The lifecycle of *Agrilus biguttatus*: the role of temperature in its development and distribution, and implications for Acute Oak Decline

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Running title: The lifecycle of *Agrilus biguttatus*

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Abstract

1. The two spotted oak buprestid, *Agrilus biguttatus* Fabricus, is implicated in oak decline events across Europe, and is strongly linked to Acute Oak Decline in the UK, although its role in the syndrome remains under investigation. In the UK, the beetle is restricted to south and central England. The aims of this study were to improve understanding of the beetle's life history and thermal requirements, in order to explain its UK distribution, and collect data for lifecycle modelling.
2. Novel methods were developed to collect and culture the beetle in the laboratory, which enabled experiments to be carried out, providing data on the beetle's sex ratio, longevity and fecundity, and the development rates of eggs, larvae, and pupae at constant temperatures.
3. On average, females lived for 63 days, and laid 82 eggs. Larvae developed through four instars. Sex ratio varied by site, with no overall trend apparent.
4. The development rates of eggs, larvae, and pupae (to adult emergence) had linear relationships with temperature, with lower developmental thresholds of 12.1, 11.9, and 15.1°C, respectively. For each life stage, day-degree values were calculated. Beetles appeared to have an obligatory prepupal diapause at all temperatures studied, up to and including 25°C.
5. The implications of the developmental findings for the beetle's current distribution, and the possible effects of climate change, are discussed. The beetle appears to be thermally limited in the UK, and if so, its distribution, and perhaps that of Acute Oak Decline, may alter under climate change.

Keywords: Acute Oak Decline, Buprestidae, climate change, day-degrees, developmental thresholds, prepupal diapause.

41 Introduction
42
43 The two spotted oak buprestid, also known as the two spotted oak borer, *Agrilus biguttatus* Fabricus
44 (Coleoptera: Buprestidae), is considered an increasingly important secondary pest of oak (Hartmann
45 & Blank, 1992; Thomas, *et al.* 2002; Sallé *et al.,* 2014). It has played a key role in large-scale oak
46 decline events across continental Europe and Russia from the 1900s onwards (Falck, 1918;
47 Starchenko, 1931), and its pest status in continental Europe and the UK appears to be increasing
48 (Moraal & Hilszczanski, 2000). Until relatively recently, *A. biguttatus* was considered rare in the UK
49 (Shirt *et al.*, 1987). Perceptions of the beetle's pest status in England, however, have been changing
50 since the 1990s, when *A. biguttatus* was first linked to Acute Oak Decline (AOD), a syndrome that
51 often leads to the rapid death of pedunculate and sessile oaks *(Quercus robur* L. and *Q. petraea*
52 (Matt.) Liebl.) (Gibbs and Greig, 1997). Buprestids like *A. biguttatus* typically develop only on
53 weakened hosts (Bellamy, 2003), or exceptionally on healthy but naïve hosts without co-evolved
54 resistance. For example, the introduced emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera:
55 Buprestidae) has caused the death of millions of susceptible ash trees in the United States and
56 Canada (Herms & McCullough, 2014).
57
58 *Agrilus biguttatus* appears to be strongly associated with AOD in the UK, although its precise role in
59 the syndrome is still under investigation. Acute Oak Decline is characterised by a number of key
60 symptoms on oak stems, in particular, the combination of fluid exudations from vertical splits between
61 the bark plates, inner bark necroses, and the larval galleries of *A. biguttatus* (Denman *et al.*, 2014).
62 Tree declines such as AOD are thought to be caused by a combination of predisposing factors (host
63 genetics or site), inciting factors (e.g. drought), and contributing factors (secondary pests or
64 pathogens) (Manion, 1981). Several pathogenic bacterial species have now been implicated in the
65 formation of the typical AOD lesions (Brady *et al.*, 2010; Denman *et al.*, 2012), but the nature of the
66 association with *A. biguttatus* remains unclear. In recent studies, exit holes of adult beetles were
67 found externally on 30-33% of symptomatic trees, but larval feeding galleries were found internally
68 adjacent to almost all examined lesions (Denman *et al.,* 2014; Brown *et al.*, 2017). *Agrilus biguttatus*
69 and AOD also share a similar UK distribution within southern and central England (Brown *et al.*,
70 2014). The beetle may be secondary, taking advantage of weakened and moribund trees, but could
71 contribute to the spread of the bacteria, the formation of necrotic lesions, or the death of trees, and so
72 may be integral to AOD (Brown *et al.*, 2014, 2017).
73
74 *Agrilus biguttatus* is cryptic and difficult to observe in nature, and as a result has been relatively little
75 studied. It has never been cultured from egg to adult in the laboratory and, therefore, its lifecycle has
76 been described only broadly (Brown *et al.*, 2014). Female beetles typically oviposit deep into bark
77 crevices on the trunk of mature oak trees, and larvae subsequently form feeding galleries at the
78 cambial interface. In continental Europe, the beetle has been reported to have a one or, more
79 commonly, two-year lifecycle, in which case the larvae overwinter as early instars and continue
80 feeding into the following summer (Klausnitzer, 1994; Moraal & Hilszczanski, 2000). Fully-grown

larvae create chambers in the outer bark, where they overwinter, and pupate in April or May. Adults emerge from D-shaped exit holes in early summer, two years after oviposition (Habermann & Preller, 2003), and feed and mate in the canopy.

Agrilus biguttatus is widespread throughout Europe, but reaches its northern-most limit in southern Sweden and, in the UK, around Manchester (Bily, 1982; Brown *et al.,* 2014). Temperature is likely to be a key limiting factor in *A. biguttatus*' distribution in the UK, with heat availability likely to be restrictive, rather than lethal summer or winter temperatures (Bale, 2002). Anecdotal evidence suggests the beetle is thermophilic, because open-grown trees are more frequently colonised, and, particularly in the early stages of host colonisation, galleries appear to be more prevalent on the warmer, south-facing side of stems (Vansteenkiste *et al.*, 2004).

