and studied in a wide variety of organisms (Jones *et al.* 2014), most research on senescence has focused on age-related changes in life history traits – especially survival and (to a lesser extent) reproduction (Finch 1994). The somatic changes that accompany and cause these declines in performance (somatic ageing) have been extensively studied in vertebrates and especially in mammals, but are poorly understood in other animals. In particular, despite the central role of insects in research on the biology of ageing, important gaps remain in knowledge of age-related somatic changes in adult insects (Promislow *et al.* 2022).

A number of studies have examined age-related changes in internal organs and tissues of adult insects, including neurons (Technau 1984; Seid *et al.* 2005; Beramendi *et al.* 2007; Münch *et al.* 2008; Liao *et al.* 2017), muscles (Baker 1976; Marden 2000), gut tissue (Miguel-Aliaga *et al.* 2018) and gonads (Ishibashi *et al.* 2020; Sepil *et al.* 2020), but much less research has addressed age-related changes in the insect exoskeleton. A few studies have investigated age-related fraying of the wings. For example, wing fraying increases with age in Hymenoptera (Mueller and Wolf-Mueller 1993) and Lepidoptera (Kemp 2001), and high rates of wing damage are associated with reduced life expectancy in *Bombus melanopygus* bumble bees (Cartar 1992) and with reduced

fecundity in *Euphaedra* sp. butterflies (Molleman *et al.* 2009). Age-related wing fraying also occurs in Diptera. In the neriid fly *Telostylinus angusticollis*, wing deterioration occurs more rapidly in individuals reared on a nutrient-rich larval diet (and therefore attaining large adult body size), especially when the flies are housed in groups and thus exposed to wear and tear from interactions with other individuals (Adler *et al.* 2016). In the house fly (*Musca domestica*), individuals that suffer high rates of wing wear exhibit reduced flight ability (Wehmann *et al.* 2022). Insects appear to possess morphological adaptations that minimise wing damage or mitigate its effects on fitness (Rajabi *et al.* 2020). Studies on other parts of the insect body have shown that the exoskeleton hardens gradually after the adult moult, reaching maximum toughness after several days (Parle and Taylor 2017; Wang *et al.* 2018), and that older insects have a reduced ability to repair experimentally induced wounds to their cuticle (O'Neill *et al.* 2019). The composition of cuticular hydrocarbons (CHCs) coating the exoskeleton also changes with age in *Drosophila melanogaster* (Cortot *et al.* 2022), and such age-related changes in CHCs could be associated with reduced capacity for hygienic grooming in old flies (Kubiak 2017), potentially leaving older individuals vulnerable to infection by the bacteria, fungi and protists that inhabit the exoskeleton surface (Douglas 2015).

Age-related changes in the exoskeleton could play an especially interesting and important role in insect somatic ageing because adult insects have very limited capacity to repair damaged cuticle, and cannot replace damaged sclerites or appendages (Parle *et al.* 2017). This means that any cuticular wear and tear associated with activities such as foraging or reproduction, wounds inflicted by predators or parasites, microbial infection, and damage resulting from exposure to sunlight, has the potential to accumulate and contribute to age-related reduction in the functionality of legs, wings and other organs (Ridgel and Ritzmann 2005). The potential for such cumulative damage and deterioration is especially great in long-lived insects. For example, whereas most insects survive for just days or weeks in the wild, individuals of some species can survive for several months or even years (Promislow *et al.* 2022). However, aside from frayed wings, very little is known about the nature or functional consequences of age-related changes and deterioration in the adult insect exoskeleton.

Many species of vertebrates undergo age-related changes in colour as adults: for example, some birds develop brighter nuptial plumage (Hawkins *et al.* 2012), some fish acquire distinct colour patterns (Sugimoto 2002), and mammals often develop grey hair (Tobin 2009). Adult insects also have the potential to change colour as they age as a consequence of changes in the cuticle or epidermis. The potential for age-related colour changes in adult insects is illustrated by increasing cuticle pigmentation in the ant *Platythyrea punctata* (Hartmann *et al.* 2019), and bleaching

of the setae in the bee *Bombus huntii* (Koch *et al.* 2014), as well as gradual loss of red hue in the wings of *Heliconius melpomene* butterflies (Dell'Aglio *et al.* 2017). Such changes could be maladaptive manifestations of senescence (e.g. resulting from the deterioration of cuticular pigments or loss of tissue through abrasion), but adaptive colour changes could also occur in some cases (e.g. to maintain effective camouflage in a seasonally changing habitat). Very few studies have investigated age-related colour change in adult insects.

