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Dim artificial light at night affects mating, reproductive output and reactive oxygen species in *Drosophila melanogaster*[†]

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Abstract

Humans are lighting the night-time environment with ever increasing extent and intensity, resulting in a variety of negative ecological effects in individuals and populations. Effects of light at night on reproductive fitness traits are demonstrated across taxa however, the mechanisms underlying these

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effects are largely untested. One possible mechanism is that light at night may result in perturbed reactive oxygen species (ROS) and oxidative stress levels. Here we reared *Drosophila melanogaster* under either dim (10 Lux) light or no light (0 Lux) at night for three generations and then compared mating and lifetime oviposition patterns. In a second experiment, we explored whether exposure to light at night treatments resulted in variation in ROS levels in the heads and ovaries of 6, 23 and 36 day old females. We demonstrate that dim light at night affects mating and reproductive output: 10 Lux flies courted for longer prior to mating, and female oviposition patterns differed to 0 Lux females. ROS levels were lower in the ovaries but not heads, of 10 Lux compared to 0 Lux females. We suggest that reduced ROS levels may reflect changes in ovarian physiology and cell signalling which may be related to the differences observed in oviposition patterns. Taken together, our results indicate negative consequences for invertebrates under more stressful, urban, lit conditions and further investigation into the mechanisms driving these changes is warranted to manage invertebrate communities in a brighter future.

Keywords

Light pollution, *Drosophila melanogaster*, reactive oxygen species, mating, oviposition, ALAN, ovaries

Introduction

The presence of artificial light at night (ALAN) is linked to species-wide shifts in behavioural and physiological traits, including courtship and mating, offspring production, growth and survival (Longcore & Rich, 2004; Navara & Nelson, 2007). These effects are evident even at relatively low levels of ALAN (≤ 30 lux), typical of the light environment in some urban and peri-urban spaces. Field studies demonstrate a negative relationship between the presence of dim ALAN (≤ 5 lux) and reproductive success and juvenile growth in birds (Dominoni, Goymann, Helm, & Partecke, 2013;

Dominoni, Quetting, & Partecke, 2013; Raap et al., 2016) and reproduction in mammals (LeTallec, Thery, & Perret, 2015; Robert, Lesku, Partecke, & Chambers, 2015), while laboratory experiments in hamsters and rats link exposure to dim ALAN (between 1 and 5 lux) with increases in tumour growth rates (Blask, Dauchy, Brainard, & Hanifin, 2009) and immune suppression (Bedrosian, Aubrecht, Kaugars, Weil, & Nelson, 2013). Similar responses to chronic exposure to dim ALAN (between 10 and 30 lux) are observed in invertebrates. Field experiments demonstrate lower mating success in moths (*Operophtera brumata*) (van Geffen, van Eck, et al., 2015) and female aphids (*Megoura viciae*) were more likely to switch to an asexual mode of reproduction under dim ALAN conditions with potential implications for winter survival (Sanders et al., 2015). Furthermore, laboratory experiments exploring the effects of dim ALAN report decreased adult longevity and reduced oviposition and egg number of young *Drosophila melanogaster* females (McLay, Green, & Jones, 2017), a decline in the sexual attractiveness of female sex pheromones and accelerated juvenile development in the moth *Mammestra brassicae* (van Geffen, Groot, et al., 2015).

A potential driver of the observed physiological effects of dim ALAN is the possibility that ALAN also affects levels of reactive oxygen species (ROS). ROS are a suite of highly reactive by-products of the oxidation-reduction (redox) reactions that occur as part of normal cell metabolism (Dowling & Simmons, 2009). Low levels of ROS are essential for cell signalling (Droge, 2002; Poli, Leonarduzzi, Biasi, & Chiarpotto, 2004), however very high levels can cause oxidative stress, leading to damage to DNA, proteins and lipids and consequent effects on organism fitness (Metcalfe & Alonso-Alvarez, 2010). ROS is so critical to normal metabolic function that it is proposed as a major mechanistic driver for the process of aging and a suite of life history constraints (Dowling & Simmons, 2009; Harman, 1956). Accordingly, male and female fertility is adversely affected by both high (for reviews see Agarwal, Aponte-Mellado, Premkumar, Shaman, & Gupta, 2012; Venkatesh, Deecaraman,

Kumar, Shamsi, & Dada, 2009), and low levels of ROS and oxidative stress (de Lamirande, Jiang, Zini, Kodama, & Gagnon, 1997). Gametes are particularly vulnerable to the negative effects of high ROS and oxidative damage to DNA can lead to transgenerational effects for offspring viability (Metcalfe & Alonso-Alvarez, 2010). Together, these observations suggest that ROS may be important mediators in the trade-off between reproduction and longevity (Dowling & Simmons, 2009; Metcalfe & Alonso-Alvarez, 2010).

Here, we investigate the potential of variation in ALAN (0 Lux or 10 Lux) to affect (i) reproductive output in the form of patterns of mating behaviour, lifetime oviposition and offspring development success and, in a second experiment, potential effects on physiology through (ii) differences in ROS levels of somatic (using the head) (Neretti et al., 2009) and reproductive tissue (ovaries) and accompanying morphological changes (measured through ovarian area). We use the model invertebrate *Drosophila melanogaster*, a species with demonstrable reduction in early reproductive effort and adult survival in the presence of 10 Lux ALAN (McLay et al., 2017). We predict that flies subjected to the presence of ALAN during juvenile development and the adult stage of the life cycle will have perturbed ROS levels compared to flies reared with no light at night (0 Lux). Furthermore, we expect a concomitant reduction in mating success, oviposition rates and offspring development.

Methods

Fly Stocks

A stock population of *Drosophila melanogaster* was created from 100 adult females and 50 adult males collected in April 2015 from Oak Ridge Winery in the Yarra Valley, Victoria, Australia (-37.686908, 145.457438). Stock flies were maintained on Bloomington's cornmeal medium (Brent & Oster, 1974) under standard conditions, at a density of approximately 50 male and 50 female flies

for 34 generations, in a retrofitted incubator at 28 ± 1°C, prior to the start of the experiment (for further details, see McLay et al. (2017)).