In the UK, damage by wood and bark-boring beetles is projected to increase with climate change (Wainhouse *et al.,* 2016). In some cases this process is already apparent; disturbance by bark beetles in Europe, for example, has been shown to have escalated in the previous century, and has been projected to increase further over the next two decades (Seidl *et al.,* 2014). Wood and bark-boring beetles in the UK are likely to benefit from an increased availability of stressed host trees, weakened by more frequent and severe droughts and storms; a decrease in generational time, due to increased heat availability; and range expansion in thermally-limited species (Williams & Liebhold, 2002; Netherer & Schopf, 2010; Stocker *et al.,* 2014; Wainhouse & Inward, 2016). Warmer temperatures appear to have contributed to recent northward range shifts in two European oak borers, *Coraebus florentinus* Herbst (Coleoptera: Buprestidae) (Buse, *et al.*, 2013; Sallé *et al.*, 2014), and *Agrilus sulcicollis* Lacordaire (Coleoptera: Buprestidae), and may have contributed to a purported increase in abundance in the UK of *A. biguttatus,* and its recent spread into Denmark (Alexander, 2003; Pedersen & Jørum, 2009).

The relationship between insect development rate and temperature is usually sigmoidal, and therefore the central, linear section of the curve may be modelled simply as the accumulation of a thermal sum, measured in day-degrees, above a lower threshold, the minimum temperature below which development ceases (Ludwig, 1928; Danks, 2000). Precise data on insect life histories and thermal requirements are required to make accurate predictions of the effects of geographical location or a changing climate on an insect's voltinism, distribution, and abundance, but these data are not available for many wood and bark boring insects, including *A. biguttatus,* due to cryptic lifecycles and long development times (Sallé *et al.*, 2014).

This study aims to improve the understanding of *A. biguttatus*' lifecycle, to define the temperature thresholds and thermal requirements regulating the beetle's development, in order to explain its ecology and distribution in Britain, and to collect empirical data for modelling both current and future life history.

Methods

Collection of overwintering larvae, adult emergence and sex ratio

Quercus robur, > 30cm diameter, demonstrating exit holes of *A. biguttatus* and with well-developed AOD lesions, were identified at five sites in central England, within the beetle's core range (Table 1). In order to obtain adult *A. biguttatus* for breeding purposes, insects were collected in the overwintering, prepupal stage, and one or two suitable trees were felled between October 2013 and April 2014. Slabs from stems and large branches comprising bark, sapwood, and some heartwood (approximately 0.5 – 2.0m x 0.4m x 0.25m), referred to as 'material' from here onwards, were removed, transported to Alice Holt, Farnham, and placed in one of three emergence facilities as follows. (1) Material from each site was placed separately into custom-built emergence cages within a glasshouse. Wooden frames (approx. 2.0 x 1.7 x 1.4 m) were covered with two layers of mesh netting and fronted with PE zipped doors cut from Walk-In Greenhouse covers (Gardman Ltd., UK). To facilitate collection of adult beetles, which orientate towards light, cage roofs were angled upwards, facing southwest. (2) Once the emergence cages were full, additional material was placed in an insulated polytunnel. Material from all sites was combined, and covered with a layer of mesh. (3) To lengthen the study period, approximately 10% of material from one site, Dudmaston, was placed in three mesh emergence traps (B&S Entomological Services, UK) within a controlled temperature room. Average temperature was controlled to ± 2°C and increased weekly for 4 weeks from 13.1 to 21.8°C, with a 12 hour light / 12 hour dark photoperiod (28 March to 3 May 2014). Temperatures in each facility were monitored using Tinytag temperature data loggers (TGP-4520; Gemini Data Loggers Ltd., UK). Adult beetles were hand-collected daily. The gender of the emerging adult beetles was determined by examining the ventral surface of the hind femur, which, in males, is covered in a row of long setae, silver-to-brownish in colour. This character is easily discernible under a dissecting microscope.

Culturing of adults, female lifespan and fecundity

Based on culturing of congeneric species, particularly *A. planipennis,* techniques were developed to culture *A. biguttatus* adults in the laboratory (Duan *et al.,* 2011; Lopez & Hoddle, 2014; J. P. Lelito, pers. comm.). Beetles were caged in 2L clear PET Round Jars (203 mm height x 110 mm diameter), (Ampulla Ltd, UK), with the base removed and the top secured with fine mesh netting. Beetles were provided with oak foliage, supported in covered containers of water, and a 20% sugar solution on cotton wool pads. They were kept in single sex groups of up to 10 beetles for three to seven days to allow the females to maturation feed (Cardenas & Gallardo, 2013). Sexes were subsequently combined, and up to 10 beetles were housed per cage, according to emergence date and site, and allowed to mate and oviposit. Pilot studies showed that females preferred to oviposit in the crevice beneath the water container, and in a layer of paper towel beneath the cages. Beetles were fed on fresh leaves, and the cages cleaned twice weekly. The laboratory temperature was maintained at 22.0 ± 2°C. Egg batches were collected twice weekly and placed in closed plastic boxes (22 x 56 x 36mm).

Because *A. planipennis* females have been shown to lay more eggs in the presence of a male of their choice (Rutledge & Keena, 2012), male-female *A. biguttatus* pairs were removed from the mixed groups when mating was observed. 26 male-female pairs from Dudmaston were monitored twice weekly to measure female lifespan and oviposition. Dead males were replaced. 19 mixed groups containing beetles that emerged on the same date were monitored twice weekly for initial oviposition.

Development of eggs, larvae and pupae

Experiments on eggs, larvae, and prepupae were performed in incubators (MIR series; Sanyo Electric Co., Ltd., Japan) at constant temperature treatments of 15, 17.5, 22.5 and 25°C, and in a constant temperature room at 20°C. These temperatures were selected using pilot studies and represent realistic field conditions in the UK. Incubator temperatures were monitored with Tinytag temperature loggers and kept within ± 0.5°C for 17.5, 20, 22.5, and 25°C and ± 1.3 for 15°C. Relative humidity in the incubators was measured with a pen-type thermo-hygrometer (ATP Instrumentation Ltd., UK) and found to range from 50% to 95%. Humidity in the constant temperature room was maintained at 65%.

To measure the effect of temperature on egg development rate, egg batches containing 12 ± 1.5 eggs (mean ± SE) < 24 hours old were placed in plastic boxes, randomly allocated across 15 replicates at each temperature, and checked daily for hatching larvae. Larvae that hatched in the egg development experiments were not used in subsequent log experiments.

