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Age determination in individual wild-caught *Drosophila serrata* using pteridine concentration

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Summary

Fluorescence spectrophotometry can reliably detect levels of the pteridine 6-biopterin in the heads of individual *Drosophila serrata* Malloch 1927. Pteridine content in both laboratory and field captured flies is typically a level of magnitude higher than the minimally detectable level ($mean_{lab}$=0.54 units, $mean_{field}$=0.44 units, minimum detectable level=0.01 units) and can be used to predict individual age in laboratory populations with high certainty (r^2=57%). Laboratory studies of individuals of known age (from 1 to 48 days old) indicate that while pteridine level increases linearly with age, they also increase in a linear manner with rearing temperature and ambient light levels, but are independent of sex. As expected, the longevity of laboratory-reared males (at least 48 days) is higher than the range of predicted ages of wild-caught males based on individual pteridine levels (40 days). However, the predictive equation based on pteridine level alone suggested that a number of wild-caught males were less than 0 days old, and the 95% confidence limits for these predictions based on the inverse regression are broad. The age of the oldest wild-caught male is predicted to fall within the range of 2 to 50 days. The significant effects of temperature and light intensity determined in the laboratory study (effect sizes ω^2=14.3 and 20.4%, respectively) suggests that the calibration of the age prediction equation for field populations would be significantly improved when combined with fine-scaled studies of habitat temperature and light conditions. The ability to determine relative age in individual wild-caught *D. serrata* presents great opportunities for a variety of evolutionary studies on the dynamics of natural populations.

Key words: age determination, pteridine, *Drosophila serrata,* survivor function.

Introduction

The age structure of field *Drosophila* populations remains relatively unknown (Powell, 1997), despite the large potential benefits of knowing field survival probabilities in an organism that is also amenable to genetic experiments (Johnston and Ellison, 1982). Estimates in the field using data from capture–release experiments suggest modal longevities of a week or less in a number of *Drosophila* species (reviewed by Powell, 1997). A direct method of age determination in *Drosophila* has been developed (Johnston and Ellison, 1982) using counts of growth layers in internal thoracic muscle attachments (apodemes). This method was used to age field-caught *D. mercatorum* (Templeton et al., 1993), and it was found that the modal age class in two replicate years was 0–3 days old, with few individuals over 12 days old. Although this method may have application for aging younger field-caught flies, the maximum number of growth layers (<18) obtainable (Johnston and Ellison, 1982) may not allow for complete characterisation of the age structure of a population in many instances. Cytoplasmic incompatibility caused by Wolbachia infection in *D. simulans* decreased with male age (Turelli and Hoffman, 1995), which was used to infer that matings in natural populations must involve males at least 2 weeks old, although this does not supply a method of age determination that could be generally applied to other *Drosophila* species.

A number of biochemical techniques have also been used to predict the age of individual insects. Pteridines, a group of fluorescent chemicals derived from a pyrimidine–pyrazine ring structure (Ziegler and Harmsen, 1969), increase with chronological age in populations of various dipteran taxa populations reared under laboratory conditions (Langley et al., 1988; Mail and Lehane, 1988; McIntyre and Gooding, 1995). The relatively widespread occurrence of pteridines in insects (Ziegler and Harmsen, 1969), the ability to detect and characterize the small quantities found in individuals *via* fluorescence spectrophotometry (Lehane and Mail, 1985), and the relative ease of sample preparation [field samples can be desiccated prior to analysis (Lehane and Mail, 1985)] make

quantifying these chemicals a potentially useful technique for age determination of field populations.

Here, we examine of the utility of 6-biopterin concentration to predict the age in individual Drosophilidae using *Drosophila serrata*. *D. serrata* and its close relatives have been adopted as a model system for investigating the evolution of stress resistance (Hoffmann et al., 2003), morphology (Hoffmann and Shirriffs, 2002) and mate choice (Higgie et al., 2000; Hine et al., 2004), but in natural populations such studies are limited by a lack of detailed knowledge of the age of individuals. We present the results of three experiments. Firstly, we determine how pteridine levels increase with age for males and females under laboratory conditions. Secondly, we examine the interaction of temperature and light intensity on individual pteridine levels, which may vary under field conditions. Finally, we explore the utility of this technique for predicting age in individual wild-caught flies.

