1 Short title: Telomere length, development and stress

2

3 **The effects of development and chronic oxidative stress on telomere length in an agricultural pest**
4 **moth, *Helicoverpa armigera***

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13 Abstract

14 Telomeres are repetitive sequences located at the end of chromosomes in eukaryotes that protect
15 against loss of important sequences during the cell replication process. Telomere length (TL) shortens
16 with every round of cell division. When a telomere becomes too short, cells can no longer proliferate
17 and this triggers the cell apoptosis process. Apart from cell replication, the length of telomeres can be
18 affected by factors such as sex, genetics, and stress levels. Oxidative stress in particular can cause
19 damage to telomeres and telomere maintenance processes, resulting in TL shortening. This
20 phenomenon occurs in humans and many vertebrates, especially endothermic species. However, the
21 ways in which various stress types affect the TL of invertebrate species remains ambiguous.

22 Here, we examined the effects of development and oxidative stress on TL in the invertebrate
23 pest moth, *Helicoverpa armigera*. In the former case, we extracted genomic DNA from three
24 developmental stages (1-day old egg, 4th instar, and first-day emerged moths) and measured TL by
25 qPCR. In the latter, we chronically exposed individuals to paraquat – an organic herbicide that induces
26 oxidative stress - and then measured TL as per our development methodology. In addition, we
27 examined TL in a subset of published whole genome short-read sequencing data of caterpillars and
28 moths using the software, Computel.

29 In our experimental work, we found that TL in *H. armigera* was significantly longer at the early
30 stages of development and shortens in later stages. However, oxidative stress does not appear to
31 shorten TL in *H. armigera* following chronic exposure to paraquat. In our Computel analysis, we found
32 that caterpillars had longer mean TL than moths but this difference was not significant due to the high
33 variation among samples.

34 Collectively, our research provides new data on TL in an underrepresented group, adding new
35 insights into the progression of TL shortening with development and the effects of oxidative stress on

36 TL, while also more generally highlighting the value of applying complementary approaches to TL
37 measurement.

38 **Introduction**

39

40 Telomeres are repetitive non-coding sequences that protect chromosomes from losing functional
 41 regions. Eukaryotes have linear chromosomes that cannot be synthesised at the very ends during the
 42 cell division process. Therefore, chromosomes lose small amounts of DNA sequence with every round
 43 of cell replication [1]. The majority of eukaryotes can partially compensate for this with an enzyme
 44 called telomerase, which can synthesise the repeat sequence [2]. Telomerase is ordinarily highly
 45 expressed during developmental periods and in certain types of cells (e.g., reproductive and malignant
 46 cells [3]); however, the activity of telomerase is negligible in stem cells and absent in normal adult
 47 somatic cells [4]. As a result, in many species, telomere length (TL) shortens over time [1]. When
 48 telomeres eventually become too short to replicate, the cell will stop proliferating and enter senescence
 49 or apoptosis [5] and this is part of the process leading to aging. Thus, shortening of telomeres is
 50 associated with several age-related diseases in humans [6, 7, 8], and also shortens the lifespan of some
 51 species (e.g., [9, 10]).

52 TL is not only dependant on the number of replication/cell division cycles, but is also affected
 53 by genetic factors, sex, and other external stimuli, such as chronic stress [11]. In particular, there is
 54 some evidence to support a correlation between chronic stress and shortened telomeres in humans [12].
 55 For example, people who have suffered from depression or anxiety may have shorter TLs [13, 14], and
 56 childhood trauma [15], bipolar disorder [16], physical and sexual assault [17], and PTSD (Post-
 57 Traumatic Stress Disorder; [18]) have also been shown to affect TL.

58 Although the mechanism remains unclear, in many organisms, stress appears to affect the
 59 length of telomeres by lowering telomerase activity or enhancing oxidative stress. In humans, early
 60 exposure in life to chronic stress may affect TL by down-regulating telomerase expression to reduce

telomerase activity [19, 20, 21]. This may account for the apparent link between stress and shorter lifespans i.e., individuals experiencing stress during early development have less telomerase activity and therefore shorter TL leading to a reduced lifespan. Chronic stress can also generate reactive oxygen species (ROS), resulting in a disruption of the antioxidant/oxidant balance and a subsequent increase in oxidative stress [22]. High levels of ROS can affect the activity of telomerase by oxidising the guanine in telomeres, preventing the telomerase from extending telomeres during development [23, 24] and ROS are also more likely to cleave repeated telomeric sequences than non-telomeric regions [25].