Light Treatments

We created two light treatments using Westinghouse incubators, as described in McLay et al. (2017). Both light treatments had an identical 12hr daytime lux (2600 Lux - equivalent to an overcast day, 6800 kelvin), followed by one of two 12hr night time lux (either 0 Lux, 0 kelvin or 10 Lux, 5900 kelvin) ALAN treatments. To ensure no incubator bias, flies and their corresponding ALAN environment were rotated between three incubators every two to three days and were randomly positioned within each incubator.

Experimental Flies

To create our experimental generation of flies, we allocated five stock bottles of approximately 50 recently emerged female and male flies to the light (10 Lux) and dark (0 Lux) ALAN treatments. We maintained flies in their designated light environment for a further two generations in the same manner as reported previously (McLay et al., 2017), after which time we collected newly emerged (< 6 hours old) virgin female and male flies under mild CO_2 anaesthesia and transferred them to individual vials to mature for two days before pairing flies to mate. Measurement of reproductive output was conducted in two blocks (N = 66 male and female pairs from generation 35; N = 70 pairs from generation 36). We used data from this experiment and our previous study (McLay et al., 2017) to inform female age classes for the ROS study. The ROS assay and ovarian area study was also run over two blocks (N = 267 flies from generation 51; and N = 259 flies from generation 52).

Effect of ALAN on reproductive output

Mating behaviour - To assess whether mating behaviour varied under the two ALAN treatments, we paired 136 female and 136 male flies (N = 66 pairs in block 1, N = 70 pairs in block 2) in vials containing standard medium. Each pair was reared under the same light treatment but originated from different vials and thus were not siblings. A pair was permitted 30 minutes to commence mating. Flies that commenced copulation within the 30-minute time interval were allowed to complete copulation after which time the male was discarded; pairs that did not commence copulation within 30 minutes were also discarded. For each trial, the time until male wing extension, defined here as time to courtship (Cobb & Jallon, 1990), the time to copulation and the duration of copulation were recorded. To explore whether the number of eggs laid and success of development from egg to adult varied between the two light environments, we transferred a random subset of the mated females from the mating behaviour assay (N = 38 females, 31 from block 1; N = 40 from block 2) to individual vials containing fresh standard medium.

Oviposition patterns - We counted the total number of eggs laid by each of the females over a maximum of eight 24-hour time points (at 3, 9, 13, 16, 20, 27, 30 and 34 days) over their adult lives. At the start of each time point, the female was transferred to a vial with standard medium, dyed blue (Queen Fine Foods, Alderley, Australia) to increase egg visibility. After 24hr the female was returned to a new vial containing standard medium until the next time point. The final time point (day 34) was chosen to cover the median lifespan of *D. melanogaster* under ALAN conditions: our previous work demonstrates that approximately half of flies reared under 10 Lux ALAN conditions are likely to have died by day 34 (McLay et al., 2017). After day 34, any surviving females were discarded, as by this time they would have almost certainly exhausted their store of sperm (Lefevre & Jonsson, 1962). The total number of eggs laid at each time point was counted twice by visual inspection under magnification.

Egg to adult success – To assess variation in the number of emerging adult offspring, we allowed eggs from the Day 3, 20 and 34 egg counts to develop to the adult stage. These time intervals represented different slopes in the survival curve of flies under these ALAN conditions (little to no mortality at Day 3, both 0 Lux and 10 Lux = 98% survival; a slow decline in survival to Day 20, 0 Lux = 96% survival, 10 Lux = 86% survival; and a sharp decline in survival to Day 34, 0 Lux = 77% survival, 10 Lux = 57% survival) (McLay et al., 2017). Vials were checked every one to two days until the first adult eclosed and then for a further seven days, after which time the vial was discarded. The number and sex of all emerging adults was recorded and the egg to adult success for each vial was determined as the number of emerging adults divided by the total number of eggs laid.

Effect of ALAN on ovarian area and comparative ROS levels in the head and ovary

In a second experiment, we investigated the effects of the different light regimens on ovarian area (as a proxy for the number of eggs remaining) and on ROS levels in heads and ovaries. A total of 526 single-mated females (N = 267 in block 1; N = 259 in block 2) were randomly allocated to one of three age cohorts (young = 6 days; medium-age = 23 days; old = 36 days post eclosion). We used heads for somatic tissue ROS levels (Neretti et al., 2009) and ovaries to determine ROS levels in female germ cells. Once a female had reached her designated age, we lightly anaesthetised her with CO_2, removed the head and dissected out the ovaries.

Ovarian area – To explore whether exposure to ALAN resulted in differences in developing gametes, we used total ovarian area as a proxy for the number of eggs remaining when the female was assessed. This assumption is justified, as in our population ovarian area correlates with the number of visible eggs (stages 9 to 14) (Bate & Martinez Arias, 1993) (mean ± SE; total eggs: 26.65 ± 2.45, N = 20 females; $F = 111.1$, $R^2 = 0.86$, $P < 0.0001$) and mature eggs (stages 13 and 14) (5.65 ± 2.33, N = 20

females; $F = 49.97$, $R^2 = 0.74$, $P < 0.0001$). Ovaries were photographed at x 64 magnification with a dissecting microscope (Olympus SZX16, Tokyo, Japan), with attached camera (SONY ILCE-QX1, Tokyo, Japan). We calculated total ovarian area of each fly (mm^2) by drawing a line around the perimeter of each ovary using Image J software (NIH, Rockville, USA).

Comparative levels of ROS – To assess whether total ROS levels varied under the different light treatments at different female ages. We measured total ROS using a 2'7'-dichlorohydrofluorescin diacetate (H2DCFDA) assay, modified from Wang and Joseph (1999), in which the non-fluorescent DCFH is oxidised by ROS to form fluorescent DCF and can be detected by fluorometry (Wang & Joseph, 1999). From each light treatment and age cohort, samples of ovaries from single-mated flies (N = 50 pools of five pairs of ovaries per pool) and individual heads (N = 108 females) were assayed. Briefly, individual samples were placed into 50 μl of cold lysis buffer (TPER, Thermo Fisher Scientific, Rockford, USA) in a 96 well PCR plate (SSI, California, USA) on ice. We then homogenised each sample for 45 secs using a micro pestle made from a modified sterile 1000 μl pipette tip (Eppendorf, Hamburg, Germany). The plate was centrifuged at 400 x *g* for 5 minutes at $4^{O}C$ and 10 μl of supernatant transferred to a 96 well black assay plate (Greiner Bio-one, Neuberg, Germany). We added 40 μl of cold 10 mM solution of 2'7'-H2DCFDA (Sigma-Aldrich, Castle Hill, Australia) dissolved in dimethyl sulfoxide (Sigma-Aldrich, St Louis, USA). The plate was incubated in darkness for 45 minutes at $37^{o}C$ and then read for fluorescence in a microplate reader (PerkinElmer, EnSpire Multimode, Waltham, USA) at 485nm excitation and 535nm emission (Wang & Joseph, 1999). We commenced all assays between 2.5 - 3hr after incubator lights on; average time between fly death and the start of the assay was 2.0 ± 0.5hr. All samples were kept on ice until dissections and photographs were complete. For all ROS assay plates, we used positive/quality control and negative control samples. The positive/quality control was prepared separately and consisted of the