Materials and methods

Measurement of pteridine content

Tissue samples from *Drosophila serrata* Malloch 1927 were collected, dried and stored in light-proof conditions prior to pteridine analysis. Individual flies were anesthetized with CO_2, their heads removed with a scalpel and the heads then placed in individual amber 2.0 ml Eppendorf tubes that were half filled with silica gel. A circular piece of brass mesh was used to separate the silica gel from the fly heads while allowing desiccation to proceed. Eppendorf tubes + fly heads were maintained at room temperature under zero-light conditions for a period of at least 14 days and head mass was measured to the nearest 0.01 mg prior to pteridine extraction. Pteridine levels and tissue dry masses are known to be stable under these conditions (Lehane and Mail, 1985) (S. K. A. Robson, unpublished).

Pteridine levels in individual fly heads were determined using methods modified from Mail and Lehane (Mail and Lehane, 1988). Pteridines were first extracted from individual heads by grinding the tissue in liquid nitrogen for 60 s, then in a solution of 0.5 ml chloroform:methanol (2:1) on ice for another 60 s, followed by sonnication for 3 min. A solution of 0.75 ml 0.1 mol l^{-1} NaOH adjusted to pH 10 with glycine (ca. 11.5 g l^{-1}) was then added to optimize pteridine extraction, vortexed for 10 s at 1800 r.p.m. then centrifuged for 5 min at 5000 ***g*** at 4°C to remove any remaining particulate material. Pteridine concentrations were determined by taking a 0.7 ml sample of the supernatant, and analyzing with a Perkin Elmer Luminescence Spectrometer LS50B (Beaconsfield, Bucks, UK). Samples were excited with a wavelength of 355 nm, the emission spectra recorded between 300 and 600 nm, and the greatest intensity of the spectra (which corresponds to an emission wavelength of 445 nm) recorded in arbitrary units. Spectral intensity at this wavelength is proportional to 6-biopterin concentration (Mail and Lehane, 1988).

Confirmation of pteridine detection and quantification protocols

A series of serial dilutions using 6-biopterin (Sigma-Aldrich: Sydney, NSW, Australia), a principle component of dipteran pteridines (Mail and Lehane, 1988; Penilla et al., 2002; Wu and Lehane, 1999), were used to confirm that the protocols used were capable of accurately detecting and quantifying the levels of pteridines found in individual *D. serrata*. Firstly, 0.7 ml of a stock solution of 6-biopterin (10 000 μg l^{-1}) was treated as a tissue sample, extracted and characterized using the methods outlined above. The shape of the emission spectra was then compared to that obtained from individual flies using the same extraction method. To confirm our ability to quantify the pteridine levels found in individual flies, the stock solution of 6-biopterin was serially diluted with samples analysed at each step, until there were no longer any changes in the intensity of the emission spectra. These results were also compared with a control (extract solution only) and the data from individual flies to ensure that individual pteridine levels could be accurately quantified.

Assay of laboratory-reared D. serrata of known age

To determine the association between pteridine content and age, virgin male and female *D. serrata* from the Forster stock described previously (Higgie et al., 2000) were sexed and placed in standard food vials, five individuals of the same sex to a vial, with three replicate vials for each sex. This procedure was conducted at 18 time periods spanning 48 days, spaced from 2 to 5 days apart, resulting in 108 vials at the end of the experiment containing flies aged between 2 and 48 days. Vials were changed every 3 days to maintain the flies in good condition, and were kept at a constant temperature of 25°C in a room with a 12 h:12 h light:dark cycle at 170 lux. After 48 days, 335 flies had their heads removed, weighed, and then prepared for the pteridine content assay. Some of the cohorts of older flies suffered significant levels of mortality by the end of the experiment. Only vials that had at least two individuals surviving were used in the analysis and vial means were taken to be used as independent data.

The analysis of pteridine content was conducted using the linear model for an analysis of covariance (ANCOVA) testing for homogeneity of slope:

$$Y_{ij} = \mu + \mathrm{Sex}_i + \mathrm{Age}_j + \mathrm{Sex}_i{*}\mathrm{Age}_j + \mathrm{error}\,, \qquad (1)$$

where age (Age) was treated as a continuous variable, sex (Sex) was a fixed factor, subscripts i and j refer to the sex and age of the particular individual Y, μ is the population mean pteridine content, and the interaction term Sex*Age tested for a difference between sexes in the slope of the relationship between age and pteridine content. In a second ANCOVA, head mass replaced age in Eqn 1 to test for the effect of head mass on pteridine content.