The Australian leaf insect *Phyllium monteithi* (Brock and Hasenpusch 2002) provides a valuable opportunity for research on exoskeletal senescence and colour changes because it is large, relatively long-lived by comparison with many other insects (Promislow *et al.* 2022; see Results), and females have a flattened body shape that facilitates quantification of cuticle colour and structure. This species is endemic to tropical rainforests in far-north Queensland, Australia, where it feeds on leaves in the tree canopy. Like many other phasmids, this species is highly camouflaged and capable of facultative parthenogenesis. Both sexes possess large wings but only males are capable of flight. We noticed that *P. monteithi* females vary considerably in the extent and pattern of brown colouration on their dorsal surface (Fig. 1), and that many individuals also appear to exhibit cuticle necrosis on the margin of the abdomen.

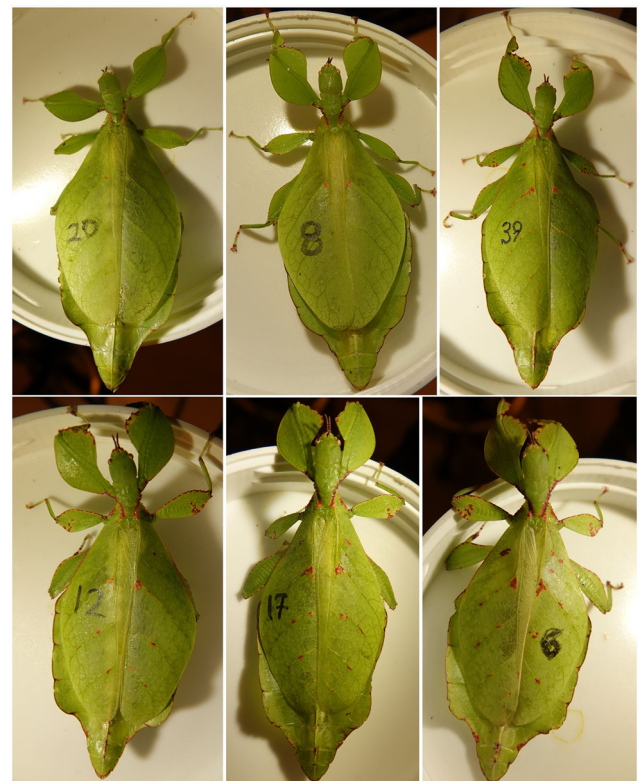


Fig. 1. Six *Phyllium monteithi* females, illustrating variation in the amount and patterning of brown pigmentation on their dorsal surface.

We investigated age-related changes in wing pigmentation (quantified as the sizes of brown patches on the forewings) and abdominal cuticle necrosis (quantified as the sizes of indentations resulting from tissue loss along the margin of the abdomen) in captive-bred *P. monteithi* females. We also asked whether increased levels of pigmentation and cuticle loss are associated with reduced fecundity or longevity.

