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Forest Research

The lifecycle and development of *Agrilus biguttatus*, and mechanisms of host resistance and annual growth in relation to Acute Oak Decline

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Abstract

1. Acute Oak Decline (AOD) affects thousands of UK oaks. The two spotted oak buprestid, *Agrilus biguttatus,* shares a similar distribution with AOD, and its larval galleries are almost always present on symptomatic trees. To determine whether the beetle is thermally limited in the UK, and collect data for lifecycle modelling, its thermal requirements were experimentally investigated, and its life history studied. To determine whether robust host defences explain the irregular emergence of adults from symptomatic trees, wound closure, a proxy for defensive ability, was measured, in monitored trees across the range of severity of AOD decline. To investigate the nature of predisposition before, and growth after AOD onset, the trees' annual increment was analysed.

2. The developmental data calculated for eggs, larvae, and pupae suggest climate limits the beetle's current UK distribution, which is likely to be further restricted to warmer microsites. Its distribution, and, potentially, that of AOD, may change under a warming climate. Beetles appeared to have an obligatory prepupal diapause at all temperatures studied.

3. After two years, wound closure was most, and least, efficient in asymptomatic trees, and trees with long-term symptoms, respectively. Adult exit hole presence was strongly correlated with reduced wound closure, suggesting robust host resistance, and particularly wound response, may result in a cessation of larval development on more vigorous hosts.

4. Trees with long-term AOD symptoms seemed predisposed by poorer growth throughout their lifespans. Surprisingly, there was no evidence of growth divergences between asymptomatic and symptomatic trees after AOD onset, although trees that experienced recent growth declines were less likely to recover when beetle exit holes were present.

5. Thinning severely declined trees may increase the vigour of remaining trees and decrease beetle emergence. Improved understanding of the role of *A. biguttatus* in AOD is urgently needed to inform management efforts.

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Selected articles and presentations

Journal articles:

Reed, K., Denman, S., Leather, S. R., Forster, J., and Inward, D. J. G. The lifecycle of *Agrilus biguttatus:* the role of temperature in its development and distribution, and implications for Acute Oak Decline. *Agricultural and Forest Entomology,* in press.

Vuts, J., Woodcock, C. M., Sumner, M. E., Caulfield, J. C., Reed, K., Inward, D. J., and Denman, S. (2016) Responses of the two-spotted oak buprestid, *Agrilus biguttatus* (Coleoptera: Buprestidae), to host tree volatiles. *Pest management science*, 72, 845–851.

Popular articles:

Reed, K. (2014) Acute Oak Decline – Research report No.2: Investigating the role of *Agrilus biguttatus* – how does this elusive native beetle fit in? *Woodland Heritage Journal,* Special Anniversary Edition, 22–23.

Reed, K. (2016) The Role of the Two Spotted Oak Buprestid in Acute Oak Decline. *Inpractice, Bulletin of the Chartered Institute of Ecology and Environmental Management*, 91: 33–36.

Reed, K. Inward, D. and Denman, S. (2015) The two-spotted oak buprestid, *Agrilus biguttatus:* new insights into its lifecycle. Report on Acute Oak Decline (AOD) research – 2014 to March 2015, *Woodland Heritage Journal.*

Selected presentations:

Denman, S., Plummer, S., Sumner, M., Reed, K., Peace, A. and Kirk, S. (2015) Simulating Key Elements of Acute Oak Decline to Produce Signs and Symptoms in Log Tests. IUFRO joint meeting, IUFRO WP7.02.02, Foliage shoot and stem diseases of forest trees, and WP7.03.04, Diseases and insects in forest nurseries Uppsala, Sweden, 7–12 June.

Lewis, E., Sapp, M., Kirk, S., Reed, K., Inward, D. Denman, S., and Elphinstone, J. (2015) Associated bacteria of *Agrilus biguttatus* sampled from English oak displaying symptoms of acute oak decline. IUFRO joint meeting, IUFRO WP7.02.02, Foliage shoot and stem diseases of forest trees, and WP7.03.04, Diseases and insects in forest nurseries Uppsala, Sweden, 7–12 June.

Reed, K., Inward, D., Leather, S., and Denman, S. (2015) New insights into the lifecycle of the jewel beetle *Agrilus biguttatus*, and its role in Acute Oak Decline. British Ecological Society Symposium 2015: The Ecology and Evolution of Emerging Plant Pests and Pathogens: Challenges to Global Food Security and Ecosystem Resilience, Penryn Campus, University of Exeter, Cornwall, UK, 13–14 July.

Reed, K., Inward, D., Leather, S., and Denman, S. (2015) New insights into the lifecycle of the jewel beetle *Agrilus biguttatus*, and its role in Acute Oak Decline. IUFRO WP7.03.05, Ecology and Management of Bark and Wood Boring Insects, and WP7.03.12, Alien Invasive Species and International Trade, Bariloche Eventos & Convenciones, Bariloche, Patagonia, Argentina, 1–4 September.

Reed, K., Inward, D., Leather, S., and Denman, S. (2015) The two spotted oak buprestid, *Agrilus biguttatus:* new insights into its lifecycle. Defra TH0108 Scientists meeting, Alice Holt Lodge, 7 May.

Reed, K., Inward, D., Denman, S., Leather, S., and Waterson, J. (2014) The role of temperature in the lifecycle of *Agrilus biguttatus*: preliminary findings*. Annual PhD Colloquium,* Harper Adams University, 27 November.

Reed, K., Inward, D., Denman, S., Leather, S., and Waterson, J. (2015) The role of temperature in the lifecycle of *Agrilus biguttatus*: preliminary findings. Royal Entomological Society Postgraduate Forum, London School of Hygiene & Tropical Medicine, 16–17 February. (Poster).

Vuts, J., Woodcock, C.M., Sumner, M.E., Caulfield, J.C., Reed, K., Inward, D.J.G., Leather, S.R., Pickett, J.A., Birkett, M.A., and Denman, S. (2016) The two-spotted oak buprestid (*Agrilus biguttatus*) in the UK: opportunities for control via chemical communication. Royal Entomological Society Forest Insects and Their Allies Special Interest Group Meeting, Peterborough, 5 April.

Vuts, J., Woodcock, C.M., Sumner, M.E., Caulfield, J.C., Reed, K., Inward, D.J.G., Leather, S.R., Pickett, J.A., Birkett, M.A., and Denman, S. (2016) Volatile aspects of oak – *Agrilus biguttatus* chemical interactions. Hungarian Plant Protection Science Days, Budapest, Hungary, 16–17 February.

Introduction and literature review

1. *Agrilus biguttatus* and oak decline in Europe and the UK

Tree decline is defined as a syndrome, in which long-term predisposing factors (e.g. site or genotype), abiotic or biotic inciting factors (e.g. drought or defoliators), and secondary organisms interact and lead to the reduction in vigour and, sometimes, death of trees (Manion, 1981; Houston, 1987). Denman & Webber (2009) recently divided oak decline into two classes, chronic and acute oak decline, in which the main distinguishing factor is time-scale. Chronic declines may last for decades in trees, and root pathogens tend to be a key factor. Acute declines develop rapidly, over only several years.

Decline of oaks (*Quercus* spp.) occurs worldwide, wherever oak species are present (Gottschalk & Wargo, 1996). Episodes of oak decline have been described for over 100 years in mainland Europe. In Slavonia, Eastern Croatia, decline of pedunculate oak (*Quercus robur* L.) was recognised as early as 1902, and symptoms, incurring widespread mortality, were recorded in 1909 (Klimesch, 1924). In Germany, oak decline was observed in 1911, becoming severe in 1916 (Falck, 1918). In England, decline was first observed from WWI. From 1923 large numbers of oaks were dying or had died (Robinson, 1927). Both native British oak species, *Q. robur* and, to a lesser extent, sessile oak (*Quercus petraea* (Matt.) Liebl.)*,* are affected by oak decline (Day, 1927; Denman & Webber, 2009).

Many of the more visible European and British decline episodes have shared a common disease progression, where defoliation and extreme weather events are key inciting factors, and these can be termed acute due to the swift onset of symptoms. Severe defoliation by biotic agents such as insect larvae, particularly in successive years, reduces trees' carbohydrate assimilation and storage pools, thus decreasing their tolerance of co-occurring extreme weather events, in particular affecting the growth of fine-roots needed to sustain water uptake in severe, prolonged summer droughts, and reducing the cold hardiness needed to tolerate severe frosts (Führer, 1998; Thomas, 2008). Common defoliators include lepidopteran insect groups such as the Lymantriidae, Geometridae, and Tortricidae (Führer, 1998). Early defoliation by lepidopteran caterpillars, followed by damage to late-growth leaves by powdery mildew (*Erysiphe alphitoides* Griffon & Maubl.), is particularly damaging to oaks' carbon budgets (Denman & Webber, 2009). Many factors may predispose trees to decline. These include poor sites, such as those with fluctuating water tables (Oosterbaan, 1991) or with soil nutritional imbalances, for example due to excess nitrogen deposition (Thomas *et al.*, 2002), and site history, including poor forest management such as overstocking, which leads to intense inter-tree competition for resources (Sonesson & Drobyshev, 2010). If the stress incurred by the biotic and abiotic

agents does not reach a critical level or duration, trees may recover (Führer, 1998). Weakened trees may, however, also be attacked and, ultimately, killed by secondary agents, including root pathogens such as *Armillaria, Collybia,* and *Phytophthera* spp. (Thomas *et al.,* 2002), and secondary wood and bark-boring insects. A phloem-mining beetle, the two-spotted oak buprestid *Agrilus biguttatus* L. (Coleoptera: Buprestidae), has been considered an important secondary agent in oak decline in Europe and Russia for over 100 years (Falck, 1918; Starchenko, 1931; Moraal & Hilszczanski, 2000; Hartmann & Blank, 1992; Thomas *et al.,* 2002).