Effect of temperature and light intensity on pteridine content

To determine how temperature and light intensity may affect pteridine content we conducted a two-way factorial experiment

using five temperatures (19, 21, 23, 25 and 27°C) and four light intensities. Light intensity was manipulated by covering the glass vials containing adult flies with neutral density polyester filters (Lee Filters, Andover, UK). Three grades of filter (209 0.3, 210 0.6, 211 0.9) were used to reduce light intensity to approximately 50%, 25% and 12.5% of ambient across a wavelength range of 400 to 700 nm, respectively. The fourth light intensity used was ambient in the temperature cabinets used for the experiment, which was generated by three 8-W fluorescent tubes. Five replicate vials, each containing five virgin 1-day-old males, were placed in each of the 20 treatment combinations, and left for 8 days. At the end of 8 days, flies had their heads removed and were prepared for the pteridine assay as above.

The analysis of pteridine content was conducted using the linear model for a two-way analysis of variance:

$$Y_{ij} = \mu + \text{Light}_i + \text{Temp}_j + \text{Light}_i*\text{Temp}_j + \text{error} , \quad (2)$$

where light intensity (Light) and temperature (Temp) were fixed effects, and subscripts i and j refer to the light intensity and temperature environments of the particular individual Y and μ is the population mean pteridine content. The effect size of each factor with pteridine content was determined by calculating ω^2, an estimate of the degree of association between each of these two fixed factors and pteridine in the population (Tabachnick and Fidell, 1989).

Survival analysis of laboratory populations

Estimates of survival were generated from the data collected during the assay of laboratory populations of known age, in order to provide a reference in which to assess the predicted ages of individual wild-caught flies. Data on the presence or absence of individual flies collected when vials were sampled represent left- and right-censored estimates of individual survival times, respectively (Allison, 1995). These estimates were converted into interval-censored data (Klein and Moeschberger, 2003) and nonparametric survival curves generated using the %ICE Macro (Peto, 1973) with SAS V8. Differences in survival between sexes was tested with the SAS procedure PROC LIFEREG with the distribution model optimized by minimizing the Akaike's information criterion (Collet, 1994).

Assay of field-caught D. serrata

Field-caught males of *D. serrata* were collected on the St Lucia campus of the University of Queensland on 20 February 2003 using banana bait, and 40 males were weighed and then prepared for the pteridine content assay on the same day. Brisbane has an average temperature of 25.3°C at 09:00 h and 27.4°C at 15:00 h during this month of the year.

To predict the ages of field-caught males, the data for laboratory males were initially placed into a multiple linear regression model, where pteridine content was predicted by age and head mass. Vial means for individual cohorts of laboratory males were used as independent units. As the beta coefficient for head mass was not significant (t=0.98, P=0.33) this term was removed and the model reduced to:

$$\text{Pteridine content} = \beta_0 + \beta_i\text{Age}_i + \text{error} . \quad (3)$$

This significant model ($F_{1,39}$=51.0, P<0.001, r^2=0.57) was then modified to form the following predictive equation for individual age of wild flies, based on pteridine content:

$$\text{Age} = (\text{Pteridine content} - 0.414) / 0.00579 . \quad (4)$$

The inverse confidence limits for the ages of individual wild-caught males predicted using inverse regression Eqn 4, were calculated by deriving the 95% fiducial limits (Draper and Smith, 1998). The derivation equation included the term '$\boldsymbol{g}$'.

Results

Confirmation of pteridine detection and quantification protocols

Fig. 1 compares the emission spectra of the 6-biopterin standard and the pteridine extracts from an individual fly head. There appears to be a significant similarity between the two, in terms of the range, shape and corresponding maximum wavelength of emission.

Fig. 2 shows the relationship between 6-biopterin concentration and emission intensities at 445 nm, for a subset of the range of concentrations examined. There was a significant linear relationship between the two variables in the range of 10 000 μg l^{-1} down to 1.2 μg l^{-1} (corresponding to an emission level of 0.01 arbitrary units, Pearson r^2=0.99, N=18). Lower concentrations of 6-biopterin (0.6–0.08 μg l^{-1}) and the control solution produced no emissions at 445 nm.

Emission intensities from individual laboratory-reared flies used in assaying the effect of age and sex on 6-biopterin levels (mean=0.54, s.d.=0.15, min.=0.13, max.=1.20, N=335) and individual field-caught flies used to predict the age of field-caught individuals (mean=0.44, s.d.=0.06, min.=0.32, max.=0.56, N=40), were all at least an order of magnitude greater than the minimum detectable level (0.01 arbitrary units, Fig. 2).