Drawing from successful techniques of culturing *A. planipennis* larvae, (Duan *et al.,* 2011; J. P. Lelito, pers. comm) methods were developed to culture *A. biguttatus* larvae on stems of oak. Oak trees (8-16 cm diameter) were felled and stems were cut into ~20cm lengths, standardised to have similar surface areas. The cut ends were placed in water, and the logs left for at least two weeks to increase the likelihood of larval establishment. To limit fungal growth, eggs were incubated at either 17.5 or 20°C until one or two days before the hatch time predicted by the egg development model. One or two small cores of outer bark (0.6 cm diameter) were removed from the logs, and a batch of eggs, on paper towel, was inserted into each of the bore-holes (totalling approximately 10-15 eggs per log). The bark core, trimmed by 1-2 mm, was then carefully replaced. Inoculated logs, with the cut ends in a tray of water, were kept at 22°C ± 2 until between one and three days had elapsed after the predicted hatch time. Fluorescent propagation lights and reflectors (SunBlaster T5 type; SunBlaster Holdings ULC, Canada) were affixed to the windows of the incubators to encourage photosynthetic activity in the epicormic shoots that emerged after the logs were cut. Incubator and constant temperature room lights were placed on 12 hour light / 12 hour dark timers, and levels monitored using a Kipp and Zonen Delft CMP3 Pyranometer (Kipp & Zonen B.V., The Netherlands). To mimic incubator conditions, the experimental area within the constant temperature room was shaded with transparent mesh until light levels were similar (mean ± SE over 20 minutes: 236 ± 13 W/m^2 in incubator, 274 ± 21 W/m^2 in the constant temperature room). Inner bark condition was monitored weekly by checking for green epicormic shoots or incising logs with a chisel to expose the inner bark

tissues. In order to standardise larval food quality, at all temperatures except for 15°C, all larval measurements used in developmental analysis were taken from logs that remained in good condition (e.g. inner bark appeared fresh and moist) until the dissection date. At 15°C, all logs deteriorated before the larvae completed development, and for the later replicates, larvae were relocated onto new logs. Undamaged larvae from deteriorating logs and from dissected logs were replaced into fresh replacement logs and allowed to finish feeding, for analysis of development from prepupa to adult emergence.

To measure larval development time, logs were allocated randomly across each constant temperature treatment. The number of logs in each treatment was based on an estimated linear model of larval development and limited by incubator space (n = 81, 60, 40, 36, and 32, at 15, 17.5, 20, 22.5 and 25°C, respectively). To determine the number of larval instars and monitor growth, at each constant temperature treatment except for 15°C, a minimum of three logs was dissected every two weeks until larvae finished feeding. At 15°C, there were insufficient remaining logs to dissect three replicates after 20 weeks. To dissect out the larvae, the bark was peeled back from the cut ends of the logs with a chisel and mallet. Larvae were traced by following their feeding galleries, and were gently removed from the cambial interface with a damp paintbrush. The width of the visible portion of the larval head capsule, or peristoma, was measured to the nearest 0.2 mm using a dissecting microscope fitted with an eyepiece and graticule. Peristomal width has been used successfully to differentiate between instars in other *Agrilus* species (Loerch & Cameron, 1983). 20 neonate larvae from five egg batches were also measured.

Techniques to monitor prepupal development (to adult emergence) were also drawn from experiments on *A. planipennis.* When the larvae were observed to finish feeding in the dissected logs, they migrated into the outer bark, excavated pupal chambers, and became inactive prepupae, often folding over in a “hairpin” or “J-shape”, as has been observed in *A. planipennis* and *Agrilus auroguttatus* Schaeffer (Coleoptera: Buprestidae) (Coleman & Seybold, 2008; Wang *et al.*, 2010). Migration to the outer bark was used to define the cessation of larval feeding, and the transition to prepupa. To simulate overwintering, prepupae within logs or bark material were chilled at 10°C for one to two weeks, and then at 5°C for a further 15 to 17 weeks (J. P. Lelito, pers. comm.). To simulate the end of winter, prepupae were transitioned to 10°C for one week, and then returned to their original temperature treatments; this marked the end of overwintering.

To measure the total development time from prepupa to adult emergence, a total of 68 logs across the constant temperature treatments, comprising replacement and undissected material, were observed twice-weekly for adult emergence. To monitor development time from the end of overwintering to pupation, surviving prepupae dissected for the larval experiments, and the deteriorating logs (= “monitored prepupae”), were placed in moist ground outer bark (n = 18), or 25 - 30 mm bark sections (n = 49), and although disturbed from their pupal cells, they were observed twice-weekly for signs of further development. It was not possible to monitor pupal development time,

241 due to high mortality of specimens when handling in this sensitive stage. To test whether prepupae
242 would develop continuously at warm temperatures without a chill period, eleven prepupae (=
243 “unchilled prepupae”) were kept at 22.5 or 25°C and observed twice-weekly for signs of further
244 development.
245
246 *Statistical analysis*
247 All analyses were carried out in R (R Core Team, 2016). All means are presented ± 1 standard error
248 unless otherwise stated.
249
250 A Chi Square test was used to determine whether the observed sex ratio deviated from the expected
251 ratio (1:1). A Welch’s two-sample t-test was used to test for differences between the initial oviposition
252 dates of single females and females in mixed groups. Pearson’s product-moment correlation was
253 used to test for correlation between adult lifespan and fecundity (total number of eggs laid).
254
255 Egg hatch, cessation of larval development, and adult emergence were all classified as binary
256 responses. As such, all three were modelled using probit regression. As the processes underlying the
257 three dichotomous responses are likely to be normally distributed across the *A. biguttatus* population,
258 probit was chosen rather than logit, as the probit model assumes errors to be normally distributed,
259 whereas logit assumes standard logistic distribution of errors. In all three cases, three basic models
260 were applied to the data, one with only the covariate “day” in the model, one with “day” and
261 “temperature” and one including the interaction between “day” and “temperature”. For eggs, larvae,
262 and prepupae (to adult emergence), respectively, “day” was defined as the time from oviposition to
263 egg hatch; from egg hatch to migration to the outer bark; and from the end of overwintering to adult
264 emergence.
265
266 In the analysis of the egg data, separate egg batches were defined as samples, and the hatching of
267 individual eggs was modelled through time, with each egg batch providing a probability of hatching on
268 each date. To prevent pseudoreplication, the probit models were set up as mixed-effects models
269 using the lme4 package in R (Bates *et al.,* 2014), and included a random effect of sample to account
270 for the repeated measurements made on an individual sample. The effects of “day” and “temperature”
271 were scaled in all three models to improve model fit, using the standard scale function in R. Analysis
272 of deviance was used to determine the best fit model. Having chosen the best fit model, model
273 predictions and confidence intervals were calculated for the fixed effects.
274
275 Larval data samples comprised larvae within logs. Each log was treated as an independent sample
276 and analysed using standard probit regression in the GLiM function in R. Analysis of model deviance
277 was used to determine the best fit model, and probability predicted from the best fit model.
278
279 All data from emerged adults were pooled. Development times, after the return of the prepupae to
280 their original temperature treatment, were analysed using standard probit regression in the GLiM

function in R. Two outliers at 20°C were considered to be erroneously skewing the model fit, and were removed. Analysis of deviance was used to determine the best fit model, and probability predicted from the best fit model.