Agrilus biguttatus*: increasing importance in European and UK oak decline*

Literature from the 1990s onwards suggests *A. biguttatus*' reputation as a damaging pest of oak has increased in both Europe and the UK from around the 1980s. Although known as a periodically important secondary pest in continental Europe since the early 1900s (Falck, 1918; Starchenko, 1931), the beetle has been cited as an increasingly key component in widespread oak declines from the 1980s onwards, as far west as France, and as far east as Russia (reviewed in Moraal & Hilszczanski, 2000). It has been named the most important European secondary pest of oak by several authors who theorise that it kills off trees that are weakened, but otherwise might recover (Hartmann & Blank, 1992; Thomas *et al.,* 2002).

In the UK, the beetle had historically been viewed as a rare, old-forest relict. It was listed as a rare and threatened red-data-list species as recently as the 1980s (Shirt *et al.*, 1987). The beetle was thought to develop on very old or dying trees, and to be confined to fragments of ancient woodland or wood pasture – e.g. The New Forest, Richmond Park, Sherwood Forest, Windsor Great Park (Shirt *et al.*, 1987; Hyman, 1992). Perceptions of the beetle's pest status have, however, been changing since a severe oak decline episode in the 1990s, when in one study, evidence of *A. biguttatus* was found at 14 of 20 sites where declining oak was present, suggesting an increasing presence of the beetle in UK oak decline (Gibbs & Greig, 1997).

Description of AOD

Agrilus biguttatus' reputation as an emergent oak pest may be attributed to its association with a specific type of acute decline, involving distinctive vertical stem exudations and lesions, newly described as Acute Oak Decline in England (AOD) (Denman & Webber, 2009). AOD may have been present in Europe since the early 1900s: Falck (1918) described black "sap-flux" on oak stems in Germany in the 1910s. AOD is thought to have been absent in England until the 1980s (Denman *et al.,* 2014). Currently, thousands of

trees are affected by AOD, of which 1.3% die each year on monitored sites (Denman *et al.,* 2016). Trees affected by AOD are characterised, externally, by the exudation of dark fluid and vertical splits between the bark plates (Figure 1), and internally, by the necrosis of inner bark tissues, and the larval galleries of *A. biguttatus* (Figure 2) (Denman *et al.,* 2014). The timespan of, and mortality levels from AOD, are not fully known, but the time for progression from first development of symptoms to tree death typically takes more than two years (Brown *et al.,* 2016). Several species of bacteria, new to science, have been consistently isolated from the lesions (Denman *et al.,* 2016). *Agrilus biguttatus* is also strongly correlated with AOD: The beetle's exit holes have been found externally on 30-33% of examined trees with AOD on monitored sites (Denman *et al.,* 2016; Brown *et al.,* 2017). Internally, its larval galleries have been found in proximity to nearly all dissected lesions (Brown *et al.,* 2017; S. Denman, pers. comm.) The beetle's distribution within England is also remarkably similar to that of AOD, with a northerly limit near Manchester (Figure 3) (Brown *et al.,* 2014). Until recently, the beetle and AOD have reportedly been absent from Wales and Cornwall, but evidence of the beetle has been newly discovered, along with AOD, along the Welsh borders and in Newport, Wales (Brown *et al.,* 2014; Denman *et al.,* 2016). AOD does not necessarily lead to tree mortality. Many trees occlude lesions with callus-like tissue and enter a state of remission or recovery, although it is not known whether the trees continue to be infected   (Denman *et al.,* 2014; Brown *et al.,* 2016).
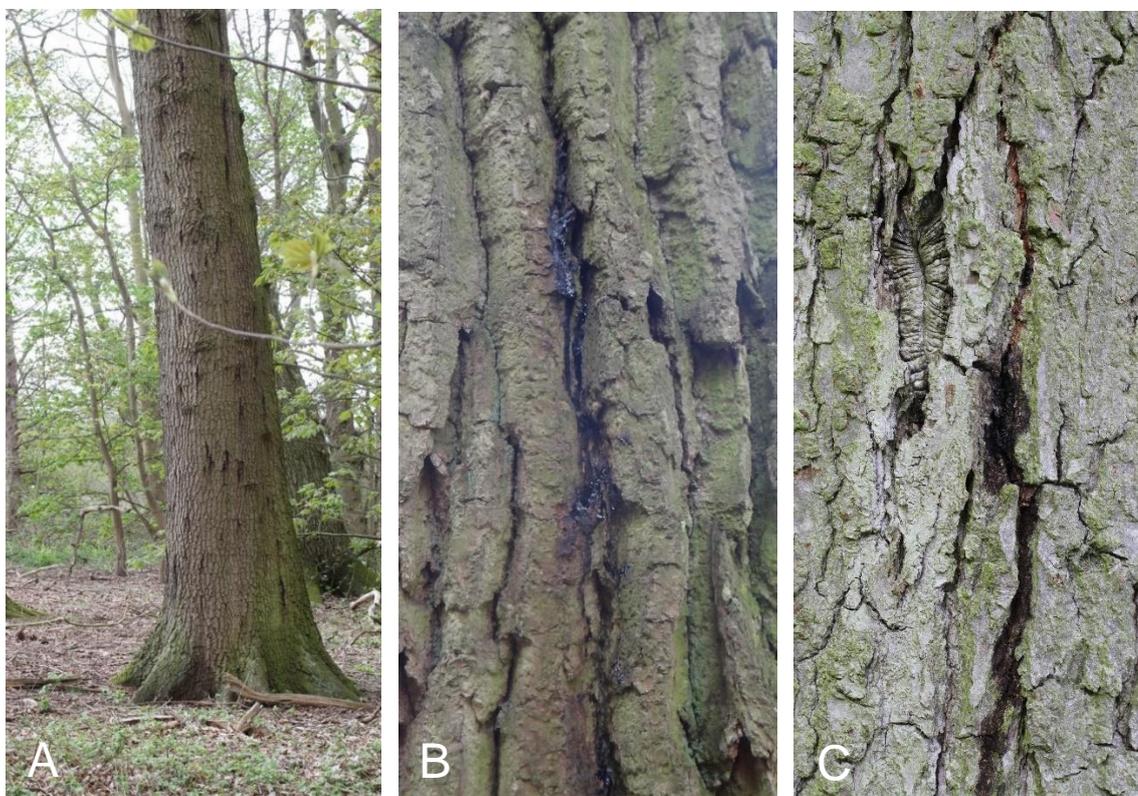
Figure 1. External AOD signs. A: multiple stem lesions; B: fluid exuding from between the bark plates; C: splits between the bark plates, with occluded lesion, left, and active lesion, right

Figure 2. Internal AOD signs. A: necrotic lesion, with early instar *Agrilus biguttatus* larval galleries below. The gallery pattern is representatitive of neonatal larvae chewing to the cambium and dispersing, forming a star-shape; all galleries appear to have been abortive. B: inactive lesion that is being covered with callus-like tissue growth (left); and cavity formed by decayed tissue (right).
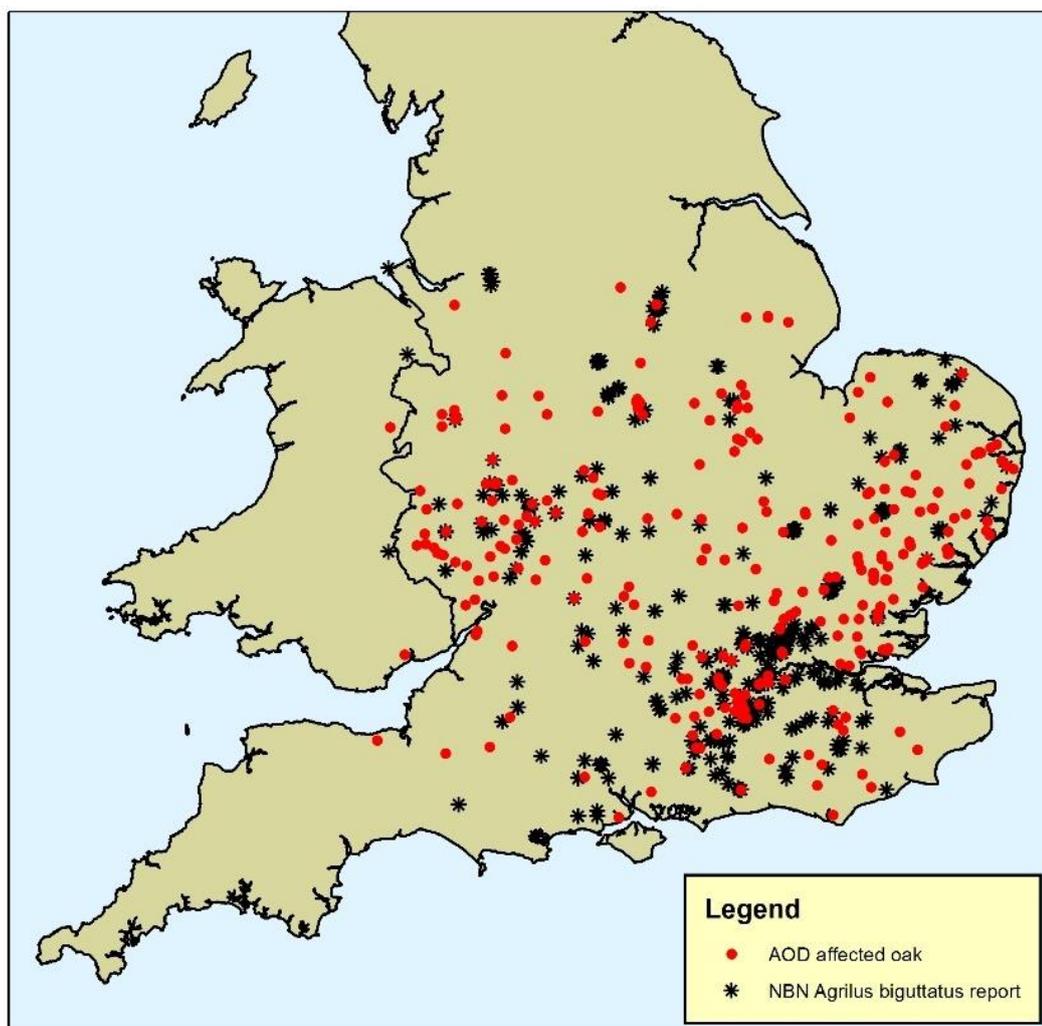
Figure 3. The current recorded distributions of AOD and *Agrilus biguttatus* in the UK*,* including verified incidence reports and data collected through systematic surveys (Data and figure: Nathan Brown, Rothamsted Research, 2017; *A. biguttatus* reports: National Biodiversity Network, 2017).