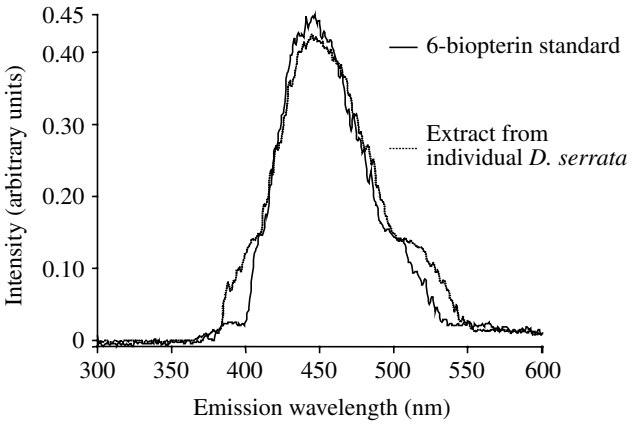

Fig. 1. Emission spectra for 6-biopterin standard and an extract from an individual *Drosophila serrata*.

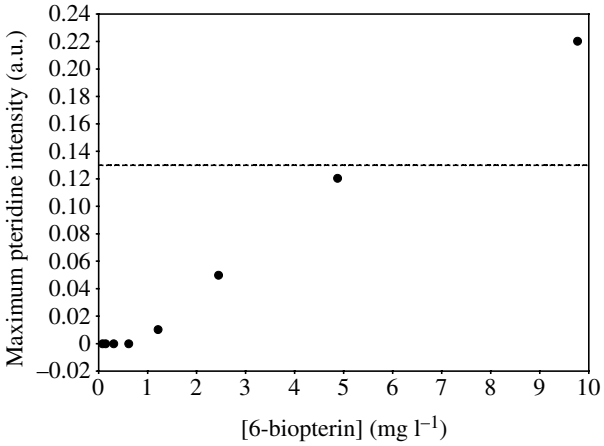

Fig. 2. The relationship between 6-biopterin concentration and emission intensity at 445 nm. The broken line indicates the minimum intensity recorded from an individual *D. serrata*. Pteridine concentrations can be detected down to emission levels of 0.01 arbitrary units (a.u.).

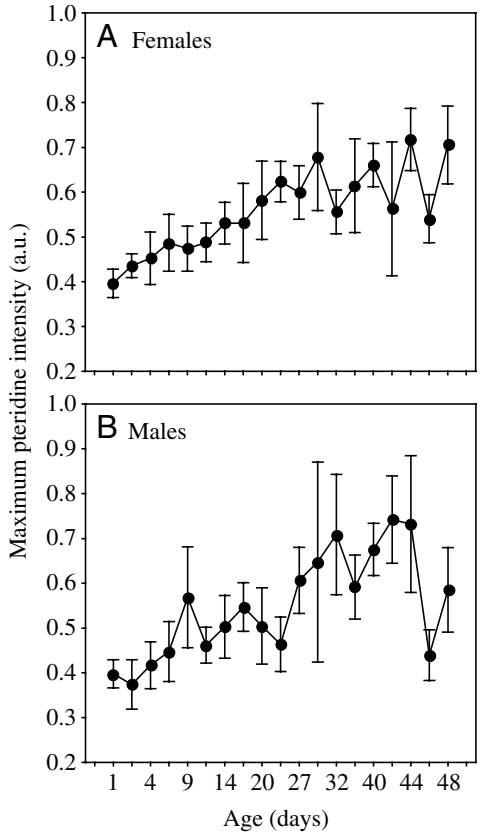

Fig. 3. The increase in pteridine content (intensity averaged over all individuals of an age class, ± 2 s.e.m.; for *N* values, see Materials and methods) with known age in laboratory-reared *D. serrata* females (A) and males (B). Linear regressions though the average values for each sex displayed here indicated that pteridine content was closely associated with age in females (r^2=0.917, $F_{1,16}$=175.8, P<0.001) and males (r^2=0.760, $F_{1,15}$=47.6, P<0.001).

Assay of laboratory-reared D. serrata of known age

Fig. 3 displays the increase in pteridine content over the 48 days of the experiment for both sexes. Pteridine content increased in both sexes by approximately 75% over the 48-day period. ANCOVA determined that pteridine content was strongly affected by age ($F_{1,87}$=153.97, P<0.001), and the linear relationship between pteridine content and age did not differ in slope between the sexes ($F_{1,87}$=0.16, P=0.688). Head mass was positively associated with pteridine content, but not significantly so ($F_{1,87}$=2.03, P=0.158), and the relationship between head mass and pteridine content did not differ significantly between the sexes ($F_{1,87}$=0.92, P=0.341).