For each life stage, times for 10%, 50%, and 90% of individuals to complete development were calculated from the best fit models for each temperature. These development times were converted to rates, and the effect of temperature, life stage, and the interaction of both variables on the rate was determined using analysis of covariance. Linear regression of rate against temperature was subsequently applied to each life stage in turn, and these models were extrapolated to determine the lower developmental threshold (i.e. development rate = day^{-1}) and thermal constant (plus/minus confidence limits) for each stage.

The calculated developmental parameters from prepupa to adult emergence, predicted by the probit regression, were overinflated because they allocated day-degrees to a diapausing (dormant) stage. To account for this overinflation, and estimate the pupal development time (to adult emergence), predicted times at each temperature treatment from a simple linear regression of the development rate of the monitored prepupae vs temperature (Figure 1), were subtracted from the calculated parameters.

To determine the number of larval instars, and to predict the expected range of peristomal widths for each instar, normal mixture models were fitted to the peristomal width data. Hartigan's dip test in the package "diptest" was first used to check for multimodality (Maechler & Ringach, 2013). A significant dip statistic (D) indicates a multimodal distribution. Subsequently, a likelihood ratio test was performed using the package "mixtools" to determine whether or not the different instars shared a common variance (Benaglia *et al.*, 2009), followed by fitting normal mixture models with multiple variances. Visually estimated medians were specified. Based on the normal mixture model parameter estimates, larvae were posteriorly assigned to instars based on a threshold probability of 0.90.

To determine whether temperature affected larval head capsule size, a linear mixed model was fitted in the lme4 package in R. The response was head capsule width, square-root transformed, and posteriorly assigned instar and constant temperature treatment were specified as fixed effects. To account for egg batch effects and multiple measurements, a random effect of log was included. First instar data were excluded from the analysis because the larvae would not yet have moulted. The significance of the fixed effects was determined from the Wald's χ^2 statistic from the analysis of deviance, in the car package in R (Fox & Weisberg, 2011). Post hoc tests, correcting for multiple comparisons by specifying the Bonferroni adjustment, were carried out in the lsmeans package in R (Lenth, 2015).

Results

Sex ratio, female lifespan and fecundity

Adult emergence dates in the glasshouse, polytunnel, and controlled temperature room were 01 June to 23 July, 08 June to 22 July, and 06 May to 16 May, respectively. The mean / mean daily minimum / mean daily maximum temperatures for the two weeks before the first beetle emerged were 14.5 / 11.5 / 18.5°C (glasshouse), 15.9 / 14.7 / 17.0 °C (polytunnel), and 20.9 / 18.4 / 24.3°C (controlled temperature room). In total, 1,561 beetles were collected and sexed (F : M = 857 : 704). There was site-level variation in the sex ratio of beetles emerging in the glasshouse (Table 1); these numbers were supplemented by beetles emerging from the additional 'polytunnel' material. Although there seemed to be a trend for female dominance, this was due to a lower proportion of males at Garnon's Estate, which contributed to a lower proportion of males overall; furthermore, emergence from the previous year indicated the opposite trend (K. Reed, unpublished). The lifespan of individually held females was highly variable (63 days ± 7.8, range = 22 to 162 days, n = 26). The average date of initial oviposition for individually held females (n = 20) was 28 ± 2.3 days after emergence, and 18 ± 2.2 days after mating was first observed, and 25 ± 2.2 days for mixed groups of up to 10 individuals (n = 19) (mating not recorded). These means were not significantly different (t = 0.69, df = 37.0, p = 0.50). The minimum dates of initial oviposition were 16 and 12 days for individually held females and mixed groups, respectively. Individually held females laid an average of 82 ± 22 eggs over their lifespan (n = 24); four females laid more than 200 eggs. The size of egg batches laid by individually held females ranged from 1 to 40, and the average batch size was 9 ± 0.5. Female lifespan and fecundity were positively correlated (t =5.77, df = 22, Pearson's r = 0.78, p < 0. 001).

Development of eggs, larvae and pupae

There were significant main effects and interactions between temperature and day on the likelihood of completion for the egg and larval developmental stages, and on the development from prepupa to adult emergence (Table 2). The best-fit probit models of the probability of completion of development vs time, for each constant temperature treatment, are shown in Figure 2, for each developmental stage, along with the raw data. Estimated times to complete development are given in Table 3. Development rate was linearly related to temperature for all three developmental stages. When the development times predicted by the best-fit models were converted to rates, there were significant effects of stage ($F_{2,9}$ = 43.3, p < 0.001), temperature ($F_{1,9}$ = 121, p < 0. 001), and their interaction ($F_{2,9}$ = 26.2, p < 0.001) on development rate. The individual linear regressions of development rate vs temperature, for each life stage, are shown in Figure 3, and the extrapolated lower developmental threshold temperatures and day degree sums are given in Table 4. Although the eggs and larvae at 20°C were cultured in a constant temperature room, rather than in an incubator, the development data appeared consistent with those at the other temperatures.

Many egg batches hatched over several days: the average hatch time across all temperatures was 3.0 ± 0.4 days (n = 61), and 20% of batches took a week or longer to hatch. Many batches of eggs failed to hatch entirely, despite appearing to develop (n = 5, 3, 2, 0, 4, of 15 batches at 15, 17.5, 20,

22.5, and 25°C, respectively). These batches may have been damaged upon removal from the oviposition substrate.

After subtraction of the estimated prepupal development times, the lower threshold temperature of pupal development (to adult emergence) was 15.1°C (95% confidence limits, 8.8, 19.0), and the day-degree sum was 76.3 (95% confidence limits, 55.7, 96.9) (Table 4, Figure 3); the broad ranges were driven by variability in prepupal development time.