supernatant from 200 whole flies from the stock population, homogenised in 3000 µl TPER, and stored at -80^{o}C in sub-aliquots until required. The negative control contained all reagents except the tissue extract for each plate. Samples and controls were run in triplicate across plates and the mean fluorescence reading (subtracting the negative control) was used for analysis, where the coefficient of variation (CV) for the triplicate reading was <15%. Inter-plate (CV of positive/quality controls) and intra-plate (CV of the mean CV of the first two and last two ovarian and head readings per plate) specific analyses were undertaken to assess the consistency of readings between plates.

Statistical analyses

Analyses were performed in R (R core team 2016) using the *lme4* software package (Bates, Machler, Bolker, & Walker, 2015). Prior to analysis, data were assessed for normality and transformed where appropriate (time to commencement of courtship and time from commencement of courtship to onset of copulation were log transformed, and time for copulation was cube root transformed). We used standard least squares linear models to explore differences in mating behaviours, total ovarian area and ROS levels; and a generalised linear model (GLM) with a binomial error distribution and logit link function to explore differences in the proportion of pairs commencing copulation in 30 minutes and numbers of females surviving to Day 34. Generalised linear mixed models (GLMM) fitted by maximum likelihood (ML) assuming a binomial error distribution and logit link function were used for all other data for reproductive output except for cumulative number of eggs, which did not include a binomial function and was fitted with restricted maximum likelihood (REML). We included light treatment and block as categorical factors in all models and other factors as appropriate: age cohort as a categorical factor in total ovarian area and ROS levels; maternal age as a continuous and polynomial variable in number of females laying at least one egg at any egg count

and cumulative number of eggs laid per female; female identity as a random effect for all oviposition and offspring development models; number of days survived as a continuous variable for number of females laying at least one egg at any egg count; number of eggs laid per female at an egg count as a continuous variable for the proportion of eggs to emerge as adults where some adults emerged and for the sex ratio of offspring. Interactions between all of these variables were included. The significance of parameters was assessed using hierarchical backwards stepwise deletion, dropping terms from the model (except the main parameter of interest, light treatment) where $P > 0.10$. *Post hoc* Tukey's tests were used to determine differences between groups. Unless otherwise stated all data presented are means ± standard errors and the level of statistical significance was taken as $P < 0.05$.

Results

Effect of ALAN on reproductive output

Mating behaviour - Flies in the 10 Lux treatment took longer from the onset of courtship to commence copulation than 0 lux flies ($P = 0.03$; Table 1c). In contrast, the probability of copulation commencing within 30 minutes; the time taken between first introduction into the vial and the onset of male courtship behaviour; and, the total duration of copulation, were comparable for the two light treatments (All $P > 0.15$; Table 1a-b, d).

Oviposition patterns and survival – Female survival to Day 34 ($\chi^2 = 0.42$, $P = 0.52$) and the number of females laying at least one egg at a given egg count ($P = 0.43$, Table 1e) were comparable for the two light treatment groups. Overall, the likelihood of an egg being laid varied with age ($P < 0.005$, Table 1e), but the relationship between maternal age and the probability of laying an egg was curvilinear ($P < 0.005$; Table 1e) and this pattern varied across the two ALAN treatments ($P = 0.01$; Table 1e;

Figure 1a). A similar pattern was observed for the cumulative number of eggs laid per female. The cumulative number of eggs laid was comparable for 0 lux and 10 lux females (P = 0.14; Table 1f), but the relationship over time was non-linear (P < 0.005; Table 1f) and varied across the two ALAN treatments (P < 0.005; Table 1f, Figure 1b).

Egg to adult success – The probability that a vial with eggs present produced adult flies was the same for both light treatments (P = 0.91; Table 1g). However, no adults emerged from the Day 34 vials (number of vials with adults emerging for 0 Lux Day 3 females = 33/36, Day 20 females = 5/22, Day 34 females = 0/0; 10 Lux - Day 3 females = 28/32, Day 20 females = 3/19, Day 34 females = 0/0; P < 0.005; Table 1h). The proportion of eggs emerging as adults (P = 0.37; Table 1h) and adult sex ratio (P = 0.97, Table 1i) were comparable between the 0 and 10 Lux treatments.

Effect of ALAN on ovarian area and comparative ROS levels in the head and ovary

Ovarian area and survival – There was no difference in ovarian area between the ALAN treatments (P = 0.75; Table 2a). The proportion of flies that survived to Day 34 was also comparable (χ^2 = 0.27, P = 0.61).

Comparative levels of ROS – The level of ROS in female's heads did not differ between the ALAN treatments (P = 0.33; Table 2b; Figure 2a), but 10 Lux females had lower ovarian ROS levels compared to 0 Lux females (P = 0.04; Table 2c; Figure 2a). ROS levels of both heads and ovaries varied with female age (heads: P < 0.005, and ovaries: P = 0.01 respectively, Table 2b-c, Figure 2b-c). *Post hoc* Tukey's tests revealed higher ROS levels in the heads of Day 23 compared with Day 36 flies, but no differences between Day 6 flies and either Day 23 or Day 36 flies. Additionally, ROS levels were higher in the ovaries of Day 6 compared with Day 36 flies but no different to flies of Day 23. The intra and inter-specific plate CVs were 14.91% and 10.50% respectively.