Effect of temperature and light intensity on pteridine content

Two-way ANOVA indicated that temperature had a significant effect on pteridine content ($F_{4,73}$=4.41, P=0.003), increasing with temperature under all four light intensities (Fig. 4). Light intensity also had a significant effect on pteridine content ($F_{3,73}$=11.45, P<0.001), with increasing levels found under higher light intensity. There was no evidence for an interaction between these two factors on pteridine content ($F_{12,73}$=0.94, P=0.509). Estimates of the association between temperature and pteridine and light intensity and pteridine (population effect size ω^2) were 14.3% and 20.4%, respectively.

Survival analysis of laboratory populations

Estimated survivor functions for female and male laboratory populations of *D. serrata* modeled with a logistic distribution are presented in Fig. 5. Median survivorship values for females and males were 39.2 and 30.7 days, respectively, and the estimated survivorship functions differed significantly between females and males (Wald χ^2=4.3, P<0.05, d.f.=1). Although it was not possible to estimate the maximum longevity of females and males, individuals of both sex remained alive at the final census date, 48 days.

Assay of field-caught D. serrata

Predicted ages of field-caught males suggest that individuals differed in age by up to 40 days, with a mean predicted age of 6 days and a minimum and maximum age of –15.6 and 25.9 days, respectively (Fig. 6). The 95% inverse regression confidence limits of the predicted ages are considerable, suggesting that the age of the oldest wild-caught fly fell within the range of 2 to 50 days (Fig. 7).

Discussion

This study confirms the presence of detectable levels of pteridines in *Drosophila serrata* and supports previous studies suggesting that pteridines are widespread in dipteran insects (Ziegler and Harmsen, 1969). Pteridines have now been detected in 28 species from at least eight dipteran families (e.g. Table 1). Confirming detectable levels of pteridines in *D. serrata* is a necessary step in evaluating their suitability as age predictors, for although pteridine concentrations can usually be

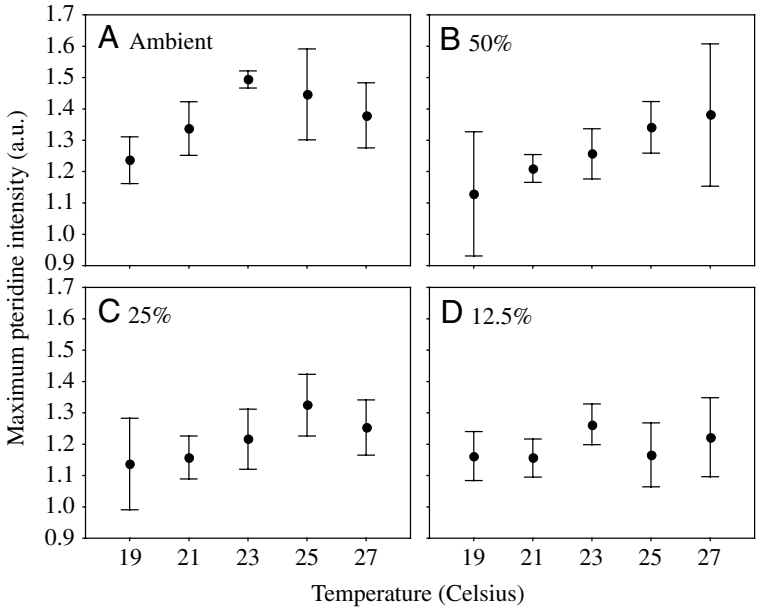

Fig. 4. The effect of temperature and light intensity on pteridine content (intensity averaged over all individuals of each age class, ± 2 s.e.m., for *N* values, see Materials and methods). (A) Cabinet ambient light intensity. (B) 50% light intensity. (C) 25% light intensity. (D) 12.5% light intensity.

measured in single individuals, this is not always the case. Lardeux et al. (Lardeux et al., 2000) were unable to detect pteridine in individuals of two very small dipteran species, *Aedes polynesiensis* and *Culex quinquefasciatus* (Table 1), and were forced to pool samples among individuals to examine the relationship between pteridine concentrations and age. The inability to detect pteridines at the individual level in these two species probably reflects in part the reduced levels found in these species and the limitations of the extraction and detection techniques used. Wu and Lehane (Wu and Lehane, 1999), for example, were able to detect pteridines in similarly sized *Anopholes gambiae* and *A. stephensi* by using reverse-phase HPLC.