2. *Agrilus biguttatus:* description, lifecycle and ecology

The genus Agrilus

Agrilus is an enormous genus, with approximately 3,000 named species worldwide (Bellamy, 2008). They are part of the buprestid family, commonly known as jewel beetles because of their bright, metallic colours. Most *Agrilus* larvae develop in the inner bark of weakened or dying woody hosts, and are species or genus specific (Bellamy, 2003; Evans *et al.,* 2007). Because most species do not attack healthy trees, only a few are economically important, but some are notable pests, especially when they encounter naïve hosts outside their native range. For example, the emerald ash borer *Agrilus planipennis* Fairmaire, a species native to Asia, has caused the death of millions of American ash trees since its accidental introduction to North America in the 1990s and discovery in 2002 (Haack *et al.*, 2002; Siegert *et al.*, 2010; Herms & McCullough, 2014). Projected economic costs of removing, treating, and replacing ash trees between 2009

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and 2019 are $10.7 billion (Kovacs *et al.*, 2010). A concerted effort is underway in the United States to study *A. planipennis* and determine effective control techniques. Other economically important species that attack and kill naïve hosts include: the goldspotted oak borer *Agrilus auroguttatus* Schaeffer, which was probably introduced to southern California in firewood from Arizona, and is devastating coastal live oaks (Coleman & Seybold, 2008, Coleman *et al.*, 2011); and the bronze birch borer *Agrilus anxius* Gory, a species which attacks and kills ornamental birches of European origin planted in its native range, the United States (Slingerland, 1906). Some species also damage and kill co-evolved host trees within their native range, although a degree of host weakening is required for hosts to become susceptible (Evans *et al.,* 2007). For example, a species with a similar ecology to *A. biguttatus* (a native species on a co-evolved host), the twolined chestnut borer *Agrilus bilineatus* Weber*,* attacks stressed native oak trees, and is thought to contribute to oak decline within its native range, the United States and Canada (Cote & Allen, 1980).

Description and lifecycle

Agrilus biguttatus is an iridescent, blue-green beetle, distinguished from other British *Agrilus* species by the pair of pubescent spots centrally located on the basal third of its elytra (Figure 4A, B). Up to 13mm long, females are usually larger than, and have less tapered bodies than males, and in colour, are usually more green / bronze than the blue / green males (Allen, 1988). *Agrilus* larvae are apodous, and have round, enlarged prothoracic segments that encase most of the head capsule, and long, flattened, telescoping bodies that terminate in a pair of pointed appendages (Figure 4D, E). Initially around 2mm long (pers. obs.), the larvae can grow up to 43mm long (Kolk & Starzyk, 1996).The beetles develop exclusively in oaks (*Q. robur* and *Q. petraea* in the UK), with rare records of development on other deciduous trees such as beech (*Fagus sylvatica* L.) and sweet chestnut (*Castanea sativa* Mill.) in Europe (Hyman, 1992). *Agrilus biguttatus'* distribution stretches across central Europe, and extends to southern Norway and Sweden to the north, to Russia in the east, and south to North Africa (Bily, 1982; Brown *et al.,* 2014).
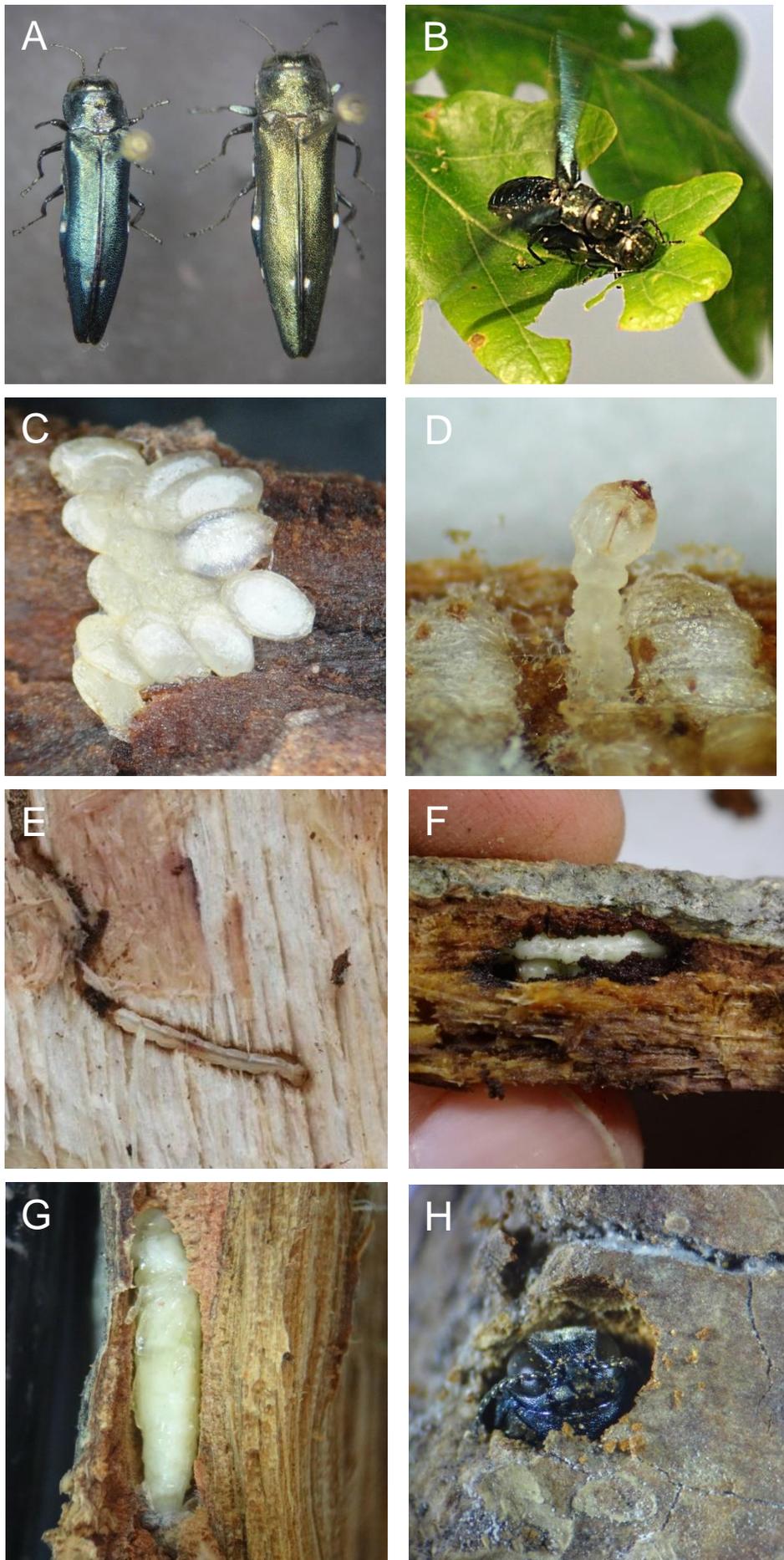
Figure 4. Life stages of *Agrilus biguttatus*, ordered chronologically*:* A: adult male (left) and female (right); B: mating adults; C: eggs; D: hatching larva; E: feeding larva, and the gallery its feeding has created; F: prepupa, in bark chamber; G: pupa, in bark chamber; H: adult emerging through D-shaped exit hole.

Agrilus biguttatus' lifecycle has been little studied because of its cryptic lifestyle and difficulties in rearing larvae, so many described biological parameters are rather vague. The adults feed on oak leaves and mate in the canopy (Figure 4B). Mating has been observed to rely on visual cues; in one study males landed from flight 1m above on pinned models of females (Domingue *et al.,* 2011). Females oviposit deep into the crevices between bark plates on the main stem with long ovipositors, laying clusters of eggs (Figure 4C) (Nathan Brown, pers. comm.). After hatching (Figure 4D), the larvae tunnel through the outer bark to feed at the cambial interface, where they chew out galleries (Figure 4E, Figure 5). Although most buprestids pass through four instars (Evans *et al.,* 2007), *A. biguttatus* has been reported to develop through five (Moraal & Hilszczanski, 2000). The lifecycle has been reported to last for one or, usually, two years in northern Germany (Klausnitzer, 1994; Moraal & Hilszczanski, 2000), with early-instar larvae over-wintering in the first year. After a second summer of feeding, larvae burrow outwards into the cork layer and create pupal chambers roughly parallel to, and 0.5-5.0mm from the surface (pers. obs.), and become inactive prepupae (Figure 4F). Prepupae overwinter folded in a hairpin-shape and, in the spring, pupate for approximately 14 days (Figure 4G) (Habermann & Preller, 2003). Adults chew through the remaining few mm of bark and emerge through D-shaped exit holes throughout the summer (Figure 4H) (Brown, 2013).