Discussion

This study provides three key findings suggesting that exposure to dim ALAN has behavioural and physiological consequences. First, chronic exposure to dim ALAN of 10 Lux was associated with an increase in the time taken between the onset of courtship and the commencement of copulation. Second, the pattern of oviposition over a female's life was different between light treatments, with both the likelihood of a female laying eggs within a 24-hour period and cumulative eggs laid over a female's life varying with the interaction between light treatment and maternal age. Finally, ROS levels of ovaries were comparatively lower in flies under 10 Lux compared to the 0 Lux treatment.

Our result that flies reared under 10 Lux spent significantly longer courting but were equally likely to eventually copulate is one of the first experimental studies to record a difference in courting and mating behaviour under dim (10 Lux) conditions. To our knowledge, these behaviours have yet to be assessed in vertebrates under dim ALAN. However, Botha, Jones, and Hopkins (2017) demonstrated interactions between light treatment and other variables in the duration of mating and number of mating bouts in crickets *(Teleogryllus commodus*). Additionally, a field study in moths (*Operophtera brumata*) demonstrated reduced mating success for females under 10 lux ALAN conditions (van Geffen, van Eck, et al., 2015). Given the peak time for mating in *D. melanogaster* is during daylight hours (Sakai & Ishida, 2001), which is when our mating assays were conducted, it is unlikely that the presence of light during a trial is responsible for the behavioural differences observed here. Our study did not aim to identify possible mechanisms for this change in behaviour, and we note that van Geffen, van Eck, et al. (2015) did not investigate courtship behaviour. However, the fact that a

key aspect of mating behaviour shifted following lifetime exposure to ALAN suggests that, in both species, its presence has the potential to interfere with mating *per se*. In another species of moth (*Mamestra brassicae)*, ALAN is related to reduction in the attractiveness of female sex pheromones, which is likely to disrupt mating cues (van Geffen, Groot, et al., 2015). *D. melanogaster* have cuticular hydrocarbons that act as sex- and species-specific pheromone cues (Howard & Blomquist, 2005) and are subject to environmental perturbation (Higgie, Chenoweth, & Blows, 2000), but whether they exhibit similar variation under ALAN is currently untested and merits investigation. Regardless of the underlying mechanism, the increased time spent courting represents a fitness cost for ALAN, due to increased competition and vulnerability to predators (Endler, 1987; Magnhagen, 1991).

Age-related declines in fecundity and oviposition rates are common across taxa (Clutton-Brock, 1988) including insects such as Coleoptera (Tanaka, 1990), Lepidoptera (Braby & Jones, 1995), Orthoptera (Carrière & Roff, 1995), Diptera (Jann & Ward, 1999), as well as *D. melanogaster* (Partridge, Fowler, Trevitt, & Sharp, 1986) and such declines in propensity to lay and egg number over time were paralleled here. In contrast, while a reduction in the number of eggs produced under ALAN conditions in young adult females was previously reported for *D. melanogaster* (McLay et al., 2017), this is the first evidence for variation in patterns of lifetime egg production and variation in dim ALAN. The interaction we observed between female age and light treatment suggests that such an investigation was warranted, as the relationship was non-linear and thus, assessing variation within a single age-class may not be representative of lifetime oviposition. It is unlikely the observed pattern of oviposition was driven by differences in survival between the two light treatments, as the likelihood of survival to Day 34 was the same for both treatments and thus it seems that this represents a real difference in the pattern of offspring production. Maternal age effects aside, our

study did not aim to disentangle male and female contributions to overall egg production. Male *D. melanogaster* transfer seminal fluids during mating, which stimulate egg production, ovulation and sperm retention (Gillott, 2003; Wolfner, 2002). Female *D. melanogaster* typically retain sperm for approximately 14 days following mating (Qazi, Heifetz, & Wolfner, 2003) and offspring production is positively related to sperm storage (Qazi et al., 2003). There is the potential that the amount or quality of sperm and/or seminal fluids transferred during mating or subsequently stored, differed between the light treatments. Such a mechanism may explain why the oviposition curves initially diverge at approximately 15 days in the current study. Therefore, further investigations into the relative contributions of females and males to egg production is warranted.

A potential underlying mechanism for the observed physiological and behavioural effects of dim ALAN is its potential to suppress endogenous melatonin production (Blask et al., 2005; Blask et al., 2009; Brainard, Richardson, Petterborg, & Reiter, 1982). The photosensitive indolamine melatonin is a key driver of circadian rhythm, as well as a powerful antioxidant (for review see Reiter, Tan, & Fuentes-Broto, 2010). The primary site of its nocturnal production across taxa is in the head (the location of primary photoreceptors) (Helfrich-Forster, Winter, Hofbauer, Hall, & Stanewsky, 2001; Vivien-Roels & Pevet, 1993) including in *D. melanogaster* (Callebert, Jaunay, & Jallon, 1991; Finocchiaro, Callebert, Launay, & Jallon, 1988) and its reduction can lead to circadian disruption and a change in levels of ROS (Pandi-Perumal et al., 2006) although this has not yet been demonstrated in *D. melanogaster.*

Our data did not support the oft-cited prediction that in the presence of ALAN, levels of ROS are likely to be increased (Jones, Durrant, Michaelides, & Green, 2015; Navara & Nelson, 2007; Pandi-Perumal et al., 2006; Reiter et al., 2003; Tan et al., 2010). On the contrary, we found no difference in

ROS levels in female heads between light treatments and comparatively lower levels of ROS in the ovaries of 10 Lux females compared to their 0 Lux counterparts. In the absence of an effect of ALAN on total ovarian area or on the cumulative number of eggs laid, it is unlikely that the difference between the two light treatments arises due to reduction in the quantity of material assayed and thus a reduced signal. Instead, we suggest that the observed variation reflects ALAN-induced changes in ovarian physiology that may explain differences in the pattern of eggs laid over a female's lifespan. A certain level of ROS is required for signalling pathways in cells (Droge, 2002). In *D. melanogaster*, ROS generated from nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) is required for normal ovulation (Ritsick, Edens, Finnerty, & Lambeth, 2007) and it is conceivable that these pathways are modulated in the presence of ALAN. The implications of this effect on fitness is currently unclear, as aside from changes for the ALAN exposed females in the oviposition pattern and cumulative egg number over time, the egg to adult success rates of offspring were unchanged in the current laboratory study.