Pteridine levels in *D. serrata* are positively correlated with individual age, rearing temperature and rearing light intensity conditions, but do not differ significantly between males and females. Increasing pteridine levels with age is commonly found within the Diptera, with a few exceptions in the Culicidae and Glossinidae (Table 1). Species within the Culicidae show either a negative relationship between age and pteridine levels (e.g. *Anopheles albimanus, A. gambiae, A. stephensidipterans*) or no relationship at all (e.g. *Aedes polynesiensis*), while in contrast to five other glossinid species, pteridine levels in *Glosinna austensi* show no relationship to individual age. Explanations for the lack of a positive relationship between pteridine levels and age in these taxa are currently lacking, and the low r^2 values in the studies using *G. austensi* indicate that pteridine levels can be poor predictors of individual age, even when a significant relationship exists between the two variables (Penilla et al., 2002). In our case, over 70% of the variation in pteridine levels was explained by variation in age in both sexes under laboratory conditions, suggesting that this approach is more promising in *Drosophila*.

The influence of sex on pteridine levels also appears variable within the Diptera, with sex effects being detected in four of the seven studies designed to test for it (Table 1). Temperature effects on pteridine levels are much more consistent. Increased adult rearing temperatures resulted in elevated pteridine levels in eight of the nine species in which this was examined (Table 1). The lack of a temperature effect in *Simulium sirbanum* is puzzling, given the predicted positive effect of temperature on pteridine levels based on enzyme kinetics (Moon and Krafsur, 1995).

The significant effect of light intensity on the pteridine levels of adults increases our understanding of the factors influencing the deposition of pteridines. Pteridines are typically concentrated in the heads of insects where they function as

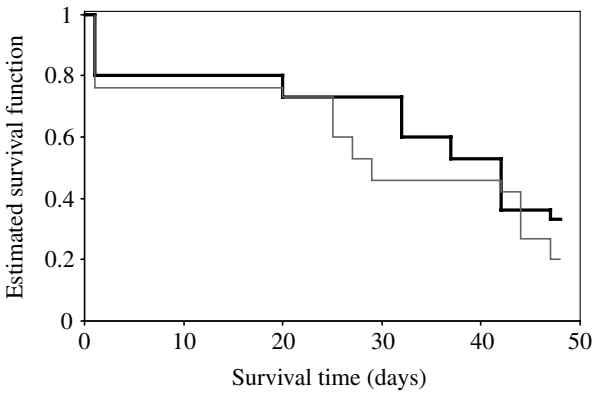

Fig. 5. Estimated survivor functions for laboratory populations of female (bold line) and male (thin line) *D. serrata*.

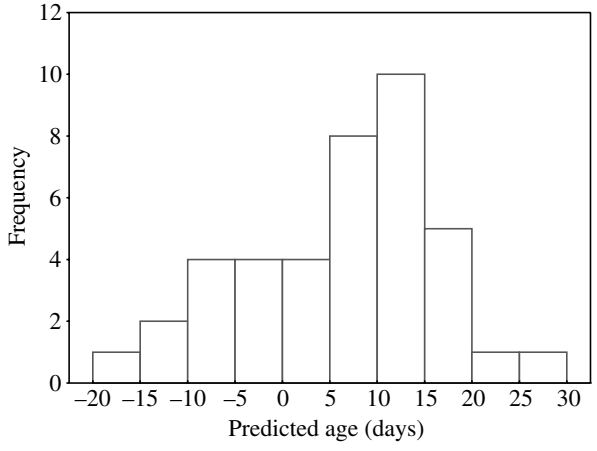

Fig. 6. Distribution of the predicted ages of field-caught *D. serrata* males.

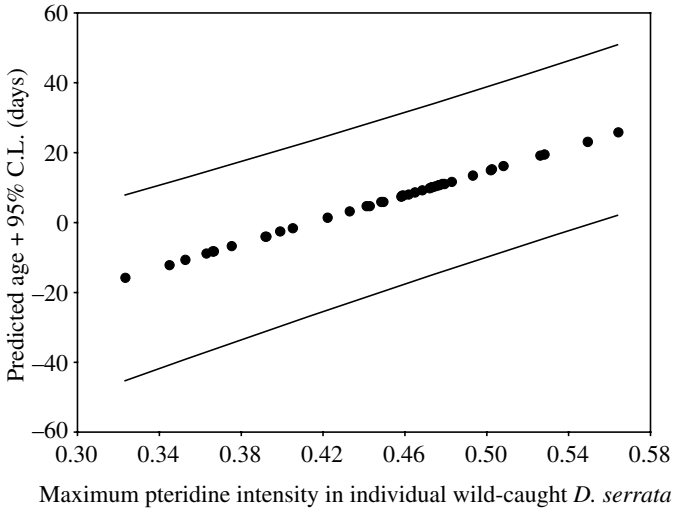

Fig. 7. Inverse regression 95% confidence limits (C.L.) for the predicted ages of field-caught *D. serrata* males (circles).

visual pigments (Ziegler and Harmsen, 1969). Yet although previous studies have claimed that pteridines accumulate in insects when stimulated by sunlight (Hanser, 1948), cited by Penilla et al. (Penilla et al., 2002) there have been limited tests of this relationship. No differences were noted in the pteridine levels of adults hatched under different ultra-violet conditions (Langley et al., 1988), but details on the duration of the exposure to the modified lighting conditions were not provided. Longer durations such as those utilized in this study of *D. serrata* may have produced comparable light intensity effects.