The relationship between TL and stress in humans has been studied for more than a decade and indications are that stress can have long-term effects on human TL that can even be passed to offspring [19, 26, 27]. However, recent research has shown that the relationship is highly complex and context dependent, with the generality of stress causing TL attrition being questioned, alongside research that shows that associations can be transient (e.g., [28]), can strongly depend on the tissue-type, life-stage, and experimental condition being tested [29, 30], and may be affected by the method of telomere measurement [31]. It is even less clear how these processes might work in other species, with telomere dynamics and maintenance systems in invertebrate and ectothermic species in particular varying or remaining unclear due to a lack of research.

Helicoverpa armigera is a pest moth that is widespread across several continents [32]. The larvae of this species damages the reproductive part of crops, leading to significant economic damage annually [33]. However, prevention and management of *H. armigera* infestation is a challenge due to their wide host range, high fecundity, and ability to migrate over long distances and evolve resistance to insecticides [33, 34, 35]. Here, we aim to reveal the effects of stress on TL in *H. armigera* following chronic exposure to paraquat (an oxidative stress producer). Paraquat (*N,N'*-dimethyl-4,4'-bipyridinium dichloride) is an organic herbicide that exerts its toxic effect by generating super oxide anions [36].

Despite being banned in several countries, paraquat is widely available throughout the world due to its efficiency and low cost [37]. As a result, paraquat is found on several plant species and may be present in the natural diet of *H. armigera* in certain parts of the world.

Oxidative stress has been shown to shorten TL in mice [38]. In addition, there is evidence that paraquat in particular has a strong impact on development, gene expression, and TL in insects. For example, in *D. melanogaster*, paraquat has been shown to increase mortality, reduce climbing ability, and result in up-regulation of several antioxidant genes [39], as well as reduce TL elongation rates [40]. In *H. armigera*, paraquat injected into pupae has been shown to extend diapause by affecting the insulin-signalling pathway [41], while our own previous research demonstrated that chronic oxidative stress exposure delays development and alters gene expression in this species [42]. Here, we use *H. armigera* as a model system to further investigate chronic oxidative stress by examining its effects on TL.

Materials and Methods

Study samples and oxidative stress exposure

Samples in this study were obtained from a laboratory colony at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Canberra, Australia. This colony was originally isolated from Northern New South Wales, Australia. *H. armigera* were exposed to paraquat, leading to chronic oxidative stress (described in detail in [42]). In brief, experiments used 2-day old fertilised eggs collected from 40 healthy moths, air-dried, and allowed to hatch. Caterpillars (1st-instar) were reared until pupation under optimal laboratory conditions ($25 \pm 1^\circ\text{C}$, $50 \pm 10\%$ relative humidity, and light

day:night 14:10 to imitate natural light) on artificial diet, which was changed weekly. Moths were fed a honey solution. Based on previous work [42], paraquat concentrations of 0.3 and 0.4 mM were chosen for our ‘stressed’ samples, which were reared as outlined above for controls and chronically exposed to paraquat through their diet (i.e., incorporation of the required concentration into the solid artificial diet before it set, or directly into the honey solution) upon hatching.

111 *DNA extraction*

To compare TL between different developmental stages and treatments, six individuals for three developmental stages (1-day old egg, 4th instar, and newly emerged moths) from control and stressed samples were collected in absolute ethanol and stored at -20°C. Second generation eggs were also collected from both controls and stressed moths to assess the spill over effect of stress on TL in the next generation.