Intriguingly, despite detectable differences in the ovaries between the two light treatments, ROS levels in the heads did not differ. This highlights the possibility of tissue-specific responses to ALAN arising through variation in the levels and pattern of ROS generation between the two tissue types. Ovaries contain rapidly dividing cells that typically contain higher numbers of mitochondria than somatic cells (Cree et al., 2015) and as ROS generation occurs primarily in mitochondria (Finkel & Holbrook, 2000), we may expect ovaries to generate greater amounts of ROS than somatic tissue. A direct link between metabolic rate and ROS levels remains controversial (Alonso-Alvarez, Canelo, & Romero-Haro, 2017; Salin et al., 2015), nonetheless, extra-mitochondrial ROS production, for example from NOX in reproductive tissues (Alonso-Alvarez et al., 2017; Ritsick et al., 2007) could also lead to discernible differences between tissues under ALAN conditions. Variation in these two ROS

generators could also explain the age-specific differences in ROS levels in both heads and ovaries between age cohorts (higher ROS levels in the Day 6 cohort compared to the Day 36 cohort). Higher metabolic activity and investment in reproduction is associated with young organisms, while older individuals undergo senescence (Frisard et al., 2007; Sohal & Weindruch, 1996), so we may expect to see shifts in ROS levels between the beginning and end of adult lifespan.

Our current study measured overall ovarian ROS at a specific timepoint (during the daylight and not during the dark period) and was not designed to identify specific signalling pathways. We note that ROS levels vary in different tissues over a 24hr period due to rhythms in circadian activity and antioxidant production (Hardeland, Coto-Montes, & Poeggeler, 2003). Therefore, we do not know whether the observed ROS levels reflect the overall diurnal changes in redox status of the ovary. Future experimentation with more sampling time points is needed to assess relative temporal, tissue-specific differences in ROS. Additionally, our experiment was undertaken in flies that had been under light treatment for three generations, it is possible (albeit not tested) that only flies with inherently lower levels of ROS progressed to the third generation. Further multigenerational studies would elucidate whether selection is occurring between generations under ALAN. Moreover, this experiment was conducted in a benign laboratory environment, with constant temperature and food *ad libitum*. While the imposed light stress was strong enough to generate detectable differences within the ovaries it may not reflect the more extreme ecological stresses imposed in nature.

In conclusion, we have demonstrated that dim anthropogenic light at night has a detrimental impact on two traits related to reproductive fitness in *D. melanogaster* (mating behaviour and oviposition patterns over a female's life). Additionally, dim ALAN lowers ovarian ROS levels compared to a no light at night treatment, which may reflect altered ovarian physiology i.e. cell signalling. Further

studies on effects of ALAN on mating cues and nocturnal/diurnal ROS loading are required to understand the underlying mechanism(s) behind and consequences of our results. Given that we are lighting the world with increasing extent and intensity (Kyba et al., 2017), understanding the mechanisms behind and the consequences of this anthropogenically induced pollutant to populations and ecosystems is critical for the effective management of our urban ecosystems and their future diversity.

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Conflict of interest

The authors declare no conflict of interest.

References

Agarwal, A., Aponte-Mellado, A., Premkumar, B., Shaman, A., & Gupta, S. (2012). The effects of oxidative stress on female reproduction: a review. *Reproductive Biology and Endocrinology, 10*(1), 49. doi:10.1186/1477-7827-10-49

Alonso-Alvarez, C., Canelo, T., & Romero-Haro, A. A. (2017). The Oxidative Cost of Reproduction: Theoretical Questions and Alternative Mechanisms. *Bioscience, 67*(3), 258-270. doi:10.1093/biosci/biw176

Bate, M., & Martinez Arias, A. (1993). *The Development of Drosophila melanogaster*: Plainview, N.Y. : Cold Spring Harbor Laboratory Press, 1993.

Bates, D., Machler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software, 67*(1), 1-48.

Bedrosian, T. A., Aubrecht, T. G., Kaugars, K. E., Weil, Z. M., & Nelson, R. J. (2013). Artificial light at night alters delayed-type hypersensitivity reaction in response to acute stress in Siberian hamsters. *Brain Behavior and Immunity, 34*, 39-42. doi:10.1016/j.bbi.2013.05.009

Blask, D. E., Brainard, G. C., Dauchy, R. T., Hanifin, J. P., Davidson, L. K., Krause, J. A., Sauer, L. A., Rivera-Bermudez, M. A., Dubocovich, M. L., Jasser, S. A., Lynch, D. T., Rollag, M. D., & Zalatan, F. (2005). Melatonin-depleted blood from premenopausal women exposed to light at night stimulates growth of human breast cancer xenografts in nude rats. *Cancer Research, 65*(23), 11174-11184. doi:10.1158/0008-5472.can-05-1945

Blask, D. E., Dauchy, R. T., Brainard, G. C., & Hanifin, J. P. (2009). Circadian Stage-Dependent Inhibition of Human Breast Cancer Metabolism and Growth by the Nocturnal Melatonin Signal: Consequences of Its Disruption by Light at Night in Rats and Women. *Integrative Cancer Therapies, 8*(4), 347-353. doi:10.1177/1534735409352320

Botha, L. M., Jones, T. M., & Hopkins, G. R. (2017). Effects of lifetime exposure to artificial light at night on cricket (Teleogryllus commodus) courtship and mating behaviour. *Animal Behaviour, 129*, 181-188. doi:http://dx.doi.org/10.1016/j.anbehav.2017.05.020

Braby, M. F., & Jones, R. E. (1995). Reproductive patterns and resource-allocation in tropical butterflies - Influence of adult diet and seasonal phenotype on fecundity, longevity and egg size. *Oikos, 72*(2), 189-204. doi:10.2307/3546221

Brainard, G. C., Richardson, B. A., Petterborg, L. J., & Reiter, R. J. (1982). The effect of different light intensities on pineal melatonin content. *Brain Research, 233*(1), 75-81. doi:10.1016/0006-8993(82)90931-3

Brent, M., & Oster, I. (1974). Nutritional substitution-a new approach to microbial control for Drosophila cultures. *Drosophila Information Service, 51*, 155-157.

Callebert, J., Jaunay, J. M., & Jallon, J. M. (1991). *Control of Drosophila biorhythms* (Vol. 5).