Numerous factors influence the level of pteridines in individual dipterans, in addition to the previously discussed factors of age, sex, rearing temperature and light intensity. Different genetic strains of *Drosophila melanogaster* accumulate pteridines at different rates as they age (McIntyre and Gooding, 1996) and diet and mating frequency may also effect pteridine accumulation. The pteridine levels of parous female *Culicoides variipennis sonorensis* that have fed on blood are less than in those of nulliparous females that have not, while pteridine levels in female *Anopheles albimanus* increase with the number of blood meals (Penilla et al., 2002). Although studies of the presence of particular pteridines in insects and the biochemical pathways associated with their accumulation continue (e.g. Fan et al., 1976; Parisi et al., 1976; Silva et al., 1991), the link between these processes and the behavioral and ecological factors that modify them unfortunately remains obscure.

How suitable are pteridine levels as predictors of age in individual wild-caught flies, particularly in light of the variety of internal and external factors that influence individual pteridine levels and the difficulty in measuring the exposure of wild individuals to these variables? Wild populations exist in a world of numerous microhabitats, for example, each with their own temperature and light intensity regimes that vary on a daily and seasonal basis (Endler, 1993). In some cases researchers have measured hourly temperatures, the number of sunlight hours and the roosting behaviour of field populations and used this to increase the predictive power of their equations (e.g. Mail et al., 1983). Individual pteridine levels have also been combined with other morphological factors such as the stage of ovarian development to improve predictive power (Wall et al., 1991). Alternatively, others have made no attempt to incorporate temperature effects into their predictive equations, based on the rationale that the actual body temperatures of wild flies could differ significantly from ambient temperatures anyway (e.g. Langley et al., 1988).

The relatively high number of flies predicted to have negative ages in this study (Fig. 6) indicates that the predictive equation derived from laboratory populations, which does not include information on rearing temperatures and light exposures, currently underestimates the age of wild-caught flies. The significant effects of temperature and light on individual pteridine intensity demonstrated in the laboratory study (14.3% and 20.4%, respectively), however, strongly suggest that the detection of the temperature and light conditions experienced by individual flies and the incorporation of this information would significantly improve age determination in wild-caught flies. Such improvements have been achieved in some studies (e.g. Mail et al., 1983), but in general we have a very limited understanding of the variation in temperature and light conditions experienced in wild populations of *Drosophila* (Feder, 1996). The small size of adult *Drosophila* (Feder et al., 2000) and the potential for dispersal over many km (Coyne and Milstead, 1987), currently preclude the direct detection of the microhabitats inhabited by individuals. However, indirect evidence suggests the realized effect of individual variability in temperature and light exposures in field populations may not be as significant as suggested by laboratory studies. For although numerous laboratory-based studies have demonstrated the significant effects of temperature on many aspects of the developmental biology of *Drosophila* (e.g. Hoffman, 1995; Partridge et al., 1995), the ability of free-living individuals to actively select and inhabit a limited range of microhabitats, and hence the ‘ecological relevance’ of such factors in free-living populations, is less clear (Feder et al., 2000). Indirect estimates involving the expression of two temperature-sensitive reporter genes, for example, suggest that free-living *D. melanogaster* experience temperature stress relatively infrequently (Feder et al., 2000) and so the individual variability in temperature and light microhabitats may be less than that of the environment as a whole. Ultimately, the ability to determine the temperature and light conditions experienced by individual flies may depend on a combination of technological advances, the extent to which temperature and light influence individual pteridine levels and the specific ecology of the target species. Rainforest specialists such a *D. serrata*, inhabiting environments with relatively reduced variability in microhabitat conditions, may prove to be more appropriate species for the calibration of real age predictive equations.