Materials and methods

Source and maintenance of *P. monteithi*

Eleven *P. monteithi* females (including adults and late-instar nymphs) were purchased from a breeder (Australian Insect Farm, Innisfail, Queensland; Jack and Sue Hasenpusch, proprietors) in April 2017. These individuals came from a stock originally derived from a small number of females collected at Kuranda and one male collected at Garradunga, Queensland, and propagated for several generations through a mix of sexual and parthenogenetic reproduction (Jack Hasenpusch, pers. comm.). It is not known precisely how these 11 females were related, or whether they developed from fertilised or unfertilised eggs. The insects were provided with live *Syzygium australe* plants as food, and sprayed daily with water. These females began to lay eggs on 1 May 2017 and ~100 eggs were collected and incubated in moist cocopeat at 27°C. Nymphs ($N = 74$) hatched from these eggs were transferred to 10 fresh *S. australe* plants (1.5–2 m tall) in the UNSW glasshouse in May 2018. When these nymphs began to moult into adults in November 2018, the plants and insects were transferred to a controlled-temperature room at 27°C. The plants were arranged with branches interlocking to allow insects to move freely between them. The room was fitted with grow-lights and the plants were sprayed daily with water. The adult moult date for each individual was recorded, and each adult was marked on one wing with a unique number using a permanent marker. A total of 40 adult females and two adult males were thus obtained. This sex ratio suggests that most of these individuals were produced via parthenogenesis (which typically results in all-female broods in phasmids), but that at least one of the females obtained from the breeder mated and laid fertilised eggs (which develop into both females and males). Both males died within a few days of their adult moult. The 40 females, which underwent their adult moult between 28 August 2018 and 12 February 2019 were used to investigate somatic ageing as described below.

Quantification of pigmentation and cuticle necrosis

The 40 females were imaged up to three times over the course of their adult life at ages (days from adult moult) ranging from

1 to 148 days. Both dorsal and ventral images were made each time using a Sony RX10 camera. The first set of images was made for all 40 females (age 9 ± 22 days, range 1–108 days) old. Because several females died over the course of the study, second images were made for 36 females (age 69 ± 23 days, range 50–172 days), and third images were made for 30 females (age 91 ± 20 days, range 45–148 days). This broad range of adult moult dates and ages enabled us to investigate senescence of individual females while reducing confounding effects of timing and seasonality.

ImageJ software (Schneider *et al.* 2012) was used to quantify pigmentation (i.e. brown patch areas) from dorsal images (Fig. 2a–c) and abdominal cuticle necrosis from ventral images (Fig. 2d–f) at successive ages for each female. To estimate the relative sizes of brown patches, the margin of one wing was traced to obtain wing area (Fig. 2b), and then the margins of all brown patches on that wing were traced to obtain their areas (Fig. 2c). The proportion of total wing area covered by brown patches was calculated by dividing the total brown patch area by total wing area. Brown patches covered <2% of wing surface in all individuals.

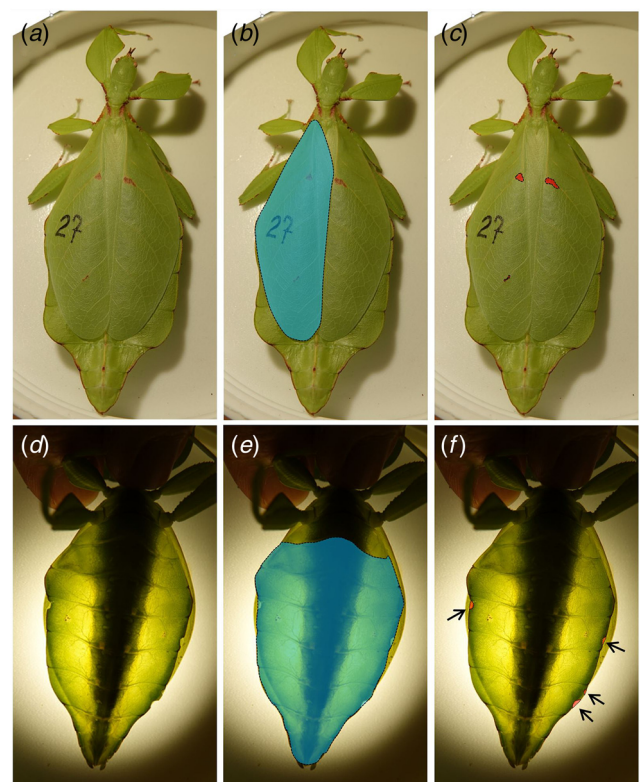


Fig. 2. The percentage of brown colouration on the forewings (a) was quantified from the total area of one wing (b) and the total area covered by brown patches (c). Percentage cuticle loss on the abdominal margin (d) was calculated from the area of the intact abdomen, with the abdominal margin extrapolated across any indentations resulting from cuticle loss (e), and the areas of indentations resulting from cuticle loss (f).