Ecological requirements

Agrilus biguttatus is reported to prefer mature trees, and has been found to require a bark thickness of at least 10mm on *Q. robur* and 13mm on *Q. petraea*, potentially the minimum for pupal chamber construction (Vansteenkiste *et al.,* 2004), although the beetles have also been found on young, 30-40 year old trees (Hilszczanski & Sierpinski, 2007). In small-diameter trees, exit holes are more often found at the base, where the bark is thickest (pers. obs.). In common with other species of the genus, *A. biguttatus* is thought to only develop on weakened, but living oak tissue. *Agrilus biguttatus* larvae are thought to be unable to develop on healthy trees, where host defences, though poorly understood, are thought to be too robust (Brown *et al.,* 2014). Two studies of declining oaks in Europe found larval establishment only occurring after a 'tipping point' of decline, quantified by approximately 25% canopy loss. In the same studies, exit holes were not found on trees with <50% canopy loss, suggesting females may oviposit onto trees that are not sufficiently weakened for complete development from egg to adult (Hartmann & Kontzog, 1994; Vansteenkiste *et al.*, 2004).

Figure 5. Panel of outer bark removed from an AOD lesion, showing decayed phloem tissue (right), adjacent larval galleries of *Agrilus biguttatus* and a dead large instar larva (left)

Mechanisms of resistance against A. biguttatus *larvae*

Although there is limited literature on host defences against *A. biguttatus*, literature on similar species suggests resistance mechanisms may include inner bark moisture and an efficient wound response. The larvae appear to have finite moisture requirements which may, in part, explain the inability of this, and similar species, to complete development on dead or healthy trees. After death, tissue only remains suitable for a short time (Evans *et al.,* 2007), probably because of drying (Brown *et al.,* 2014). In two studies in the US, *Agrilus bilineatus* struggled to complete development on experimentally xylem-girdled trees, with surviving larvae confined to isolated patches of still-moist tissue (Cote & Allen, 1980; Dunn et all, 1986). Conversely, high moisture levels may also be detrimental to larval survival. Hanks *et al.* (2005) found neonate eucalyptus longhorned borer

Phoracantha semipunctata Fabricius larvae were unable to establish in freshly-cut eucalyptus logs, with a high inherent inner-bark moisture level, but were able to colonise older, dryer logs. No difference was found, however, when the inner-bark moisture of trees with low and high colonisation of *Enaphalodes rufulus* Haldeman were compared (Fierke & Stephen, 2008). Moisture has been hypothesized to play a more active role as a host defence by some European authors, who attributed AOD stem bleeds to a host defence aimed at drowning *A. biguttatus* larvae (Falck, 1918; Hartmann & Blank, 1992; Vansteenkiste *et al.,* 2004). Denman *et al.* (2014) questioned this, as fluid exudation, which is presumably carbohydrate-expensive, often continues long after larval colonisation ceases. More vigorous trees may simply have a higher inherent moisture content that inhibits colonisation by neonate larvae, and may reduce larval survival to adulthood, e.g. through drowning (Hanks *et al.,* 2005; Brown *et al.,* 2014).

A robust wound response has also been hypothesized to play a role in host resistance to phloem-mining larvae. When bark tissues, including the cambium, are wounded, deciduous trees such as oaks quickly form internal "boundary zones" adjacent to damaged areas, strengthened by suberin and lignin deposition to reduce water loss and limit the spread of pathogens (Biggs *et al.,* 1984), and, potentially, inhibit further larval feeding (Brown *et al.,* 2014). To further limit the spread of decay, oaks also fill adjacent vessels with tyloses (Jacquiot, 1976; Vansteenkiste *et al.,* 2004). To reform the protective bark tissues, phellogen regeneration occurs through the growth of callus-like tissue, or wound periderm, which is lignified to increase its resistance to further injury; this is followed by the regeneration of the cambium and sapwood (Biggs *et al.,* 1984; Smith, 1988; Armstrong *et al.,* 2007; Denman *et al.,* 2014). The host wound response to feeding larvae may also include the production of defensive chemicals. Studies of the chemicals induced by feeding *A. planipennis* larvae in resistant vs susceptible ash species have shown differences in groups such as lignans, that may be directly toxic or act as growth inhibitors (Chakraborty *et al.,* 2014; Villari *et al.,* 2015). The wound responses of trees have been experimentally quantified by measuring the rate of closure of mechanical wounds, and the results of two studies indicate that a vigorous wound response limits larval success: the rate of wound closure was shown to vary with the level of colonisation of both *Agrilus bilineatus* and *Enaphalodes rufulus* (Dunn *et al.,* 1990; Fierke & Stephen, 2008). Oaks with lower colonisation by *Enaphalodes rufulus* also had smaller larvae-induced wound lesions, indicating faster compartmentalization of feeding damage (Haavik & Stephen, 2011).

Agrilus biguttatus' *physiological effects on trees*

Damage to oak trees occurs to the inner bark tissues, during *A. biguttatus*' larval feeding stage (Figure 5). The adults are not thought to feed on leaves in sufficient quantity to cause significant damage to the canopy. Upon hatching, the neonate larvae tunnel through the outer bark to the cambium; larval galleries initially disperse from a central point to form a star-shape (Figure 2) (Hartmann & Blank, 1992). The earliest instar galleries are "zig-zag" or step-like appearance, and the largest are straighter, spiralling around the stem or appearing tangled (Wachtendorf, 1955; Denman *et al.*. 2014). Galleries increase in width from <1mm (1st instar) to 3.5mm across, and reach a total length of around 1.5m (Wachtendorf, 1955). Most galleries occur at the cambial interface, with larvae reportedly feeding 1mm into the outer face of the xylem and inner face of the phloem (Vansteenkiste *et al.*, 2004), although galleries have been observed to penetrate 5mm into the sapwood, which, in other species, is thought to occur during moulting, or when host defences in the phloem are particularly vigorous (Anderson, 1944; Dunn *et al.,* 1990; pers. obs.).

Larval feeding is thought to cause both immediate and delayed damage to trees' vascular systems. Immediate damage occurs when feeding into the xylem interrupts the tree's upward flow of water and nutrients from the roots, and feeding into the phloem impacts upon the tree's downward photosynthate transport. Jacquiot (1976) also claimed larvae caused delayed damage to host tissue through the excretion of a necrotic compound in the frass. Toxic to cells in its immediate vicinity, cambial death also stimulated the nearby proliferation of callus-like tissue with reduced water-carrying vessels, further decreasing water transport (Denman *et al.,* 2014). A chemical extracted from larval frass produced a similar effect when injected into trees (Gouvernel-Guillemain, 1953), but the described effects have not been conclusively shown (Denman *et al.,* 2014). A host wound response may further reduce hydraulic conductivity: oaks plug vessels adjacent to galleries with tyloses in response to feeding *A. biguttatus* larvae (Jacquiot, 1976; Vansteenkiste *et al.*, 2004).

3. *Agrilus biguttatus*: relationship with AOD

Causes of AOD

The causes of AOD appear to be complex, and remain under investigation, but experiments suggest trees may be affected by an interaction between several species of bacteria and, potentially, *A. biguttatus,* in combination with abiotic predisposing factors (Denman *et al.,* 2014). A number of bacterial species are consistently isolated from the

margins of AOD lesions. Through whole genome sequencing and pathology experiments on logs and standing trees, adhering to Koch's postulates, several of these bacterial species have been shown to be capable of causing the oak tissue necrosis and lesion formation characteristic of AOD (Koch, 1891; Denman *et al.,* in press). These species include *Gibbsiella quercinecans* gen. & sp. nov. and *Brenneria goodwinii* sp. nov.*,* and, potentially, *Lonsdalea quercina* ssp. *Britannica* subsp. nov. and *Rahnella Victoriana* sp. nov. (Brady *et al.,* 2010, 2016; Denman *et al.,* 2012, 2014, 2016, in press). Experimental replication of AOD has proved challenging. Difficulties include the size and value of the affected hosts: mature oak trees often have very high monetary and cultural values, limiting opportunities to conduct damaging investigations. In another complication, other biotic agents, such as root pathogens, may also be important in ultimate tree mortality (Denman *et al.,* 2014). Experimentally reproducing any decline syndrome is complicated because, by the definition of tree declines, multiple abiotic and biotic factors must act in combination to produce symptoms (Manion, 1981).