After approximately 100 days, none of the unchilled prepupae showed signs of pupation. These individuals suffered 100% mortality, indicating that the beetle requires a period at cold temperatures in order to complete its development. Overall, relatively few beetles survived from egg to adult, particularly at the cooler temperatures (n = 1, 6, 26, 14, 3 at 15, 17.5, 20, 22.5, 25°C, respectively). Replicates were particularly low in the final stages of development, due to the deterioration of logs and fragility of the exposed larvae. Of the monitored prepupae (18 in ground bark and 49 in bark sections), 10 reached the pupal stage, and only three eclosed successfully.

Hartigan's dip test indicated that the larval peristomal width data were at least bimodal ($D = 0.08$, $p < 0.001$) (Figure 4). Application of normal mixture models indicated that *A. biguttatus* had four larval instars (Table 5, Figure 4).

Posteriorly-assigned instar, temperature, and their interaction were all significant predictors of head capsule width (instar: $\chi^2 = 8003.6$, df = 2, $p < 0.001$; temperature: $\chi^2 = 44.9$, df = 4, $p < 0.001$; instar : temperature: $\chi^2 = 37.0$, df = 8, $p < 0.001$). Post hoc testing found an effect of temperature treatment that was significant in third and fourth instar larvae. Larvae at 15 and 17.5°C were smaller than larvae at 20°C in the third instar, and smaller than larvae at 20, 22.5 and 25°C in the fourth instar (Figure 5). In the second instar, head capsule size was smallest at the highest temperatures (22.5 and 25°C), although this pattern was not significant (Figure 5).

Discussion

This study's developmental findings suggest *A. biguttatus*' lower threshold temperatures are likely to restrict the beetle to its current distribution in England under current climatic parameters. Host range is clearly not the limiting factor, as oaks are present throughout the UK. For an area to be suitable for *A. biguttatus*, sufficient day-degrees must be available, above the lower threshold temperature, for each life stage to develop within an appropriate period. In particular, pupation and adult emergence must occur early enough in the summer for females to maturation feed, mate and oviposit, and subsequent egg development must then complete early enough for neonate larvae to become established in the host before the winter (Régnière, 2009). The pupal development time (to adult emergence) at 15°C (e.g. 10.6 weeks for 10% completion from initiation of pupation to adult emergence) appears particularly limiting. South-central England, at the centre of the species' UK

distribution, currently experiences mean daily air temperatures of just 11-12°C in May and 14-15°C in June (UK Climate, 2016), although it is important to note that sun-warmed stems are likely to be significantly warmer than air temperatures (Vermunt *et al.,* 2012). Pupation early enough in the summer to allow for mating, egg maturation and hatch, even within the beetle's core range in England, must depend on warm, sunny days where temperatures rise well above 15°C. Although the confidence intervals surrounding the lower threshold for egg development appear to be broad, examination of the predicted development times shows protracted egg development at 15°C (e.g. only 10% completion after 6.2 weeks). At colder temperatures, development time would be prohibitively long, as the eggs must hatch before winter. The broad ranges in confidence limits for the lower threshold for egg development (12.1°C (95% confidence limits, 7.4 and 14.9)) and day-degree sum (157.1 DD (95% confidence limits, 126.1 and 188.1) (Table 4) were driven by an apparent deviation from a straight-line developmental relationship with temperature (Figure 3), although insufficient temperatures were studied to adequately compare the fit of models with more than two parameters. As insect development is typically characterised by a straight line under optimal temperatures, the departure from a straight line suggests the lowest temperature, 15°C, may be suboptimal (Danks, 2000).

The beetle's restrictive lower threshold temperatures may, in part, clarify several aspects of the beetle's UK ecology, including characteristics of its association with AOD. A relatively low incidence of adult *A. biguttatus* exit holes has been reported on AOD-symptomatic trees (Denman *et al.*, 2014; Brown *et al.*, 2017), including on severely declined and even dead trees (pers. obs.), despite the presence of larval galleries in the phloem. Although host resistance is likely to be important, successful development may be inhibited on trees at sites of marginal thermal suitability, for instance where the canopy density or the understorey of the woodland is too dense for sunlight to reach and warm otherwise-suitable tree stems (Brown, 2013). *Agrilus biguttatus*' thermal requirements appear to explain its reported preference for open-grown, south-facing tree stems, where under-bark temperatures are likely to be warmer than in closed forests (Starchenko, 1931; Brown *et al.*, 2014).

Although the number of replicates used to generate *A. biguttatus*' lower threshold temperatures and thermal requirements was relatively low, the threshold temperatures reported in this study for the egg stage, and the threshold temperatures and day-degree sums for egg and pupal development, were similar to those reported for another temperate *Agrilus* species, *A. planipennis* (Lyons & Jones, 2005; Duan *et al.,* 2013). *Agrilus biguttatus*' day-degree values for the egg and larval stages were also in line with those of two damaging European bark boring pests, *Hylobius abietis* Linnaeus (Coleoptera: Curculionidae) and *Dendroctonus micans* Kugelmann (Colepotera: Scolytidae), as found in comparable studies (Inward *et al.,* 2012; Gent *et al.,* 2017). *Agrilus biguttatus'* egg and larval threshold temperatures, however, were considerably higher than those of *H. abietis,* which has egg and larval thresholds of 8 and 4.5°C respectively, or *D. micans,* which has egg and larval thresholds of 7.4 and 6.6°C, respectively. Their development at lower temperatures allows the two insects to

colonise much cooler parts of the UK than *A. biguttatus*: *H. abietis* is found throughout the UK (CAB International, 2003), and *D. micans'* range includes parts of southern Scotland (Gent *et al.*, 2017).

The cessation of further development and mortality of all individuals that were not subjected to a chill period suggests *A. biguttatus* has an obligatory prepupal diapause at all temperatures studied, up to and including 25°C. After larval feeding is complete, all prepupae enter diapause, and require a period of cold temperatures (overwintering) before development may resume (Saunders *et al.*, 2002). An obligatory diapause has also been reported in *A. planipennis* (Duan *et al.*, 2013; Liang & Fei, 2014). The diapause forces larvae that finish feeding at any time after late spring to overwinter and emerge the following year, which is advantageous for three reasons: it prevents sensitive pupae from exposure to cold temperatures, and it synchronises the lifecycle, which may be particularly important given *A. biguttatus*' typically small populations (Saunders *et al.*, 2002). Finally, due to the relatively high threshold temperatures of each life stage, it ensures that the adult beetles do not emerge too late in the summer and have insufficient time to maturation feed and reproduce, and that the eggs can complete development before autumn.