To estimate the relative extent of abdominal cuticle necrosis, the total ventral surface area of the intact abdomen was obtained by tracing around the abdominal margin, with the abdominal contour extrapolated across any indentations in the margin (Fig. 2e). The margins of all indentations were then traced to obtain their areas (Fig. 2f). The proportion of abdominal area lost to cuticle necrosis was then calculated by dividing the summed areas of the indentations by the total area of the intact abdomen. The proportion of the cuticle lost was <1% in all cases, except for three individuals that showed signs of abdominal damage caused by other individuals chewing on the margins of their abdomen. Data from images where such damage was apparent ($N = 4$ images from three females) were excluded from analysis.

Wing length (in mm) was measured for each female as an index of body size.

Quantification of longevity and fecundity

Fecundity was estimated by transferring each female to an individual container (20 cm wide \times 40 cm high) with a mesh lid and collecting eggs over 14 days. Containers were sprayed daily with water, and fresh *S. australe* branches (inserted in 250 mL containers filled with water) were provided every few days for food. Females were 38 ± 15 days old at the start of egg collection (range 31–111 days).

The plants on which focal females were feeding were inspected daily and dates of death were recorded. In addition, we censused the population on six occasions at intervals of approximately two weeks, attempting each time to locate each live individual and bodies of any missing individuals. Nonetheless, because *P. monteithi* females are extremely cryptic on their host plants, lifespan could not be determined with accuracy for 20 females. For these individuals, the ages at final sighting were included in the lifespan analyses as censored data.

Statistical analysis

We investigated the effect of age on pigmentation (i.e. the proportion of the wing covered by brown spots) and cuticle necrosis (i.e. the proportion of cuticle lost) by fitting separate Gaussian mixed models of these response variables, with female age, body size (wing length) and moult date as fixed effects and female identity as a random effect to account for repeated measures of individual females. Because the response variables were proportions, they were arcsine-transformed for the analysis. These models were fitted using the *lmer* function in the *lme4* package (Bates *et al.* 2015) in R ver. 3.5.1 (R_Core_Team 2013). Fixed effects were tested using *F*-tests based on Satterthwaite's degrees of freedom using the *lmerTest* package (Kuznetsova *et al.* 2017).

We used the *lm* function in base R to test for effects of female age at the start of egg collection, moult date, and wing length as fixed effects on female fecundity (eggs laid per day). To determine whether increased pigmentation or increased abdominal cuticle loss were associated with reduced fecundity, wing pigmentation (mean across all ages) and cuticle necrosis (mean across all ages) were then added to the model of female fecundity.

We used the *coxph* function in the *Survival* package (Therneau 2022) in R to test for effects of moult date and wing length on hazard (mortality) rate. To determine whether increased pigmentation or increased cuticle necrosis were associated with reduced longevity, these variables were then added to the cox model of female hazard rate.

Results

Mean wing pigmentation (i.e. percentage wing surface area covered by brown patches) was 0.28 ± 0.25 in the first set of images (age = 9 ± 23 days, $N = 39$), 0.43 ± 0.35 in the second set of images (age = 70 ± 23 days; $N = 37$) and 0.44 ± 0.36 in the third set of images (age = 90 ± 20 days; $N = 27$). Wing pigmentation increased with age in individual females (estimate = 0.00012, s.e. = 0.000023, $t = 5.40$, $P < 0.0001$) (Fig. 3). The mean amount of pigmentation was not affected by wing length (estimate = -0.00028 , s.e. = 0.00095, $t = -0.298$, $P = 0.768$) or moult date (estimate = -0.000017 , s.e. = 0.00016, $t = -0.111$, $P = 0.912$).