Predisposition is thought to be important for the development of AOD. Trees become susceptible to attack by secondary pests and pathogens when severe or progressive stress reduces their carbohydrate reserves, leaving them with insufficient resources to mount effective defences (Führer, 1998; Bréda *et al.,* 2006). The putative causal agents of AOD are thought to be at least to some extent secondary. *Agrilus biguttatus* is not thought to develop on healthy trees, and pilot studies suggest the bacterial species, if they do indeed form the characteristic AOD lesions, require a degree of host weakening to establish (Denman *et al.,* 2014; Brown *et al.,* 2014, 2017; S. Denman, pers. comm.). AOD notably occurs without the severe defoliation thought to be an inciting factor in most historic European decline events (Denman & Webber, 2009). A predisposition model linking AOD prevalence to environmental variables found the syndrome occurred on warm sites with low rainfall (Brown *et al.,* 2018). If *A. biguttatus* is essential to AOD, the syndrome will be limited to sites suitable for beetle development, and in the UK the beetle is probably limited to warmer areas (Brown *et al.,* 2014). Changing climatic conditions, including increasingly dry and warm summers, may be predisposing trees to decline; droughts are thought to be a major trigger of oak decline, both in Europe and in North America (Thomas *et al.,* 2002; Coleman *et al.,* 2011). Warm temperatures exacerbate the physiological impacts of drought in trees through increased water loss and carbohydrate expenditure (Allen *et al.,* 2010). Poor soil quality, including insufficient moisture retention or drainage, will also exacerbate drought stress (Denman *et al.,* 2014). The predisposition model created by Brown *et al.* (2018) also found that AOD was more likely to occur on sites with high levels of deposition of nitrogen and base cations, and low levels of deposition of sulphur. Excess nitrogen deposition is thought to lead to nutritional imbalances in oaks, decreasing their resilience to drought, frost, and insect defoliation,

and may also exacerbate soil nutritional imbalances (Thomas *et al.,* 2002; Thomas, 2008), while the authors suggest sulphur deposition may act in a pest / disease control capacity (Brown *et al.,* 2017 in prep.). Although trees that develop AOD are presumed to be predisposed, or to have had lower defences than trees that do not develop AOD, no examination of the long-term health of asymptomatic and symptomatic trees has taken place. Techniques such as dendrochronology, in which cores are taken from tree stems with increment borers, and the annual tree rings are measured and cross-dated, allow for comparisons of long-term growth trends and individual differences in growth rates (Cook, 1987). Predisposition may manifest as lower mean stem growth in weaker trees (McDowell *et al.,* 2008).

The very strong association between the larval galleries of *A. biguttatus* and AOD lesions suggests the beetle is integral to AOD, but current evidence remains circumstantial and, largely, anecdotal. The high co-occurrence of larval galleries and bark lesions was observed from non-systematic removal of outer bark panels to study lesion formation (S. Denman, pers. comm.). While AOD lesions without adjacent larval galleries have not been observed, it is important to note that *A. biguttatus* is not restricted to trees with AOD: trees with exit holes, but no AOD symptoms, are infrequently found on AOD sites (Brown *et al.,* 2017). Several possible roles and chronologies of *A. biguttatus* and the pathogenic bacterial species within the AOD syndrome have been described, as follows (Brown *et al.,* 2014, 2017). 1. The bacteria may colonise trees first, and *A. biguttatus* secondarily, with the beetle attracted by the bacteria or host volatiles to oviposit on trees that, weakened by AOD, have become suitable for larval development. 2. Both may colonise host tissues simultaneously if *A. biguttatus* vectors the bacteria, either between or within trees, and if the beetle and the bacteria interact to degrade tissues, forming lesions. 3. The beetle may attack the tree first, and the bacteria may secondarily colonise areas of damaged tissue created by larval feeding.

Mechanisms by which A. biguttatus *may be essential to AOD*

Agrilus biguttatus may be an essential component in AOD if it is directly involved in the instigation of the fluid exudations and necrotic lesions that are key symptoms of AOD; this role has, however, yet to be proved conclusively experimentally. Denman *et al.* (2014) have deduced the chronological progression of an AOD lesion: the first sign is the external exudation of a small amount of fluid from intact bark. Necrosis develops in the phloem, spreading towards the sapwood; as the tissues beneath decay, the outer bark cracks; a cavity may be created when the necrosis advances to the sapwood. Affected trees may have just one lesion, or more than 20 (Denman *et al.,* 2014). Brown *et. al.* (2017) presented the first quantitative evidence on the co-occurrence of AOD symptoms and *A.*

biguttatus exit holes. On sites monitored for AOD lesions and *A. biguttatus* exit holes, their presence was strongly correlated. The incidence of stem bleeding increased through the summer months, peaking from May to August, coinciding with the highest period of beetle larval activity and oviposition (Brown *et al.,* 2017). At most UK sites monitored for AOD, symptoms were more extensive on the south sides of stems, a pattern consistent with *A. biguttatus*'s preference for warmer, southerly stem faces (Brown *et al.,* 2016). The same study found an apparently positive relationship between the mortality of monitored trees and the co-occurrence of bleeds and exit holes, although there was insufficient data to test this. In a study in the Netherlands, galleries were often found to run adjacent to or through lesions, suggestive of a role of larvae in lesion formation (Vansteenkiste *et al.,* 2004), although in the UK, Denman *et al.,* (2014) found that larvae avoided direct contact with lesions, potentially due to a risk of drowning or because the food quality of degraded tissues would be inferior.

Several European authors have described possible mechanisms in which *A. biguttatus* larvae could contribute to lesion formation in attempts to describe what appears to be a very similar condition to AOD in Europe, without reference to the AOD bacteria. Small necroses induced by the toxic component in larval frass could, theoretically, form when a vigorous host responds to neonate larvae tunnelling into the phloem (Hartmann & Blank, 1992), or when larval feeding kills sections of cambium (Jacquiot, 1976). In the United States, *Enaphalodes rufulus'* larval feeding results in vertical lesions on *Quercus rubra* L.; the lesion shape is thought to be a result of compartmentalisation, the combined processes of phellogen and vascular cambium regeneration and the formation of tyloses that wall off damage to the xylem (Shigo *et al.,* 1977; Biggs *et al.,* 1984; Haavik & Stephen, 2011). Alternatively, lesions could be created when tissue, patch-girdled through dense larval feeding, dies (Hartmann & Blank, 1992), or when swollen callus tissue, formed to cover over galleries, splits the bark, providing a pathway for weak microbial pathogens that decay the tissue underneath (Vansteenkiste *et al.,* 2004). Another agent (e.g. the implicated bacterial species (Denman *et al.,* in press) would seem to be required to explain the spreading nature of the lesions (Denman *et al.,* 2014); particularly in vigorous trees, rapid isolation of small areas of necrosis would be expected to occur (Shigo, 1977).

Agrilus biguttatus also may be essential to AOD if the lesions are the result of bacterial (or other microbial) activity, if the pathogen is vectored by the beetle. A study on the epidemiology of AOD found the highest clustering of infected trees at 10-25m, supporting the role of a vector, rather than very local transmission (e.g. water splash) or a larger-scale, environmental cause (Brown *et al.,* 2016). Trees with stem bleeds also clustered around trees with *A. biguttatus'* exit holes (Brown *et al.,* 2017). A map produced by

Hämmerli and Stadler (1988) that tracked the progression of a wave of oak decline reports beginning in Western Russia in 1967, spreading across central Europe in the 1970s and 80s, and finally arriving in the UK in 1989, appeared to suggest a pathogenic cause of decline (Führer, 1998), and a vector such as *A. biguttatus* could be instrumental in spreading the pathogen across large distances. *Agrilus* are strong fliers, and are likely to be able to fly considerable distances, although the flight capabilities and dispersal behaviour of *A. biguttatus* have not been tested, *A. planipennis* has been shown to be capable of flying more than 7 km in a day in flight mill experiments (Taylor *et al.,* 2010). Extensive fluid exudation is characteristic of AOD, and adult *A. biguttatus* may come into contact with the exudate, for example during emergence or oviposition (Brown *et al.,* 2014). No *Agrilus* species has, however, been shown experimentally to vector a pathogen (Brown *et al.,* 2014). As adult beetles do not burrow into the bark to lay eggs, the transmission pathway is not straightforward; bacteria could either be introduced into the leaves during maturation feeding, or could be harboured on, or within the eggs, or on the outer bark adjacent to egg batches, allowing neonatal larvae to carry it through to the phloem and cambium (Brown *et al.,* 2014). Lesions do seem to progress inwards from the phloem to the cambium, which would be the case if larval entry was providing a pathway for the bacteria (Denman *et al.,* 2014). Recent experimental work has shown that bacteria may travel along larval feeding galleries, resulting in lesion area expansion, and this suggests the beetle may be implicated in the formation of new lesions within symptomatic trees (Denman *et al.,* in press).

Finally, *A. biguttatus* may be an essential component of AOD if it kills trees that might otherwise recover. The mechanisms and ability of *A. biguttatus* larvae to kill oak trees have not been tested. There is only limited experimental evidence of the ability of larvae of similar species of *Agrilus* to kill weakened trees; in one study on oaks in the US, experimentally phloem-girdled trees died only if colonised by *Agrilus bilineatus* (Dunn *et al.,* 1986). Two hypotheses, created to explain the mechanisms by which drought kills trees, and which have also been applied to declining trees, suggest trees die when they experience hydraulic failure, carbon starvation, or a combination of the two (McDowell *et al.,* 2008; Sala *et al.,* 2012; Haavik *et al.,* 2015). Hydraulic failure occurs when water flow in the xylem is so impaired that desiccation of tree tissues occurs, whereas carbon starvation results when carbon reserves are so reduced that they can no longer meet the tree's metabolic demands (McDowell *et al.,* 2008; Haavik *et al.,* 2015). One mechanism by which hydraulic failure could be induced is xylem-girdling (Moraal & Hilszcaanski, 2000; Brown *et al.,* 2014). Stressed oaks produce thinner annual growth rings that contain a smaller volume of water-carrying vessels, and are more likely to be severed by larval feeding (Wargo, 1996). The plugging of vessels adjacent to galleries with tyloses, produced as a host response to wall off the damage caused by *A. biguttatus* wounding,

may also considerably reduce water transport in the presence of a high density of larval galleries: if a critical volume of vessels are made non-functional through compartmentalization, the tree will die (Shigo *et al.,* 1977; Smith, 1988; Brown *et al.,* 2014). High concentrations of larval galleries, and tyloses, may be more likely to kill trees when water stress is exacerbated by drought (Hartmann & Blank, 1992; Thomas *et al.*, 2002; Vansteenkiste *et al.*, 2004). Although there is no experimental evidence of *A. biguttatus'* ability to kill trees through girdling, evidence suggests *A. planipennis* kills susceptible ash trees through xylem-girdling (Flower *et al.,* 2013).