The day-degree parameters given in this paper may be used for detailed modelling of *A. biguttatus'* lifecycle and distribution when combined with appropriate temperature data. Although it is not possible to give a single lower developmental threshold temperature, because each life stage has a different value, the total day-degree sum required for *A. biguttatus* to complete its development may be calculated by summing the estimated values for eggs, larvae, and pupae (to adult emergence) (Tables 3, 4), and assuming a cessation of temperature-related development during the diapausing stage. For example, at an average temperature of 20°C, the average length of the lifecycle from egg to adult emergence was 20.9 + 60.6 days, followed by an obligatory chilling / overwintering period (15-17 weeks at 5°C in this study), followed by a final 12.7 days (Table 3). For modelling in the field, the estimated developmental parameters may be combined with under-bark temperatures, with the assumption that temperature-related development ceases during the overwintering (early instar overwintering / prepupal diapause) periods, and resumes when temperatures rise above the larval or pupal (to adult emergence) thresholds. Modelling with air temperatures may not yield a true representation of the beetle's under-bark microhabitat. The beetle is known to prefer sun-warmed tree stems, which may achieve significantly higher temperatures than air temperatures, and its current distribution may be dependent on seeking out these more suitable microhabitats (Vermunt et al, 2012; K. Reed, unpublished). Although these parameters apply to the UK population of *A. biguttatus,* they may also be used to model European development of the species, with the caveat that countergradient variation in development times has been shown in other insects, when genetic plasticity opposes environmentally-induced variance (e.g. Mitten & Ferrenberg, 2012).

Anecdotal observations of larval ecology in this laboratory study support the body of literature suggesting *A. biguttatus* may only develop on weakened, but living hosts, taking advantage of a narrow window of opportunity before host death (Moraal & Hilszczanski, 2000; Vansteenkiste *et al.*,

2004; Brown *et al.*, 2014). Moisture within the inner bark tissues seemed very important to larval success. Larvae frequently desiccated if inner bark tissues dried. Conversely, dead larvae were also often found in pockets of free liquid, suggesting a role of host moisture content in drowning larvae, especially during moulting. In a separate experiment, no larvae survived past the first instar on larger-diameter logs cut 10 days before larvae hatched. Residual host defences in these logs appear to have been prohibitive to larval establishment even ten days after tree felling. Similarly, colonisation of *Phoracantha semipunctata* Fabricius (Coleoptera: Cerambycidae) on newly-cut, as opposed to aged eucalyptus logs was inhibited, potentially by high inner bark moisture (Hanks *et al.,* 2005). Host defences likely to be employed against *A. biguttatus* are reviewed in Brown *et al.* (2014), and include moisture content, a rapid callusing response, and chemical defences such as feeding inhibitors and defensive proteins.

In conjunction with the developmental work, the novel culturing methods employed in this study permitted detailed and unprecedented observation of all life stages of *A. biguttatus*. As far as the authors are aware, these experiments represent the first laboratory study of the development of *A. biguttatus* from egg to adult. Of the Agrilinae*,* which include some of the most economically important wood and bark-boring forest pests, to our knowledge only *A. planipennis* has also been successfully cultured. New findings on *A. biguttatus*' biology included observations of sex ratio, female lifespan and fecundity, and larval biology. The sex ratio of emerging beetles varied by site, a pattern that has been similarly reported in *A. planipennis,* along with year-to-year variation (Lyons & Jones, 2005; Wei *et al.*, 2007). The mechanism behind this variation is unclear, and may simply be the result of limited replication, but temperature seemed to influence the sex ratio of laboratory-reared *Ips typographus* L. (Coleoptera: Curculionidae) in one study (Wermelinger, 1999). Many *A. biguttatus* females were able to live for two months in the laboratory, and to lay multiple batches of eggs. Although seemingly maladaptive, the mean 28 day period from emergence to initial oviposition observed in females was similar to findings of 23 days before initial oviposition, and 18-24 days before the maturation of eggs, for *A. planipennis* (Lyons & Jones, 2005; Ryall *et al.*, 2013). Some females did lay eggs approximately two weeks after emergence. Initial oviposition in the laboratory may have been inhibited by a lack of host cues, and initial segregation of males and females may also have lengthened this period. Also, at the warmer temperatures that are more optimal for the species, *A. biguttatus* may mature their eggs more rapidly. The long lifespans and correlated high egg productivity of females were probably influenced by the addition of the sugar solution in their diet. In a separate study, females fed only leaves and water had shorter lifespans (M. Sumner, unpublished). There was within-batch variation in hatching time of a week or longer in many egg batches. This temporal variability in hatching time may be a "bet hedging" or risk-spreading strategy, hedging against temporal weather variation (Hopper, 1999). Individual variability is a standard feature of insect development (Danks, 2000).

In this study, *A. biguttatus* developed through four larval instars, in contrast with existing literature, which reports five (Moraal & Hilszczanski, 2000). Most buprestid larvae develop through four instars (Evans *et al.*, 2007), and four have been reported in several congeneric species (Cote & Allen, 1980;

519 Loerch & Cameron, 1983; Lyons & Jones, 2005; Haavik *et al.*, 2013; Orlova-Bienkowskaja &
520 Bieńkowski, 2016). The pattern of smallest head capsule size at the highest temperatures in second instar
521 larvae, although not significant, and largest head capsule size at the highest temperatures in fourth instar
522 larvae, suggests a shifting thermal optimum (Atkinson, 1996), with early instar larvae attaining optimal
523 growth at lower temperatures, and later instar larvae attaining optimal growth at higher temperatures
524 (Figure 5). After hatching in late summer, optimal growth at low temperatures would allow early instar
525 larvae to take advantage of cooler conditions in the early autumn. Conversely, fourth instar larvae,
526 developing during the following summer, would be able to take advantage of warmer summer
527 temperatures. This provides further support that the lower experimental temperatures chosen in this study,
528 15 and 17.5°C, although representative of summer temperatures within the beetle's range in England, are
529 suboptimal for the development of *A. biguttatus;* the finding may, however, have been compounded by an
530 effect of reduced food quality due to longer development times at these temperatures.
531
532 *Conclusions*
533 The results of the present study of *A. biguttatus*' development suggest its thermal requirements limit
534 its UK distribution. The beetle's relatively long pupal and egg development times, at temperatures
535 similar to current mean daily summer temperatures in the UK, appear to restrict the beetle to the
536 warmer parts of England. The increasingly warmer summer temperatures expected under climate
537 change may allow the beetle to spread to new areas of the UK. If *A. biguttatus* proves an essential
538 component of AOD, the area affected by that disease syndrome is also expected to increase, and
539 indeed, evidence of the beetle, and AOD, have been newly discovered in Wales (Denman *et al.,*
540 2016). The influence of climate change upon the *A. biguttatus* lifecycle may also allow the beetle to
541 become more damaging in its own right. Warmer summers may provide more frequent opportunities
542 for the beetle to complete development, and additionally increase host availability, as more frequent
543 stress events, such as droughts, impact oak trees (Lindner *et al.*, 2010; Netherer & Schopf, 2010). As
544 host death, irrespective of AOD, is reportedly dependent on the density of larval galleries, with
545 increasing abundance and opportunity, the beetle may become more harmful (Hartmann & Blank,
546 1992). The new insights into the beetle's life history found in the present study, including its thermal
547 requirements, the presence of an obligatory diapause, and its adult lifespan and fecundity, are
548 important for modelling the beetle's lifecycle and role in AOD, and how this may alter under a warming
549 climate.
550
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Tables