Individual females varied considerably in the extent of cuticle necrosis (i.e. percentage abdominal cuticle lost) on the abdominal margin (Fig. 4). The mean percentage cuticle necrosis was 0.07 ± 0.12 (including 16 females that exhibited no cuticle loss at all) in the first set of images (age = 9 ± 23 days, $N = 39$), 0.14 ± 0.20 (including six females that exhibited no tissue loss at all) in the second set of images (age = 70 ± 23 days; $N = 35$) and 0.25 ± 0.24 (including one female exhibiting no tissue loss at all) in the third set of images (age = 90 ± 20 days; $N = 25$). The extent of cuticle necrosis increased with age in individual females (estimate = 0.00026, s.e. = 0.000043, $t = 6.00$, $P < 0.0001$) (Fig. 4). The mean extent of cuticle necrosis was not affected by wing length (estimate -0.00021 , s.e. = 0.00079, $t = -0.027$, $P = 0.979$) or the extent of wing pigmentation (estimate 0.028, s.e. = 0.12, $t = 0.241$, $P = 0.810$). However, females that underwent their adult moult later exhibited greater cuticle necrosis (estimate 0.00028, s.e. = 0.00013, $t = 2.10$, $P = 0.042$).

Females laid an average of 18 ± 6.3 eggs (range 2–35, $N = 37$) over 14 days (1.3 ± 0.45 eggs per day). Females that underwent their adult moult later laid fewer eggs (estimate -0.0134 , s.e. = 0.0052, $t = -2.67$, $P = 0.015$). However, female fecundity was not related to female age at

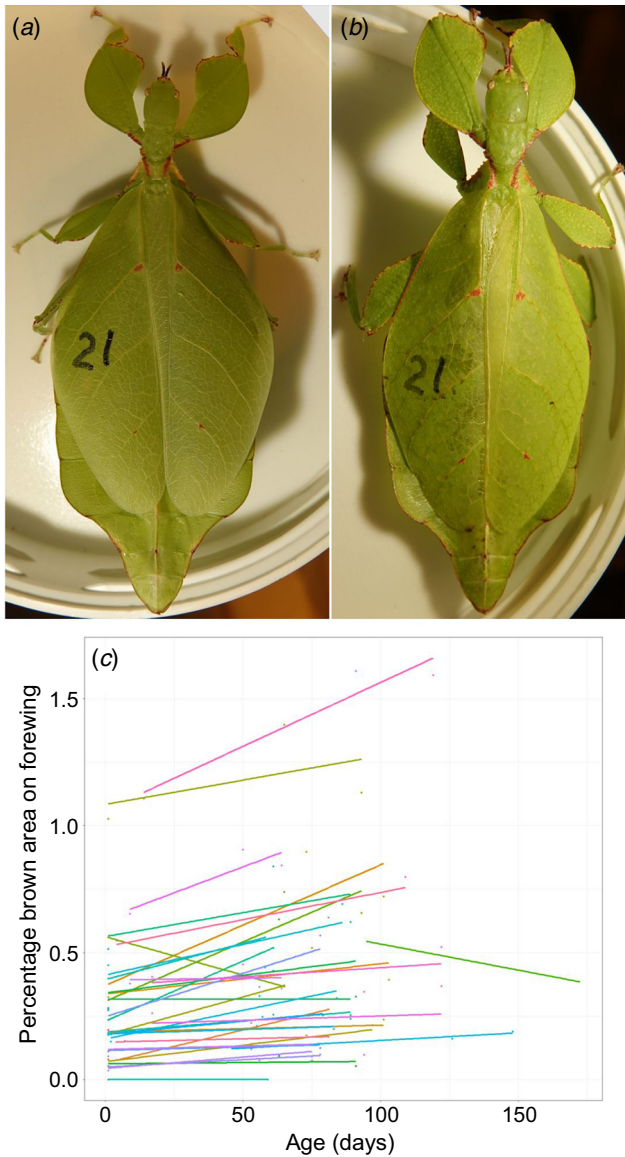


Fig. 3. Dorsal views of female #21 at 2 (a) and 84 (b) days adult age. Linear trajectories of age-related changes in brown patch size on the forewings for individual females (c).

the start of egg collection (estimate = 0.0292, s.e. = 0.0162, $t = 1.793$, $P = 0.890$) or to wing length (estimate = -0.0149, s.e. = 0.0294, $t = -0.508$, $P = 0.617$). Female fecundity was also not clearly related to the extent of wing pigmentation (estimate = -0.0645, s.e. = 0.2639, $t = -0.244$, $P = 0.810$) or cuticle necrosis (estimate = -1.185, s.e. = 0.6306, $t = -1.879$, $P = 0.076$) (Fig. 5).