In order to isolate genomic DNA (gDNA), samples were air-dried, allowing ethanol to evaporate at room temperature. Because TL might shorten differently in different tissue types, the midgut of 4th-instar caterpillars was isolated, as this tissue has high contact with paraquat after ingestion. Leg tissue of moths was chosen to avoid the reproductive system in adults. Dried tissue was placed into 1.5 mL Eppendorf tubes. Lysis buffer was added and samples were ground with DNase-free plastic pestles before 20 µl of proteinase K was added and samples were incubated overnight at 58°C. DNA extraction was then conducted following the JetFlex™ Genomic DNA Purification Kit DNA (Cat no. A30701) protocol to isolate gDNA from tissue samples. Subsequently, gDNA was re-suspended by adding 20 µl (eggs and moth legs) or 50 µl (4th-instar midguts) of TE buffer and incubating overnight at room temperature before storage at -20°C.

127 *Telomere length measurement via qPCR*

TL in both control and stressed samples (six biological replicates each) for both the current (eggs, 4th-
 instar, moths) and 2nd generation (eggs) was determined by qPCR following the Luna® Universal
 qPCR Master Mix Protocol (M3003, New England Biolabs) using 2 µl of DNA template. The primers
 were designed based on telomeric sequence of other Lepidopteran species (TTAGG)_n [43, 44, 45].
 This sequence was also confirmed by mining it from existing long-read sequencing (minION) data of
H. armigera obtained from the Australian National Insect Collection (ANIC; Dr. Luana S. F. Lins). For
 comparison, elongation factor 1-alpha (EF1, GenBank accession No. U20129) was chosen as a
 housekeeping gene not expected to have changed in length over time or treatment. The primers used
 were Tel F: GGTTTTTTGGGTTGGGTTGGGTTGGGTTGGGTT, Tel R:
 GGCTTGCCTTACCTTACCTTACCTTACCTTACCT, EF1 F: GACAAACGTACCATCGAGAAG,
 and EF1 R: GATACCAGCCTCGAAGTCAC.

Mean T/S ratio (i.e., the ratio of the telomere repeat copy number to the single-copy -
 elongation factor 1-alpha – number) was calculated following Livak's equation [46, 47] for each
 developmental stage and treatment.

Telomere length computation with Computel

The software, Computel ver. 1.2 (available at: <https://github.com/lilit-nersisyan/computel>; [48]) was
 used to compute mean telomere length from whole genome re-sequencing data for 50 individuals of *H.*
armigera collected from ten countries around the world (Australia, Brazil, China, France, India,
 Madagascar, New Zealand, Senegal, Spain, Uganda), as larvae from wild and crop host plants or as
 adult moths via light/pheromone traps [49]. This included 23 caterpillars and 27 moths, although we
 lack information as to the ages of any of the samples beyond this broad categorisation. The software
 was run in default mode on paired-end fastq files obtained from [49], with the number of
 chromosomes, genome length, and telomere repeat pattern specified.

151 *Statistical analysis*

152 In order to evaluate differences in TL between developmental stages and between control and stressed
 153 samples, results were statistically analysed in SPSS ver. 22 [50]. Means and standard deviations (SD)
 154 were calculated for all measures and one-way ANOVA with Tukey's Honestly Significant Difference
 155 (HSD) was used to assess differences between groups at a 95% confidence interval. In this analysis,
 156 samples that are statistically similar (in terms of mean and variance for TL) group together into
 157 homogeneous subsets. Thus, if samples are categorised into different groups (referred to as 'a' or 'b'
 158 see Results), they are considered significantly different from each other. Data visualisation was
 159 performed using the ggplot2 ver. 3.2.1 [51] package in R ver 3.6.1 [52].

160 To evaluate differences in mean TL between caterpillars and moths, as computed in Computel,
 161 an unpaired t-test was performed in R. A Pearson's product-moment correlation test was also
 162 performed using the cor.test command in R to examine whether estimates of telomere length were
 163 associated with base coverage [48]. Finally, mean computed telomere length per sample, and the
 164 correlation between mean telomere length and base coverage, were plotted with ggplot2 in R.

165

166 **Results**

167

168 *Telomere length during development*

169 Differences in the amount of relative telomeric sequence (i.e. T/S ratio) of *H. armigera* at different
 170 developmental stages and across experimental treatments are illustrated in Figure 1.