Carrière, Y., & Roff, D. A. (1995). The evolution of offspring size and number: a test of the Smith-Fretwell model in three species of crickets. *Oecologia, 102*(3), 389-396. doi:10.1007/BF00329806

Clutton-Brock, T. (1988). *Reproductive success: studies of individual variation in contrasting breeding systems* (T. H. Clutton-Brock Ed.). Chicago, USA: University of Chicago Press.

Cobb, M., & Jallon, J. M. (1990). Pheromones, mate recognition and courtship stimulation in the Drosophila melanogaster species subgroup. *Animal Behaviour, 39*, 1058-1067. doi:10.1016/s0003-3472(05)80778-x

Cree, L. M., Hammond, E. R., Shelling, A. N., Berg, M. C., Peek, J. C., & Green, M. P. (2015). Maternal age and ovarian stimulation independently affect oocyte mtDNA copy number and cumulus cell gene expression in bovine clones. *Human Reproduction, 30*(6), 1410-1420. doi:10.1093/humrep/dev066

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., & Gagnon, C. (1997). Reactive oxygen species and sperm physiology. *Reproduction, 2*, 48-54. doi:10.1530/ror.0.0020048

Dominoni, D., Goymann, W., Helm, B., & Partecke, J. (2013). Urban-like night illumination reduces melatonin release in European blackbirds (Turdus merula): implications of city life for biological time-keeping of songbirds. *Frontiers in Zoology, 10*, 10. doi:10.1186/1742-9994-10-60

Dominoni, D., Quetting, M., & Partecke, J. (2013). Artificial light at night advances avian reproductive physiology. *Proceedings of the Royal Society B-Biological Sciences, 280*(1756), 8. doi:10.1098/rspb.2012.3017

Dowling, D. K., & Simmons, L. W. (2009). Reactive oxygen species as universal constraints in life-history evolution. *Proceedings of the Royal Society B-Biological Sciences, 276*(1663), 1737-1745. doi:10.1098/rspb.2008.1791

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews, 82*(1), 47-95. doi:10.1152/physrev.00018.2001

Endler, J. (1987). Predation, light intensity and courtship behaviour in Poecilia reticulata (Pisces: Poeciliidae). *Animal Behaviour, 35*(5), 1376-1385. doi:http://dx.doi.org/10.1016/S0003-3472(87)80010-6

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature, 408*(6809), 239-247. doi:10.1038/35041687

Finocchiaro, L., Callebert, J., Launay, J. M., & Jallon, J. M. (1988). Melatonin biosysnthesis in Drosophila - its nature and its effects. *Journal of Neurochemistry, 50*(2), 382-387. doi:10.1111/j.1471-4159.1988.tb02923.x

Frisard, M., Broussard, A., Davies, S., Roberts, I., Rood, J., de Jonge, L., Fang, X., Jazwinski, S., Deutsch, W., & Ravussin, E. (2007). Aging, Resting Metabolic Rate, and Oxidative Damage: Results From the Louisiana Healthy Aging Study. *The Journals of Gerontology: Series A, 62*(7), 752-759. doi:10.1093/gerona/62.7.752

Gillott, C. (2003). Male accessory gland secretions: Modulators of female reproductive physiology and behavior. *Annual Review of Entomology, 48*, 163-184. doi:10.1146/annurev.ento.48.091801.112657

Hardeland, R., Coto-Montes, A., & Poeggeler, B. (2003). Circadian rhythms, oxidative stress, and antioxidative defense mechanisms. *Chronobiology International, 20*(6), 921-962. doi:10.1081/cbi-120025245

Harman, D. (1956). Aging - A theory based on free-radical and radiation-chemistry. *Journals of Gerontology, 11*(3), 298-300. doi:10.1016/0921-8734(92)90030-S

Helfrich-Forster, C., Winter, C., Hofbauer, A., Hall, J. C., & Stanewsky, R. (2001). The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron, 30*(1), 249-261. doi:10.1016/s0896-6273(01)00277-x

Higgie, M., Chenoweth, S., & Blows, M. W. (2000). Natural selection and the reinforcement of mate recognition. *Science, 290*(5491), 519-521. doi:10.1126/science.290.5491.519

Howard, R. W., & Blomquist, G. J. (2005). Ecological, behavioral, andbiochemical aspects of insect hydrocarbons. *Annual Review of Entomology* (Vol. 50, pp. 371-393). Palo Alto: Annual Reviews.

Jann, P., & Ward, P. I. (1999). Maternal effects and their consequences for offspring fitness in the Yellow Dung Fly. *Functional Ecology, 13*(1), 51-58. doi:10.1046/j.1365-2435.1999.00269.x

Jones, T. M., Durrant, J., Michaelides, E. B., & Green, M. P. (2015). Melatonin: a possible link between the presence of articial light at night and reductions in biological fitness. *Philosophical Transactions B, 370*(1667), 1-10. doi:10.1098/rstb.2014.1022

Kyba, C. C. M., Kuester, T., Sánchez de Miguel, A., Baugh, K., Jechow, A., Hölker, F., Bennie, J., Elvidge, C. D., Gaston, K. J., & Guanter, L. (2017). Artificially lit surface of Earth at night increasing in radiance and extent. *Science Advances, 3*(11). doi:10.1126/sciadv.1701528

Lefevre, G., & Jonsson, U. B. (1962). Sperm transfer, storage, displacement, and uutilization in Drosophila melanogaster. *Genetics, 42*(12), 1719-&.