The utility of the pteridine method for age determination in individuals from the wild therefore depends on the type of

Table 1. *Studies examining the relationship between pteridine levels and age in Dipterans*[1]

Family (Genus species)	Sex effect[2]	Temp effect[3]	Age effect[4]	Max age (days)[5]	Study
Calliphoridae					
Chrysomya bezziana		Yes	+*		(Wall et al., 1990)
Cochliomyia hominivorax		Yes	+	25	(Thomas and Chen, 1989)
Lucilia sericata					
Male	Yes	Yes	+*	18	(Wall et al., 1991)
Female	Yes	Yes	+*	15	(Wall et al., 1991)
Ceratopogonidae					
Culicoides variipennis sonorensis	Yes		+	5	(Mullens and Lehane, 1995)
Culicidae					
Aedes polynesiensis			None	12	(Lardeux et al., 2000)
Anopheles albimanus			–*	25	(Penilla et al., 2002)
Anopheles gambiae			–*	40	(Wu and Lehane, 1999)
Anopheles stephensi			–*	40	(Wu and Lehane, 1999)
Culex quinquefasciatus			None	12	(Lardeux et al., 2000)
Drosophilidae					
Drosophila serrata	No	Yes	+	48	This study
Glossinidae					
Glossina austeni			None	90	(Langley et al., 1988)
Glossina morsitans morsitans			+	94	(Langley et al., 1988)
Glossina morsitans morsitans					
Male	No	Yes	+	63	(Lehane and Mail, 1985)
Female	No	Yes	+	140	(Lehane and Mail, 1985)
Glossina morsitans morsitans			+	86	(McIntyre and Gooding, 1996)
Glossina pallidipes			+	93	(Langley et al., 1988)
Glossina palpalis palpalis			+	102	(Langley et al., 1988)
Glossina tachinoides			+	103	(Langley et al., 1988)
Muscidae					
Hematoba irritans irritans			+	15	(Krafsur et al., 1992)
Musca autumnalis	Yes	Yes	+	12	(Moon and Krafsur, 1995)
Musca domestica		Yes	+*	17	(McIntyre and Gooding, 1995)
Stomoxys calcitrans	No	Yes	+	24	(Mail et al., 1983)
Simulidae					
Simulium damnosum			+	13	(Millest et al., 1992)
Simulium sanctipauli			+	9	(Millest et al., 1992)
Simulium sirbanum		No	+	22	(Millest et al., 1992)
Simulium soubrense			+	17	(Millest et al., 1992)
Simulium squamosum			+	16	(Millest et al., 1992)
Simulium yahense			+	11	(Millest et al., 1992)
Tephritidae					
Ceratis capitata			+	28	(Camin et al., 1991)
Anastrepha ludens			+*	28	(Tomic-Carruthers et al., 1996)
Bactrocera cucurbitae	Yes		+*	37	(Mochizuki et al., 1993)

[1]Table refers to studies looking for a relationship between age and pteridine levels only.

[2]Empty cells in the Table mean that the relevant effect was not examined or could not be resolved.

[3]Yes, positive relationship between temperature and pteridine levels. No, no relationship between temperature and pteridine levels.

[4]+, positive linear effect of age. +*, positive asymptotic effect of age; None, no effect of age.

[5]Maximum age of individuals used to test the relationship between age and pteridine levels. Separate maximum ages are provided for males and females when they differed.

questions asked and the degree of precision required. Most pteridine studies have involved species of significant agricultural or medical importance due to their role as vectors of animal or human disease (e.g. Langley et al., 1988; Tomic-Carruthers et al., 1996; Wu and Lehane, 1999). The goal of these studies has therefore been to determine the exact age of individuals in the wild to better understand life history and transmission dynamics of the diseases they carry. In these studies the influence of such factors as ambient temperature, sex and light on absolute pteridine levels can make predicting

absolute age difficult and require the formulation of species-specific predictive equations.

For many general evolutionary questions in such areas as mate choice and life-history evolution, however, where individuals choose from available options, it is knowledge of an individual's relative rather than its absolute age that is important. In these cases, the pteridine methods outlined here represent a significant improvement on other approaches. Even if temperature and light regimes differ on a daily basis, the linear and non-interacting effect of these two variables means that the pteridine levels of older flies should be greater than that of younger flies.

Greater certainty may also be obtained by 'trading-off' the ability to predict the age of single *versus* a group of individuals against the certainty of these predictions. The confidence limits associated with the inverse regression predictions decrease significantly when the dependent variable used to predict the independent variable, in this case pteridine concentration predicting age, is based on the mean of a sample of individuals rather than a single individual. In some cases it might be worth combining the pteridine concentrations of subgroups of individuals, in order to calculate predicted ages with greater certainty.

The age of *Drosophila* in the field has typically been suggested to be less than 2 weeks in a variety of species (Powell, 1997). The possibility that flies under field conditions survive for over 4 weeks, as suggested by our analysis, suggests that quantifying life-history traits and mating success at ages greater than those commonly used in laboratory studies may be important.

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