Agrilus biguttatus is also likely to contribute to the depletion of carbohydrate reserves of trees affected by AOD. Carbon starvation theoretically occurs over time, whereas hydraulic failure may be immediate: while trees tend to recover from phloem-girdling, xylem-girdling is an effective way to kill oaks (Dunn *et al.,* 1986; McDowell *et al.,* 2008). Feeding into the phloem disrupts within-tree carbohydrate transport. The maintenance of host responses to *A. biguttatus* and AOD (e.g. the formation of tyloses, the compartmentalization of tissues damaged by galleries, and the production of defensive compounds) is likely to be expensive in terms of carbohydrate utilisation, and may progressively deplete reserves over time (Haavik *et al.,* 2015). On trees with severe AOD symptoms, a large volume of the tree's hydraulic transport may be compromised by necrotic lesions, and a large portion of the tree's carbohydrate reserves may have been exhausted by host defence mobilisation, while lesions also impair phloem carbohydrate transport (Denman *et al.,* 2014). The development of extensive networks of *A. biguttatus'* large-instar larval feeding galleries may deal the death blow, through xylem-girdling, progressive exhaustion of the carbon budget, or both in combination.

Thermal requirements of A. biguttatus*, and implications for AOD*

If *A. biguttatus* does prove to be essential to AOD, the geographic range of AOD will be limited to the geographic range of the beetle (Figure 3). Insect species are usually limited geographically to areas where host range intersects with suitable temperatures for development. The beetle's narrow distribution within the UK, restricted to the southern, warmer parts of England, and the fact that the UK is on the northern edge of its range, suggest it may be thermally limited in the UK (Brown *et al.,* 2014). As oaks are present across the UK, host availability is not the limiting factor. Although critical lower and upper thresholds may restrict insect distributions through the mortality of certain life stages, many species' distributions are simply limited by summer heat availability (Bale, 2002). Temperature is a key regulator of the insect lifecycle: in order to complete development of each life stage, insects must develop for a certain amount of time at temperatures above a lower threshold (Ludwig, 1928; Danks, 2000). Environmental temperatures must be

conducive to adaptive seasonality, or the appropriate developmental timing of each life stage (Netherer & Schopf, 2010). A precise understanding of the beetle's life history and thermal requirements would be required to model the beetle's lifecycle, to determine its current suitable temperature range in the UK and whether this matches its recorded UK distribution.

Knowledge of the beetle's life history and thermal requirements is particularly important in order to predict the effects of climate change on the beetle's ecology, abundance, and distribution, as a warmer climate is likely to result in increased damage by many wood and bark-boring species (Sallé *et al.,* 2014). A greater number of stressed host trees, weakened by increasingly frequent and severe storms and droughts, may interact with reduced generational times to allow population increases of wood and bark-boring beetles in the UK (Wainhouse & Inward, 2016). The warmer summer temperatures projected under climate change may also allow the beetle to expand northwards, into new parts of the UK (Wainhouse *et al.,* 2016). Increased destructiveness and a northward range expansion due to climate change has already been documented in another European oak buprestid, *Coraebus florentinus* Herbst (Sallé *et al.,* 2014; Buse *et al.,* 2013). The recent establishment of *Agrilus sulcicollis* Lacordaire in the UK may also have been facilitated by climate warming (Alexander, 2003). Warmer summers, along with an increased number of stressed oaks, may also have contributed to a reported increase in both abundance and distribution for *A. biguttatus* (although there has also been increased recognition of the species), and may explain its recent establishment in southern Denmark (Pedersen & Jørum, 2009; Alexander, 2003; Brown *et al.,* 2014).

Summary and justification for the work

A precise understanding of how the predisposing, inciting, and contributing factors interact to cause AOD is necessary, in order to determine where management interventions may be most usefully made. Although *A. biguttatus* is considered to be an important component of oak decline in Europe and in the UK, because its lifecycle is cryptic and difficult to observe, it has been relatively little studied. AOD currently affects thousands of oak trees in the UK, yet numerous questions about the condition remain. This thesis aims to address three of these research gaps. First, the distributions of *A. biguttatus* and AOD are limited to the southern parts of the UK. The beetle's distribution is probably limited by temperature, but the beetle's life history, and the role of temperature in its development, are not well understood. A better understanding of the beetle's lifecycle, and thermal requirements, are required, in order to explain its distribution, and how this may be affected by climate change. Second, the larval galleries of *A. biguttatus* are nearly always found adjacent to AOD lesions, but adult exit holes are only found on some trees.

Although all symptomatic trees may be attractive to ovipositing females, the irregular emergence of adults may be explained if host resistance prevents the beetle from completing its development on vigorous trees. A better understanding of the current vigour of symptomatic trees is necessary to understand the impact of host defences on beetle success in trees with AOD. Third, although predisposition is assumed to be a prerequisite for AOD onset and colonisation by *A. biguttatus* larvae, the vigour of trees before AOD onset has not been evaluated. Knowledge of the vigour of trees before AOD onset is necessary to understand the role of predisposition in the syndrome.

<u>Aims and objectives of the thesis:</u>

1. To collect data on the life history of *A. biguttatus* and experimentally investigate the role of temperature in its development. This can be used to understand the current distribution of the beetle in the UK, and perhaps that of the AOD syndrome. The data can then be utilised for modelling the future influences of a warming climate upon these distributions.

2. To quantify the current vigour of trees in various states of decline by AOD, and with and without *A. biguttatus* exit holes, by measuring their response to mechanical wounding. These data will be used to determine whether host condition explains the irregular emergence of adults from AOD-symptomatic trees, and to improve the understanding of wound closure as a host defence against beetle larvae. The use of mechanical wound closure as a monitoring tool for identifying the healthiest trees will also be evaluated.

3. To examine the stem growth record of symptomatic and asymptomatic trees, in order to determine whether predisposition, as measured by reduced or declining growth, is evident in trees with AOD. An examination of the recent growth trends of trees at various stages of decline by AOD, including those in remission, should show the impact of AOD on stem growth. Finally, the growth of symptomatic trees with and without *A. biguttatus* exit holes will be compared, to determine the relationship between stem growth and the successful development of the beetle.

Paper 1:

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

by Reed, K., Denman, S., Leather, S.R., Forster, J. and Inward, D.J.G.

Paper 1*: The lifecycle of *Agrilus biguttatus*: the role of temperature in its development and distribution, and implications for Acute Oak Decline

Abstract

1. The two spotted oak buprestid, *Agrilus biguttatus* Fabricius, 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.

Introduction

 The two spotted oak buprestid, also known as the two spotted oak borer, *Agrilus biguttatus* Fabricius (Coleoptera: Buprestidae), is considered an increasingly important secondary pest of oak (Hartmann & Blank, 1992; Thomas, *et al.* 2002; Sallé *et al.,* 2014). It has played a key role in large-scale oak decline events across continental Europe and Russia from the 1900s onwards (Falck, 1918; Starchenko, 1931; Hartmann & Blank, 1992; Moraal & Hilszczanski, 2000; Thomas, *et al.* 2002; Sallé *et al.,* 2014), and its pest status in continental Europe and the UK appears to be increasing (Moraal & Hilszczanski, 2000). Until relatively recently, *A. biguttatus* was considered rare in the UK (Shirt *et al.*, 1987). Perceptions of the beetle's pest status in England, however, have been changing since the 1990s, when *A. biguttatus* was first linked to Acute Oak Decline (AOD), a syndrome that often leads to the rapid death of pedunculate and sessile oaks *(Quercus robur* L. and *Q. petraea* (Matt.) Liebl.) (Gibbs and Greig, 1997). Buprestids like *A. biguttatus* typically develop only on weakened hosts (Bellamy, 2003), or exceptionally on healthy but naïve hosts without co-evolved resistance. For example, the introduced emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) has caused the death of millions of susceptible ash trees in the United States and Canada (Herms & McCullough, 2014).

Agrilus biguttatus appears to be strongly associated with AOD in the UK, although its precise role in the syndrome is still under investigation. Acute Oak Decline is characterised by a number of key symptoms on oak stems, in particular, the combination of fluid exudations from vertical splits between the bark plates, inner bark necroses, and the larval galleries of *A. biguttatus* (Denman *et al.*, 2014). Tree declines such as AOD are thought to be caused by a combination of predisposing factors (host genetics or site), inciting factors (e.g. drought), and contributing factors (secondary pests or pathogens) (Manion, 1981). Several pathogenic bacterial species have now been implicated in the formation of the typical AOD lesions (Brady *et al.*, 2010; Denman *et al.*, 2012), but the nature of the association with *A. biguttatus* remains unclear. In recent studies, exit holes of adult beetles were found externally on 30-33% of symptomatic trees, but larval feeding galleries were found internally adjacent to almost all examined lesions (Denman *et al.,* 2014; Brown *et al.*, 2017). *Agrilus biguttatus* and AOD also share a similar UK distribution within southern and central England (Brown *et al.*, 2014). The beetle may be secondary, taking advantage of weakened and moribund trees, but could contribute to the spread of the bacteria, the formation of necrotic lesions, or the death of trees, and so may be integral to AOD (Brown *et al.*, 2014, 2017).