Table 1. Sources of oak trees infested with *Agrilus biguttatus*, which were subsequently used for experiments, with sex ratios of emerging adults.

Site	Latitude	Longitude	Female	Male	X^2 (df = 1)	*p*
Dudmaston, Shropshire	52.496603	-2.375157	189	187	0.00	1.000
Garnon's Estate, Herefordshire	52.089785	-2.881768	106	68	3.93	0.048
Grafton Wood, Worcestershire	52.198769	-2.042427	1	4	1.80	0.180
Richmond Park, London	51.455423	-0.270892	39	30	0.36	0.550
Runs Wood, Norfolk	52.672009	0.410581	7	8	0.07	0.796

Table 2. Analysis of deviance output from the best fit models applied to *Agrilus biguttatus* egg development, larval development, and prepupal development (to adult emergence). Test statistics vary based on model type (mixed-effects uses Wald's chi-square, GLiMs use likelihood ratio chi-square.)

Egg Development			
Variable	**df**	**Wald's χ^2**	***p***
Temperature	1	600	<0.001
Day	1	14,900	<0.001
Temperature:Day	1	615	<0.001
Larval Development			
Variable	**df**	**χ^2**	***p***
Temperature	1	177	<0.001
Day	1	151	<0.001
Temperature:Day	1	95.2	<0.001
Development from prepupa to adult emergence			
Variable	**df**	**χ^2**	***p***
Temperature	1	1,170	<0.001
Day	1	5,300	<0.001
Temperature:Day	1	650	<0.001

Table 3. Predicted time for 10%, 50%, and 90% of individual *Agrilus biguttatus* to complete development, by temperature and life stage. The development times from pupa to adult emergence represent a correction of the overinflated calculated development times from prepupa to adult emergence, by subtracting the estimated prepupal development times.

		Predicted development time (days)			
Percent completion	**Temperature**	**Egg**	**Larva**	**Prepupa**	**Pupa (to adult emergence)**
10%	15.0 °C	43.1	153.0	-	73.9
	17.5 °C	29.7	86.9	-	27.0
	20.0 °C	20.9	60.6	-	12.7
	22.5 °C	14.9	46.4	-	6.2
	25.0 °C	10.4	37.6	-	2.7
50%	15.0 °C	46.0	188.2	21.9	97.9
	17.5 °C	31.8	107.1	20.1	38.7
	20.0 °C	22.7	74.6	18.6	20.5
	22.5 °C	16.4	57.3	17.3	12.0
	25.0 °C	11.9	46.4	16.2	7.3
90%	15.0 °C	48.8	223.5	-	-
	17.5 °C	33.9	127.1	-	50.5
	20.0 °C	24.6	88.7	-	28.3
	22.5 °C	18.0	68.0	-	17.8
	25.0 °C	13.2	55.2	-	11.9

Table 4. The lower developmental thresholds and thermal sums in day degrees (DD) for each life stage of *Agrilus biguttatus*.

Stage	**Lower Threshold**	**-95% CLI**	**+95% CLI**	**DD**	**-95% CLI**	**+95% CLI**
Egg	12.1 °C	7.4 °C	14.9 °C	157.1	126.1	188.1
Larva	11.9 °C	11.7 °C	12.0 °C	615.9	613.8	618.0
Pupa (to adult emergence)	15.1 °C	8.8 °C	19.0 °C	76.3	55.7	96.9

Table 5. Mean peristomal widths, by instar, of *Agrilus biguttatus* larvae, as predicted by the normal mixture models, and actual data ranges following posterior instar allocation.

Instar	**Peristomal width**	
	Mean ± SE	Range
1	0.19 ± 0.04	0.15 to 0.23
2	0.33 ± 0.05	0.25 to 0.38
3	0.60 ± 0.05	0.44 to 0.82
4	1.13 ± 0.06	0.92 to 1.46

Figures

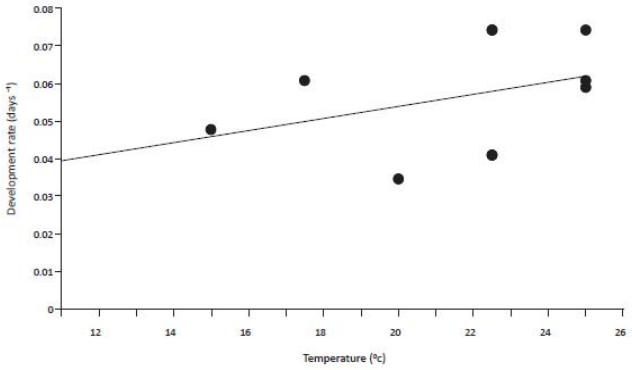
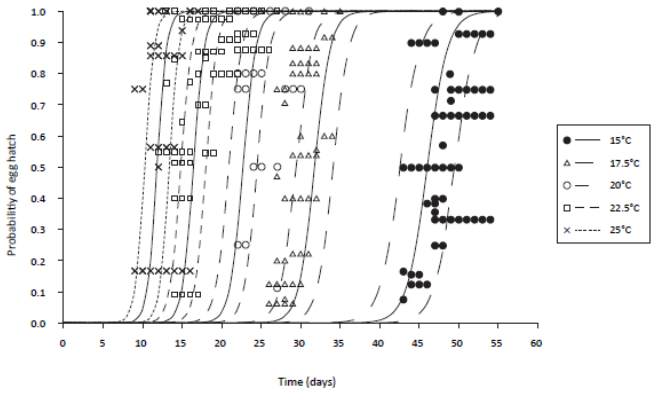


Figure 1. Development rate (days^{-1}) of monitored prepupae of *Agrilus biguttatus* from cessation of overwintering to pupation. Data points are the actual development times of individual prepupae, and a linear regression line is fitted.