Among the 20 females for which the date of death was known, mean lifespan was 109 ± 46 days (range 34–192; Fig. 6). Female hazard (mortality rate) increased with moult date (estimate = 0.0376, s.e. = 0.0133, $z = 2.835$, $P = 0.005$), but hazard was not strongly associated with wing length (estimate = 0.1001, s.e. = 1.105, $z = 1.809$,

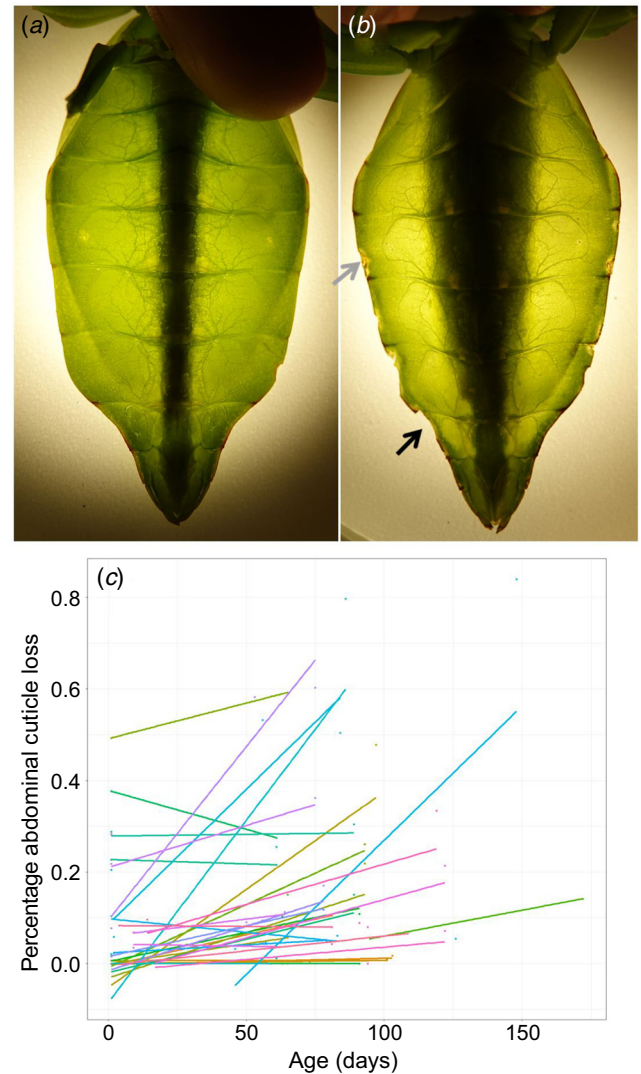


Fig. 4. Ventral views of female #39 at 9 (a) and 64 (b) days adult age. Cuticle loss on the abdominal margin (black arrow) is preceded by local tissue necrosis (grey arrow). Linear trajectories of age-related cuticle loss for individual females (c).

$P = 0.070$). Hazard was not related to the extent of wing pigmentation (estimate = 1.572, s.e. = 4.814, $z = 0.798$, $P = 0.425$) or cuticle necrosis (estimate = -1.422, s.e. = 4.777, $z = -0.298$, $P = 0.766$).