Agrilus biguttatus is cryptic and difficult to observe in nature, and as a result has been relatively little studied. It has never been cultured from egg to adult in the laboratory and, therefore, its lifecycle has been described only broadly (Brown *et al.*, 2014). Female beetles typically oviposit deep into bark crevices on the trunk of mature oak trees, and larvae subsequently form feeding galleries at the cambial interface. In continental Europe, the beetle has been reported to have a one or, more commonly, two-year lifecycle, in which case the larvae overwinter as early instars and continue 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.

<u>Methods</u>

In order to experimentally examine the role of temperature in the development of *A. biguttatus,* and to collect data on the life history of the beetle for lifecycle modelling, novel methods were developed to collect and culture the beetle in the laboratory.

Collection of overwintering larvae, adult emergence and sex ratio

Quercus robur, > 30cm diameter at breast height (1.3m), demonstrating exit holes of *A. biguttatus* and with 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, one or two suitable trees were felled between October 2013 and April 2014, when insects were likely to be in the overwintering, prepupal stage within the outer bark. Slabs from stems and large branches comprising bark, sapwood, and some heartwood (approximately 0.5 – 2.0m high x 0.4m wide x 0.25m deep), 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. 2.0m high x 1.7m wide x 1.4 m deep) 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).

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

Site	Latitude	Longitude	Elevation (m a.s.l.)	Description
Dudmaston, Shropshire	52.496603	-2.375157	60m	*Quercus robur* plantation within parkland
Garnons Estate, Herefordshire	52.089785	-2.881768	70m	Parkland, mainly *Quercus robur*
Grafton Wood, Worcestershire	52.198769	-2.042427	40m	Semi-natural ash and oak woodland
Richmond Park, London	51.455423	-0.270892	20m	*Quercus robur* plantation within urban parkland
Runs Wood, Norfolk	52.672009	0.410581	10m	Plantation, mainly *Quercus robur*

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 housed 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 jar, 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 jars. Beetles were fed on fresh leaves, and the jars 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 placed in individual jars and 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. Replicates were limited by incubator space, and allocation was based on an assumption of longer development times at lower temperatures (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, due to high mortality of specimens when handling in this sensitive stage. To test whether prepupae would develop continuously at warm temperatures without a chill period, eleven prepupae (= "unchilled prepupae") were kept at 22.5 or 25°C and observed twice-weekly for signs of further development.

See Appendix B, Figures B.1 and B.2, for photographs of the experimental set-up.

Statistical analysis

All analyses were carried out in R (R Core Team, 2016). All means are presented ± 1 standard error unless otherwise stated.

A Chi Square test was used to determine whether the observed sex ratio deviated from the expected ratio (1:1). A Welch's two-sample t-test was used to test for differences between the initial oviposition dates of single females and females in mixed groups. Pearson's product-moment correlation was used to test for correlation between adult lifespan and fecundity (total number of eggs laid).

Egg hatch, cessation of larval development, and adult emergence were all classified as binary responses. As such, all three were modelled using probit regression. As the processes underlying the three dichotomous responses are likely to be normally distributed across the *A. biguttatus* population, probit was chosen rather than logit, as the probit model assumes errors to be normally distributed, whereas logit assumes standard logistic distribution of errors. In all three cases, three basic models were applied to the data, one with only the covariate "day" in the model, one with "day" and "temperature" and one including the interaction between "day" and "temperature".

In the analysis of the egg data, separate egg batches were defined as samples, and the hatching of individual eggs was modelled through time, with each egg batch providing a probability of hatching on each date. To prevent pseudoreplication, the probit models were set up as mixed-effects models using the lme4 package in R (Bates *et al.,* 2014), and included a random effect of sample to account for the repeated measurements made on an individual sample. The effects of "day" and "temperature" were scaled in all three models to improve model fit, using the standard scale function in R. Analysis of deviance

was used to determine the best fit model. Having chosen the best fit model, model predictions and confidence intervals were calculated for the fixed effects.

Larval data samples comprised the number of larvae within logs. Each log was treated as an independent sample and analysed using standard probit regression in the GLiM function in R. Analysis of model deviance was used to determine the best fit model, and probability predicted from the best fit model.

All data from emerged adults were pooled. Development times, after the return of the prepupae to 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 and temperature, times for 10%, 50%, and 90% of individuals to complete development were calculated from the best fit models. The 50% 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).

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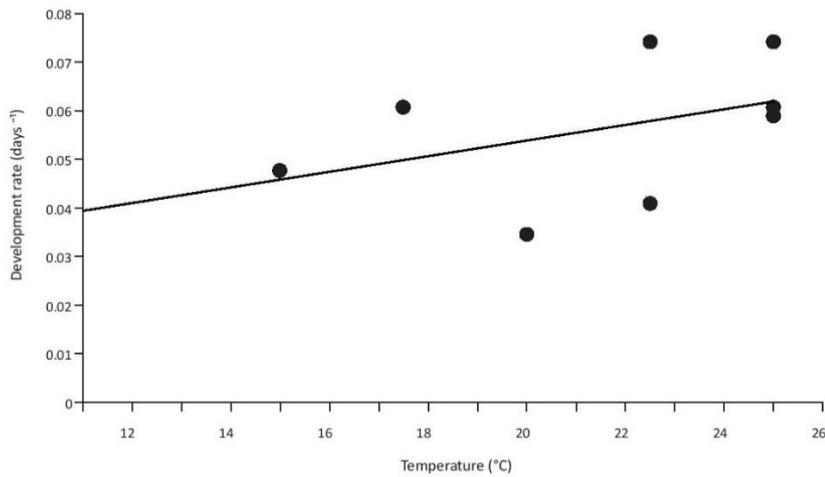
Figure 1. Development rate (days^{-1}) of monitored prepupae of *Agrilus biguttatus* from cessation of overwintering to pupation. Data points are the actual development times of individual prepupae, and a linear regression line is fitted.

To determine whether temperature affected larval peristomal width, a linear mixed model was fitted in the lme4 package in R. The response, peristomal width, was square-root transformed, and posteriorly assigned instar and constant temperature treatment were specified as explanatory variables. 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 (Table 2); 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 Garnons 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 from each other (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).

Table 2. Sex ratios of emerging adults within the emergence cages.

Site	Female	Male	p	Significance
Dudmaston, Shropshire	189	187	0.96	
Garnon's Estate, Herefordshire	106	68	< 0.01	*
Grafton Wood, Worcestershire	1	4	0.38	
Richmond Park, London	39	30	0.34	
Runs Wood, Norfolk	7	8	1.00	

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 3). 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 completion of development of selected percentages of individuals, chosen to represent the variability in developmental time, are given in Table 4. 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 5. 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.

Table 3. 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	600.00	<0.001
Day	1	14,900	<0.001
Temperature:Day	1	615.00	<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	<0.001
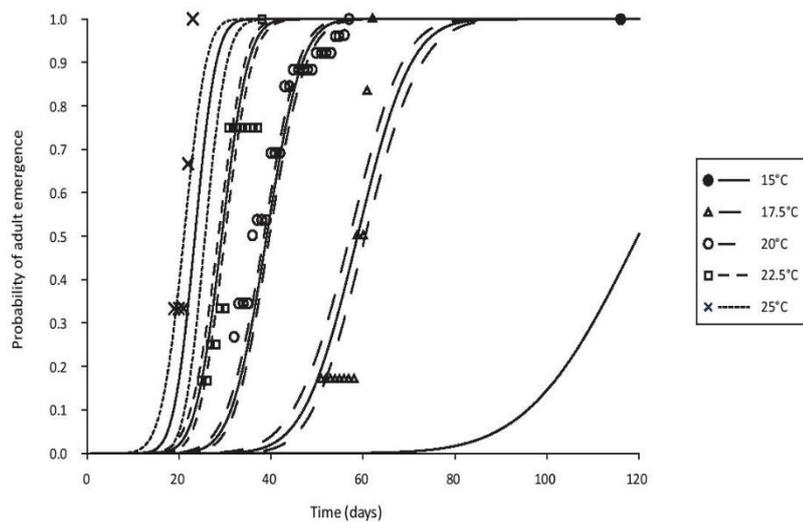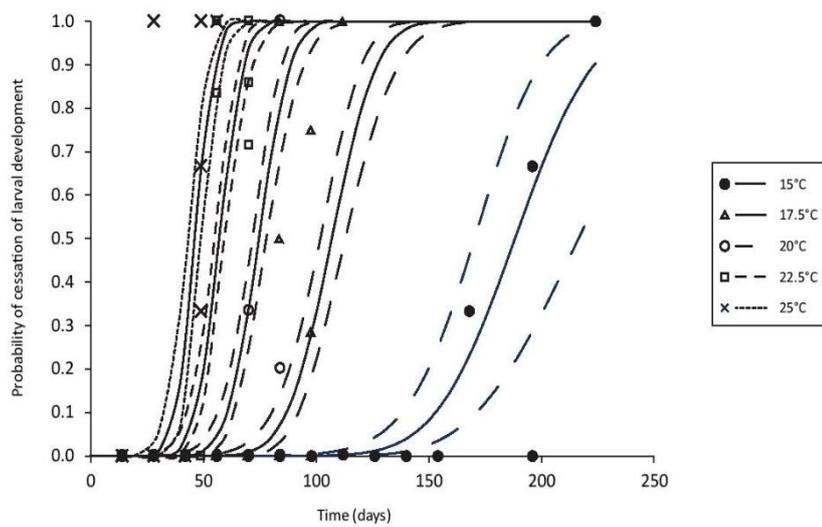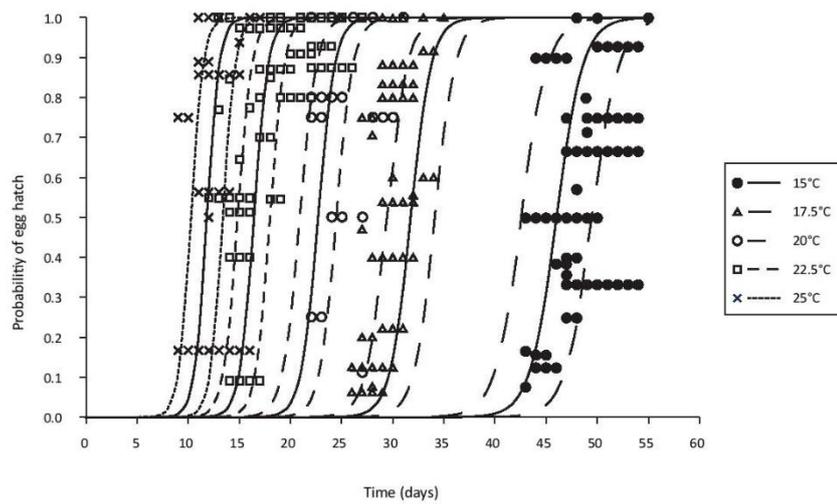
Figure 2. Probit models estimating the probability of: (A) *Agrilus biguttatus* egg hatch, signifying the length of egg development from oviposition to egg hatch; (B) cessation of larval development, signifying the length of larval development from egg hatch to migration to the outer bark; and (C) adult emergence, signifying the length of pupal development, after overwintering, (to adult emergence). Data points are the proportions of individuals that had completed the relevant developmental stage, and the dotted lines represent the 95% confidence intervals.