LeTallec, T., Thery, M., & Perret, M. (2015). Effects of light pollution on seasonal estrus and daily rhythms in a nocturnal primate. *Journal of Mammalogy, 96*(2), 438-445. doi:10.1093/jmammal/gyv047

Longcore, T., & Rich, C. (2004). Ecological Light Pollution. *Frontiers in Ecology and the Environment, 2*(4), 191-198. doi:10.2307/3868314

Magnhagen, C. (1991). Predation risk as a cost of reproduction. *Trends in Ecology & Evolution, 6*(6), 183-185. doi:10.1016/0169-5347(91)90210-o

McLay, L. K., Green, M. P., & Jones, T. M. (2017). Chronic exposure to dim artificial light at night decreases fecundity and adult survival in Drosophila melanogaster. *Journal of Insect Physiology, 100*, 15-20. doi:10.1016/j.jinsphys.2017.04.009

Metcalfe, N. B., & Alonso-Alvarez, C. (2010). Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Functional Ecology, 24*(5), 984-996. doi:10.1111/j.1365-2435.2010.01750.x

Navara, K. J., & Nelson, R. J. (2007). The dark side of light at night: physiological, epidemiological, and ecological consequences. *Journal of Pineal Research, 43*(3), 215-224. doi:10.1111/j.1600-079X.2007.00473.x

Neretti, N., Wang, P. Y., Brodsky, A. S., Nyguyen, H. H., White, K. P., Rogina, B., & Helfand, S. L. (2009). Long-lived Indy induces reduced mitochondrial reactive oxygen species production and oxidative damage. *Proceedings of the National Academy of Sciences of the United States of America, 106*(7), 2277-2282. doi:10.1073/pnas.0812484106

Pandi-Perumal, S. R., Srinivasan, V., Maestroni, G. J. M., Cardinali, D. P., Poeggeler, B., & Hardeland, R. (2006). Melatonin - Nature's most versatile biological signal? *Febs Journal, 273*(13), 2813-2838. doi:10.1111/j.1742-4658.2006.05322.x

Partridge, L., Fowler, K., Trevitt, S., & Sharp, W. (1986). An examination of the effects of males on the survival and egg-production rates of female Drosophila melanogaster. *Journal of Insect Physiology, 32*(11), 925-929. doi:10.1016/0022-1910(86)90140-x

Poli, G., Leonarduzzi, G., Biasi, F., & Chiarpotto, E. (2004). Oxidative stress and cell signalling. *Current Medicinal Chemistry, 11*(9), 1163-1182.

Qazi, M. C. B., Heifetz, Y., & Wolfner, M. F. (2003). The developments between gametogenesis and fertilization: ovulation and female sperm storage in Drosophila melanogaster. *Developmental Biology, 256*(2), 195-211. doi:10.1016/s0012-1606(02)00125-2

Raap, T., Casasole, G., Costantini, D., AbdElgawad, H., Asard, H., Pinxten, R., & Eens, M. (2016). Artificial light at night affects body mass but not oxidative status in free-living nestling songbirds: an experimental study. *Scientific Reports, 6*, 35626. doi:10.1038/srep35626

Reiter, R. J., Tan, D. X., & Fuentes-Broto, L. (2010). Melatonin: a multitasking molecule. *Neuroendocrinology: The Normal Neuroendocrine System, 181*, 127-151. doi:10.1016/s0079-6123(08)81008-4

Reiter, R. J., Tan, D. X., Mayo, J. C., Sainz, R. M., Leon, J., & Czarnocki, Z. (2003). Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. *Acta Biochimica Polonica, 50*(4), 1129-1146.

Ritsick, D. R., Edens, W. A., Finnerty, V., & Lambeth, J. D. (2007). Nox regulation of smooth muscle contraction. *Free Radical Biology and Medicine, 43*(1), 31-38. doi:10.1016/j.freeradbiomed.2007.03.006

Robert, K. A., Lesku, J. A., Partecke, J., & Chambers, B. (2015). Artificial light at night desynchronizes strictly seasonal reproduction in a wild mammal. *Proceedings of the Royal Society B-Biological Sciences, 282*(1816), 7.

Sakai, T., & Ishida, N. (2001). Circadian rhythms of female mating activity governed by clock genes in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America, 98*(16), 9221-9225. doi:10.1073/pnas.151443298

Salin, K., Auer, S. K., Rudolf, A. M., Anderson, G. J., Cairns, A. G., Mullen, W., Hartley, R. C., Selman, C., & Metcalfe, N. B. (2015). Individuals with higher metabolic rates have lower levels of reactive oxygen species in vivo. *Biology Letters, 11*(9). doi:10.1098/rsbl.2015.0538

Sanders, D., Kehoe, R., Tiley, K., Bennie, J., Cruse, D., Davies, T. W., van Veen, F. J. F., & Gaston, K. J. (2015). Artificial nighttime light changes aphid-parasitoid population dynamics. *Scientific Reports, 5*, 7. doi:10.1038/srep15232

Sohal, R. S., & Weindruch, R. (1996). Oxidative Stress, Caloric Restriction, and Aging. *Science, 273*(5271), 59-63.

Tan, D. X., Hardeland, R., Manchester, L. C., Paredes, S. D., Korkmaz, A., Sainz, R. M., Mayo, J. C., Fuentes-Broto, L., & Reiter, R. J. (2010). The changing biological roles of melatonin during evolution: from an antioxidant to signals of darkness, sexual selection and fitness. *Biological Reviews of the Cambridge Philosophical Society, 85*(3), 607-623. doi:10.1111/j.1469-185X.2009.00118.x

Tanaka, Y. (1990). Ae specificity of inbreeding depression during a lify-cycle of Callosobruchus chinensis (Coleoptera, Bruchidae). *Researches on Population Ecology, 32*(2), 329-335. doi:10.1007/bf02512567

van Geffen, K. G., Groot, A. T., Van Grunsven, R. H. A., Donners, M., Berendse, F., & Veenendaal, E. M. (2015). Artificial night lighting disrupts sex pheromone in a noctuid moth. *Ecological Entomology, 40*(4), 401-408. doi:10.1111/een.12202

van Geffen, K. G., van Eck, E., de Boer, R. A., van Grunsven, R. H. A., Salis, L., Berendse, F., & Veenendaal, E. M. (2015). Artificial light at night inhibits mating in a Geometrid moth. *Insect Conservation and Diversity, 8*(3), 282-287. doi:10.1111/icad.12116

Venkatesh, S., Deecaraman, A., Kumar, R., Shamsi, M. B., & Dada, R. (2009). Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. *Indian Journal of Medical Research, 129*(2), 127-137.

Vivien-Roels, B., & Pevet, P. (1993). Melatonin - Presence and formation in invertebrates. *Experientia, 49*(8), 642-647. doi:10.1007/bf01923945

Wang, H., & Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology and Medicine, 27*(5-6), 612-616. doi:10.1016/s0891-5849(99)00107-0

Wolfner, M. F. (2002). The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in Drosophila. *Heredity, 88*, 85-93. doi:10.1038/sj/hdy/6800017

Table 1: Statistical models exploring the effect of artificial light at night (ALAN) on reproductive output in *D. melanogaster.* Besides the main variable of light treatment only those variables contributing to the minimal adequate model are reported. All statistics are mean ± standard error except where indicated.