171

Figure 1. Effects of development and stress on telomere length in *Helicoverpa armigera*. Mean TS ratio (i.e., the ratio of the telomere repeat copy number to the single-copy - elongation factor 1-alpha - number) following chronic oxidative stress exposure for developmental stage and treatment as shown by the *x-axis* and legend, respectively. Labels on the *x-axis* correspond to: ‘1_Egg’ = 1st generation eggs; ‘2_Egg’ = 2nd generation eggs; 4th = 4th-instar. PQ0.3 and PQ0.4 correspond to use of paraquat doses of 0.3 mM and 0.4 mM, respectively. Significant differences among developmental stages/treatments are indicated by non-overlapping characters (‘a’, ‘b’), and error bars indicate standard deviation.

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The relative TL of whole eggs (‘1_Egg’) and other developmental stages (‘4th’ and ‘Moth’) were significantly different (as indicated by ‘a’ and ‘b’ subgroups in the figure), with TL in 4th-instar individuals shorter than TL of eggs for both control and stressed samples ($F_{8,49}=38.679$, $P < 0.001$). However, 4th-instar and moth TLs of controls were not significantly different from each other (both in ‘b’ subset).

These results were consistent with the results of the Computel analysis, where mean TL was found to be longer in caterpillars compared to moths, though there was a lot of variability in mean TL among samples and this result was not significant (mean of caterpillar minus moth = 959.000; 95% confidence interval of difference: -683.608:2601.608; $T_{48} = 1.175$; $P = 0.246$; SE of difference = 816.462) (Fig. 2A). A correlation analysis found that mean telomere length was significantly associated with base coverage per sample ($T_{48} = -2.330$; $P = 0.024$; 95% confidence interval: -0.548:-0.044; $R = -0.319$), such that lower estimates of base coverage resulted in higher estimates of telomere length (Fig. 2B).

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Figure 2. Computed mean telomere length in *Helicoverpa armigera*. Whole genome re-sequencing data was processed in the software, Computel, to compute mean telomere length for samples collected from the field and broadly classified as ‘caterpillar’ and ‘moth’. (A) The boxplots show the distribution of mean telomere lengths across samples for each of these categories (with the mean indicated by a black diamond inside the boxplot), indicating a general but non-significant ($T_{48} = 1.175$; $P = 0.246$), trend of longer telomeres in caterpillars compared to moths; (B) The correlation between mean telomere length and base coverage per sample ($T_{48} = -2.330$; $P = 0.024$; 95% confidence interval: -0.548:-0.044; $R = -0.319$). Raw data points are plotted, along with the linear regression trend line.

Telomere length in response to oxidative stress

The relative TLs of control and paraquat 0.3 mM and 0.4 mM stressed samples were not significantly different for the 4th-instar and moth stages (all ‘b’ subset in Fig. 1), or for the eggs of the next generation (‘2_Egg’; all ‘a’ subset in Fig. 1) ($F_{3,20}=2.645$, $P = 0.077$).

Discussion

There is a large body of work that suggests that stress can have a negative effect on TL in various species. However, our results suggest that, while aging negatively correlates with TL in *H. armigera*, chronic oxidative stress does not, at least under the experimental conditions we applied.

In particular, we found that TL is dramatically shorter in 4th-instar individuals compared to eggs, though not between 4th-instar and moths. This finding may relate to the caterpillar stage being when *H. armigera* undergoes significant growth (see [42]), and therefore cell division and proliferation

217 compared to the other developmental stages. As a pupa and adult, there is also less nutritional uptake
218 (pupae are non-feeding and adults consume only small amounts of honey solution), leading to less
219 exposure to ROS production and its associated effects on telomerase inhibition and telomere damage
220 [23, 24].

221 This experimental result was consistent with our computation of mean TLs from whole genome
222 re-sequencing data, which generally indicated that mean TL is shorter in moths compared to
223 caterpillars. Although this trend was not significant for our data, there was a lot of variation in the
224 dataset and we lack information as to the specific ages of the tested individuals beyond their broad
225 categorisation as ‘caterpillar’ and ‘moth’ as they were all field-collected vs. controlled for development
226 under laboratory conditions. In addition, the estimates of mean telomere length were significantly
227 correlated with estimates of per sample base coverage. In general, increasing coverage is known to
228 improve the accuracy of mean TL estimation in Computel, however performance comparisons with
229 synthetic data have shown that the accuracy of Computel was not affected for 100 bp read lengths and
230 coverage values of 0.2x, 2x and 10x [48] – our read length was 90 or 101, and our base coverage
231 ranged from 3x to 31x. Overall, both our experimental and computational results are consistent with the
232 hypothesis that TL shortens as a result of aging, as has been shown in other species, including black
233 garden ants [53], humans [1], and birds [54].