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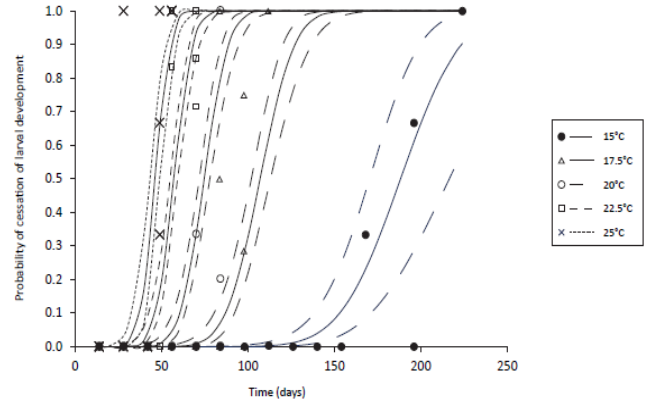

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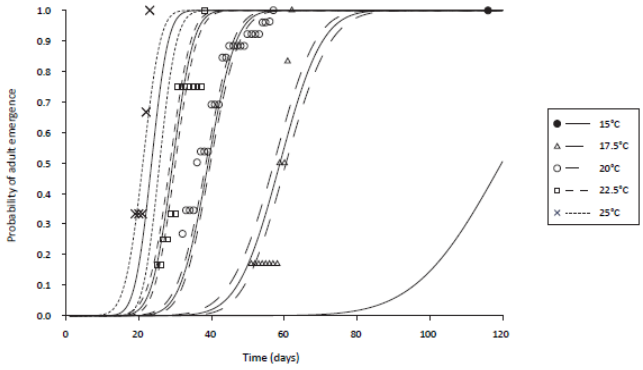
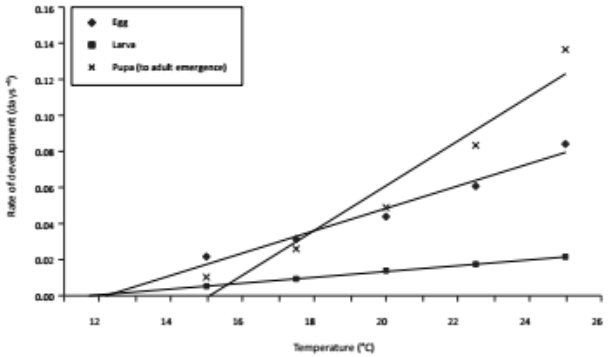


798

799 **Figure 2**. Probit models estimating the probability of completion of: (A) *Agrilus biguttatus* egg
800 development, from oviposition to egg hatch; (B) larval development, from egg hatch to migration to
801 the outer bark; and (C) pupal development, after overwintering, (to adult emergence). Data points are
802 the proportions of individuals that had completed the relevant developmental stage, and the dotted
803 lines represent the 95% confidence intervals.
804
805

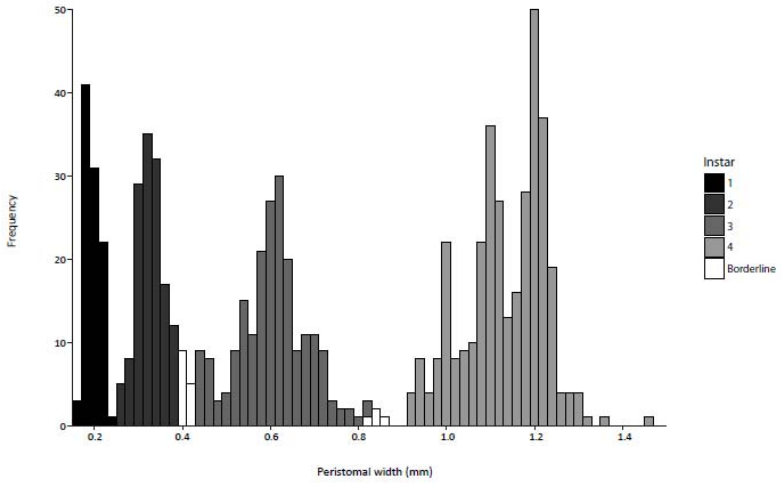


806

807 **Figure 3**. Temperature-dependent development rates (days^{-1}) of *Agrilus biguttatus* eggs, larvae, and
808 pupae (to adult emergence), as predicted by probit regression. Data points are the predicted 50%
809 completion times.

810

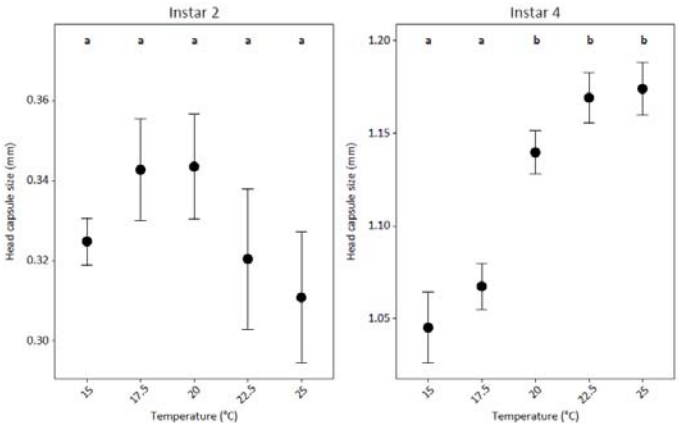
811



812

813 **Figure 4**. Histogram of *Agrilus biguttatus* larval peristomal widths, showing the four instars predicted
814 by the normal mixture model.

815



816
817

818 **Figure 5**. Head capsule size (mean ± SE) of second and fourth instar larvae of *Agrilus biguttatus* at
819 each constant temperature treatment, suggestive of a shifting thermal optimum, wherein early instar
820 larvae attain optimal growth at lower temperatures (third instar larvae not shown).