Model Parameters	Mean ± SE or Proportions		Statistic, P value
	0 Lux	10 Lux	
Mating Behaviour			
a) Number of pairs commencing copulation in 30 minutes			
Light treatment	62/68	58/68	$\chi^2 = 1.14$, P = 0.28
b) Time to commencement of courtship (log secs)			
Light treatment	4.60 ± 0.11	4.86 ± 0.14	$F_{1, 111} = 2.10$, P = 0.15
c) Time from commencement of courting to onset of copulation (log secs)			
Light treatment	4.51 ± 0.16	5.02 ± 0.17	$F_{1, 120} = 4.65$, **P = 0.03**
d) Time for copulation (cube root secs)			
Light treatment	9.60 ± 0.07	9.63 ± 0.07	$F_{1, 116} = 0.08$, P = 0.78

Model Parameters	Mean ± SE or Proportions		Statistic, P value
	0 Lux	10 Lux	
Oviposition and offspring development			
e) Number of females laying at least one egg at any egg count			
Light treatment	185/249	132/199	χ^2 = 0.62, P = 0.43
Maternal age			χ^2 = 28.40, **P < 0.005**
Maternal age x Maternal age			χ^2 = 17.45, **P < 0.005**
Light treatment x (Maternal age x Maternal age)			χ^2 = 6.89, **P = 0.01**
f) Cumulative number of eggs laid per female			
Light treatment	81.25 ± 2.79	72.88 ± 3.12	χ^2 = 2.15, P = 0.14
Maternal age			χ^2 = 127.31, **P < 0.005**
Maternal age x Maternal age			χ^2 = 15.78, **P < 0.005**
Light treatment x (Maternal age x Maternal age)			χ^2 = 12.79, **P < 0.005**
g) Egg to adult success – proportion of vials where eggs were laid that had any adults emerge			
Light treatment	38/65	31/54	χ^2= 0.01, P = 0.91
Maternal age			χ^2= 39.90, **P < 0.005**

Model Parameters	Mean ± SE or Proportions		Statistic, P value
	0 Lux	10 Lux	
h) Egg to adult success – proportion of eggs to emerge as adults where any adults emerged			
Light treatment	0.51 ± 0.03	0.48 ± 0.03	$\chi^2 = 0.81$ P = 0.37
i) Sex ratio of offspring (Days 3 and 20)			
Light treatment	0.50 ± 0.03	0.48 ± 0.03	$\chi^2 = 0.001$ P = 0.97

Table 2: Statistical models exploring the effect of artificial light at night (ALAN) on reactive oxygen species (ROS) levels and ovarian area. a) Total ovarian area; b) ROS levels in female heads; and c) ROS levels of ovaries; Besides the main variable of light treatment only those variables contributing to the minimal adequate model are reported. All statistics are mean ± standard error.

Model Parameters	Mean ± SE		Statistic, P value
	0 Lux	10 Lux	
Ovarian area			
a) Total ovarian area			
Light treatment	1.24 ± 0.06	1.27 ± 0.06	$F_{1, 110} = 0.10$, P = 0.75

Model Parameters	Mean ± SE		Statistic, P value
	0 Lux	10 Lux	
ROS			
b) ROS levels - female head			
Light treatment	234.76 ± 8.78	222.22 ± 9.93	$F_{1, 108} = 0.97$, P = 0.33
Female age			$F_{2, 108} = 5.64$, **P < 0.005**
c) ROS levels - ovaries			
Light treatment	111.07 ± 11.14	87.25 ± 6.88	$F_{1, 50} = 4.49$, **P = 0.04**
Female age			$F_{2, 50} = 5.05$, **P = 0.01**

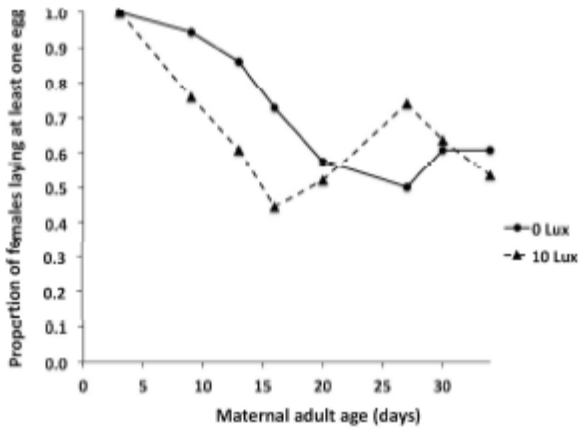

Figure 1 a) Proportion females ovipositing varying with maternal age (significant interaction term of treatment x maternal age x maternal age, P = 0.01 N = 348); and b) Mean cumulative number of eggs varying with maternal age (significant interaction term of treatment x maternal age x maternal age, P < 0.005, N = 305).

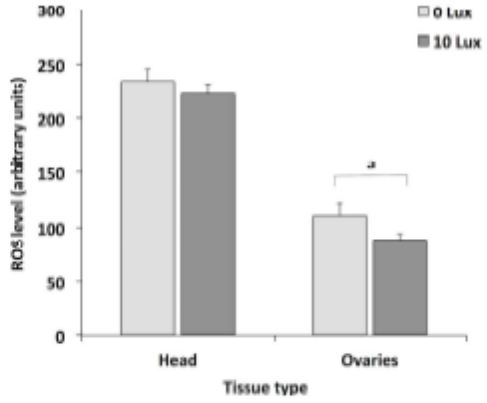

Figure 2 Comparative mean ROS levels (arbitrary relative units) in a) Heads and pooled ovarian samples (five pairs of ovaries per pool) combined for all ages for each light treatment (heads P = 0.33, N = 108); ovaries P = 0.04, N = 50); b) Heads varying with female age for each light treatment (P < 0.005, N = 108; and c) Pooled ovarian samples varying with female age for each light treatment (P = 0.01, N = 50). Different superscripts denote differences (P < 0.05) between; a) light treatment for ovarian samples; and b) & c) between female ages.