Discussion

We found that *P. monteithi* females exhibit increasing forewing pigmentation and abdominal cuticle necrosis as they age. To our knowledge, increased wing pigmentation and necrotic loss of abdominal cuticle have not been reported previously as manifestations of somatic ageing in adult insects. Neither pigmentation nor cuticle necrosis

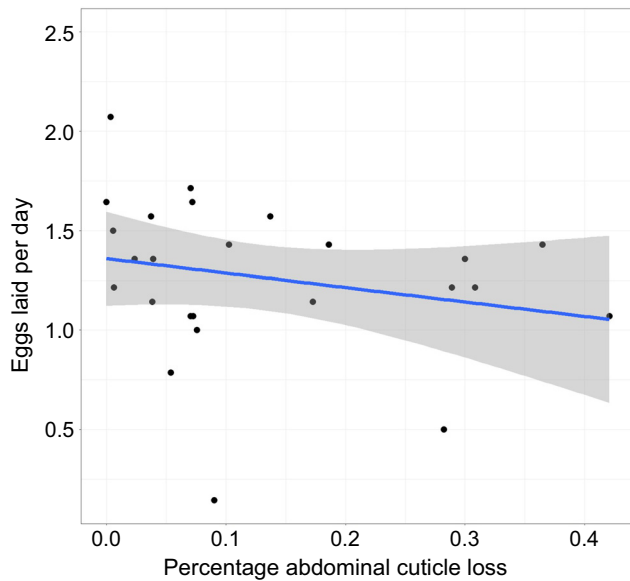


Fig. 5. Female fecundity (eggs laid per day) as a function of abdominal cuticle necrosis (% cuticle lost). Points show values for individual females. The blue line is a linear regression, and the shaded area is the 95% confidence interval for the regression line.

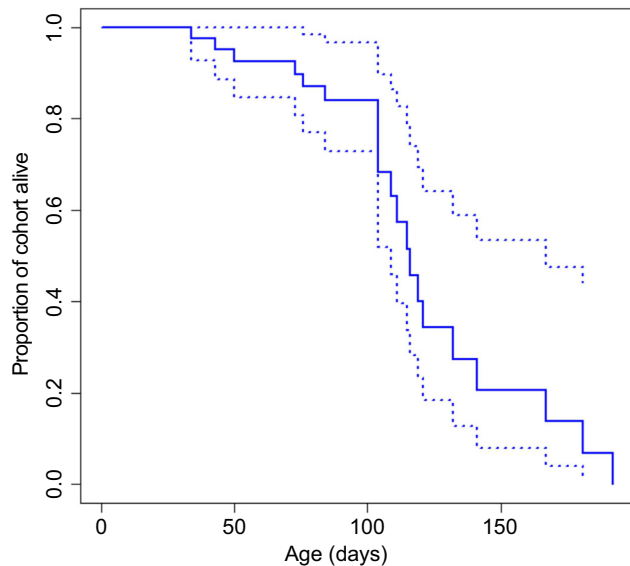


Fig. 6. Kaplan–Meier survival curve for 40 adult *Phyllium monteithi* females. The dotted lines represent 95% confidence limits.

were clearly related to fecundity or longevity in the laboratory environment, but it remains unknown whether these phenotypes have functional significance in the wild.

The adult insect cuticle cannot be replaced, and the capacity for wound healing appears to be far more limited in insects than in vertebrates. Insects can heal wounds by regenerating damaged epidermis and thickening the cuticle around the wound (Parle *et al.* 2017), but adult insects

cannot fully repair or replace damaged cuticle (Parle *et al.* 2016). This suggests that the adult insect cuticle is susceptible to accumulating damage from a variety of factors, such as mechanical abrasion and injury, solar radiation, and corrosion caused by chemicals in the environment or substances secreted by parasites and pathogens.

Brown pigmentation on the wings could increase with age as a result of senescent deterioration of the wing cuticle or underlying epidermis (Malek 1958). In the flies *Drosophila melanogaster* and *Sarcophaga bullata*, brown cuticle colour results from cross-linking of cuticular proteins and chitin in response to compounds that act as sclerotising precursors, as well as the synthesis of eumelanin and pheomelanin (Barek *et al.* 2018). It is not known whether brown pigmentation in *P. monteithi* is formed in a similar way. Fluorescent pigments are known to change with age in several insect species, and such changes have even been used to estimate the age of wild-caught individuals (Křemenová *et al.* 2020). Following the adult moult and full sclerotisation, the pigmentation and colouration of the adult insect body is generally assumed to remain constant. However, there is evidence of age-related changes in pigmentation in the exoskeletons of ants and bees (Koch *et al.* 2014; Hartmann *et al.* 2019), and of age-related colour fading in butterfly wings (Dell’Aglia *et al.* 2017) and exoskeleton colours of salticid spiders (Lim and Li 2006; Taylor *et al.* 2014). Our results show that age-related changes in pigmentation can also occur on the wings of adult phasmids.