234 Conversely, TL was not found to be shortened in response to chronic oxidative stress in the
235 current study and we saw no spillover effects in second generation eggs. According to the literature,
236 various types of stress can shorten TL in a variety of species, including humans [55, 56, 57], rodents
237 [58, 38], birds [59, 54, 60, 61, 62, 63], fish [64, 65], lizards [66], toads [67], flies [40], plants [68], and
238 yeast [69, 70]. However, other studies have found no such correlation (nine-spined stickleback, [71];
239 young brown trout, [72]; desert agama lizard, [66], Western spadefoot toad, [67]; garden snails, [73]),
240 or even elongated TL with various stress types (e.g., in yeast; [69]).

241 Although the reason for the lack of relationship in the fish remains unclear, for yeast and the
 242 desert lizard, the authors each suggested that effects of stress on TL may depend on previous exposure
 243 to the particular stress applied. For example, [69] found that *S. cerevisiae* experienced TL elongation
 244 under alcohol stress, and [66]) found that consistent thermal stress in the desert lizard led to an up-
 245 regulated thermal-regulatory ability to protect TL integrity. Because alcohol (yeast) and consistent
 246 thermal stress (desert lizard) are regularly faced by these species in their natural environments, they
 247 may have mechanisms in place to quickly mitigate these stresses. Similarly, our finding of no
 248 significant differences in TL between control and stressed samples of *H. armigera* may be due to the
 249 fact that oxidative stress is induced through the plant defence mechanisms that *H. armigera* naturally
 250 faces in the wild [74]. Thus, *H. armigera* may have a well-developed and effective mechanism to cope
 251 with chronic oxidative stress. It is unclear what such a mechanism may be, however in our previous
 252 work using the same experimental samples to examine gene expression, we found that telomerase
 253 genes were not up-regulated in paraquat stressed samples [42]. It is possible that acute severe stress,
 254 such as pesticide exposure, would lead to shortening of telomeres in *H. armigera*, but this remains to be
 255 investigated.

256 There is some also evidence to suggest that different levels of stress may affect TL differently
 257 in some species. For example, [40] stressed *Drosophila melanogaster* with paraquat at different
 258 concentrations and then measured the telomeric retroelements (NB: *Drosophila* has a different
 259 telomeric system to *H. armigera*; [75]). These authors found that only certain non-/sub-lethal
 260 concentrations of paraquat increased TL in *Drosophila*, whereas too much or too little paraquat resulted
 261 in no significant effects. Interestingly, some TL shortening effects in *D. melanogaster* were also shown
 262 in this study to occur only after multi-generational exposure to paraquat, suggesting that the effects of
 263 such stress on TL might be too minor to determine in a single generation [40]. This is an exciting result
 264 that could be explored with future work.

265   Thus, the lack of a relationship between chronic oxidative stress and TL found here may be due
 266 to the chosen experimental design (type or concentration of stress applied). It is also possible that the
 267 effects of such stress on TL in *H. armigera* are very minor or transient and that the TL measurement
 268 approaches used in this experiment were not sensitive enough to detect them. However, because the
 269 methods applied did see the expected decrease in TL with age, we believe this latter explanation to be
 270 less likely. More generally, as noted in the Introduction, the relationship between stress and TL is
 271 known to be highly complex and context (tissue-type, life-stage, stress-type, TL measurement method,
 272 etc.) dependent [29, 31, 30].

273   In summary, we provide new evidence of TL shortening with development in *H. armigera* that
 274 is consistent with the general hypothesis that aging is associated with TL attrition. Chronic oxidative
 275 stress, however, does not appear to shorten TL in *H. armigera* in the current or second generation under
 276 the conditions of chronic paraquat exposure used here. This is consistent with recent research that calls
 277 the generality of TL attrition with stress into question and highlights the context-dependent nature of
 278 TL dynamics.

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Figure 1

Figure 2a