Deterioration of the insect cuticle often results from the degradation or depletion of chitin (Muthukrishnan *et al.* 2020), and it is possible that such structural changes are involved in abdominal cuticle necrosis and loss in *P. monteithi*. However, it is not clear why this process might occur most rapidly on the margin of the abdomen. One possibility is that the abdominal cuticle of *P. monteithi* females deteriorates as a result of bacterial or fungal infection, but we did not find a relation between the degree of abdominal cuticle loss and either fecundity or longevity. Nonetheless, it is possible that cuticle loss is associated with reduced performance in natural environments. Insects harbour a rich variety of microorganisms on their exoskeletons (Douglas 2015), and cuticle necrosis could allow pathogenic bacteria or fungi to gain access to the body cavity and haemolymph, thus exposing females to increased risk of systemic infection.

Increasing forewing pigmentation and especially abdominal cuticle necrosis are most plausibly interpreted as deleterious manifestations of somatic ageing. However, an alternative possibility is that these changes serve adaptive functions in enhancing camouflage. Young leaf insects resemble fresh, healthy leaves, but the enlargement of pigmented spots and development of necrotic patches on the abdominal margin might increase resemblance to old or damaged leaves. Such changes could enhance camouflage if

P. monteithi females typically moult into the adult stage at a certain time of year, and if host plant leaves tend to deteriorate over the course of the female lifespan. Testing this possibility would require better understanding of the ecology and seasonality of *P. monteithi* in its natural rainforest habitat.

Interestingly, individual females varied considerably throughout their lives in the extent of brown pigmentation on their dorsal surface (Fig. 1), and also appeared to vary in the rate of increase in pigmentation with age (Fig. 3). Females also appeared to vary in the extent and rate of accumulation of abdominal cuticle necrosis (Fig. 4). Further research is needed to determine the contribution of genetic and microenvironmental differences to this variation. In particular, the individuals used in this study were derived from a stock originally founded with a small number of wild-collected individuals, and therefore probably lacking in genetic diversity. Further research on other genotypes sourced from across the natural range of *P. monteithi* in northern Queensland is needed to determine whether the patterns of morphological senescence reported here are genotype-specific. Larval nutrition appears to affect the susceptibility of the neriid fly (*Telostylinus angusticollis*) cuticle to mechanical damage (Adler *et al.* 2016), and it is possible that such environmental effects influence properties of the *P. monteithi* cuticle.

Our findings highlight the need for more research on somatic ageing of the insect exoskeleton. Just as very little is known about the mechanical properties and wound-healing capacity of the insect cuticle (Parle *et al.* 2017), knowledge of how exoskeletons wear and deteriorate with age is very limited. Studies on both captive and wild insects are needed to understand how the cuticle ages, and the functional implications of such changes. Research is also needed to understand the sources of individual variation in cuticle properties and susceptibility to age-related deterioration.

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Data availability. The data that support this study are available in Dryad (<https://doi.org/10.5061/dryad.sqv9s4n5x>).

Conflicts of interest. The authors declare no conflicts of interest.

Declaration of funding. This research was funded by Australian Research Council Discovery Grant DP170102449 to RB.

Acknowledgements. We thank Pearl Lei, Emma Dawson-Glass, and Amalyn Bonduriansky for assistance with data collection and insect maintenance. Jack Hasenpusch kindly provided information on the source of the stock from which our *Phyllium monteithi* females were derived.

Author contributions. RB designed the study, collected the fecundity and longevity data, carried out the analysis, and wrote the paper. CC collected the morphological data.

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