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Table 4. Predicted time for 10%, 50%, and 90% of individual *Agrilus biguttatus* to complete development, by temperature and life stage; these centiles were chosen to represent the variability in development times. 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.

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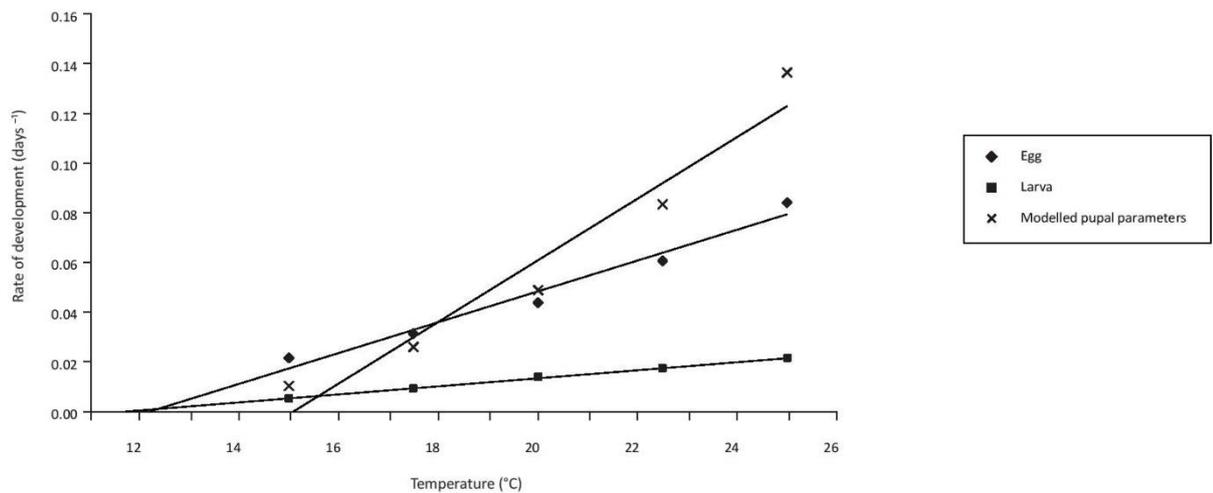
Figure 3. Temperature-dependent development rates (days^{-1}) of *Agrilus biguttatus* eggs, larvae, and pupae (to adult emergence), as predicted by probit regression. Data points are the predicted 50% completion times.

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 5, Figure 3); the broad ranges were driven by variability in prepupal development time.

Table 5. 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

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 (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 6, Figure 4). Posteriorly-assigned instar, temperature, and their interaction were all significant predictors of peristomal 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, peristomal width was smallest at the highest temperatures (22.5 and 25°C), although this pattern was not significant (Figure 5).
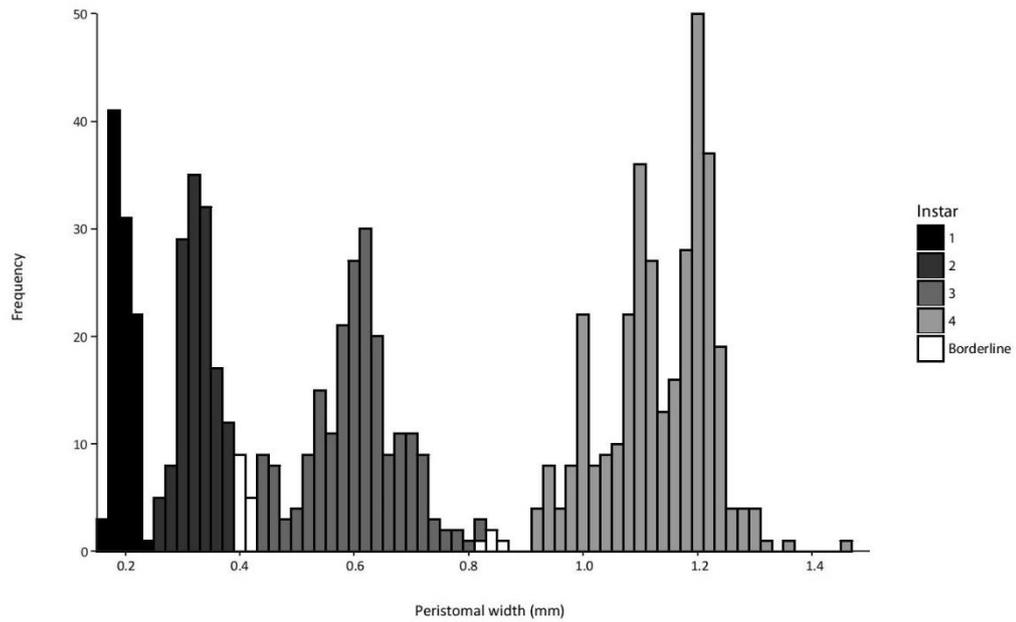
Figure 4. Histogram of *Agrilus biguttatus* larval peristomal widths, showing the four instars predicted by the normal mixture model.

Table 6. 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
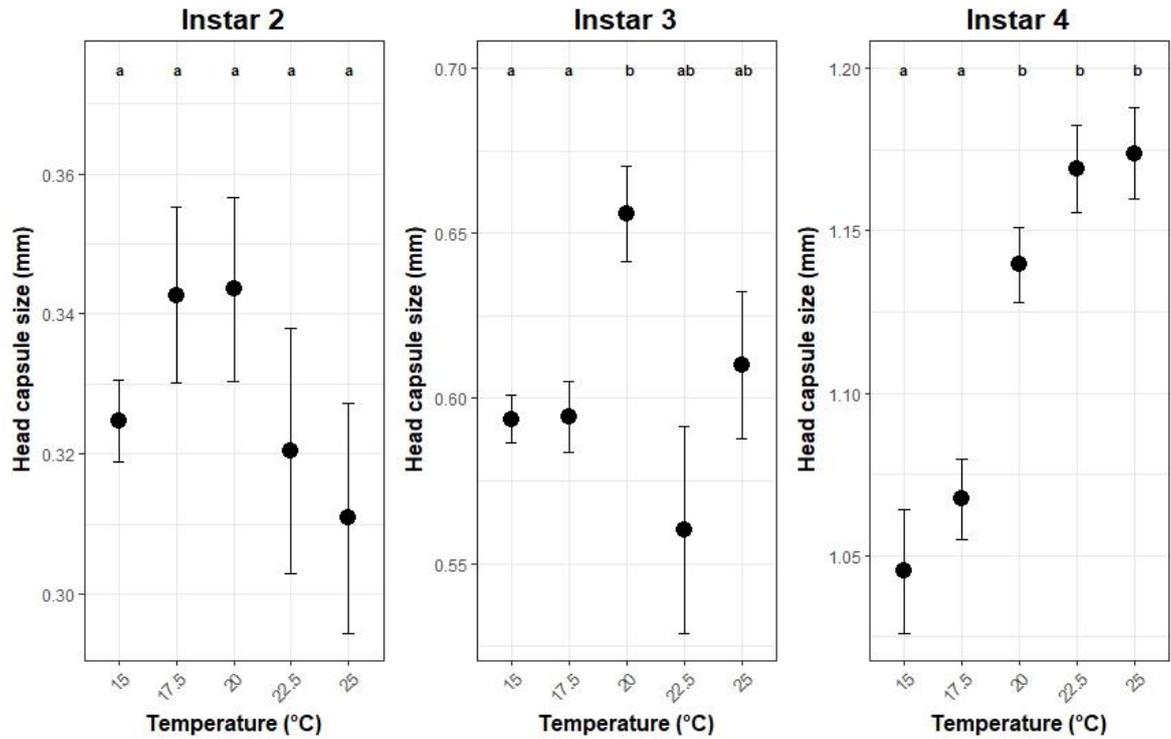
Figure 5. Peristomal width (mean ± SE) of second, third and fourth instar larvae of *Agrilus biguttatus* at each constant temperature treatment, suggestive of a shifting thermal optimum, wherein early instar larvae attain optimal growth at lower temperatures.

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

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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 5) 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 of larvae 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 (Coleoptera: 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 4, 5), 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 4). 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 pupal (to adult emergence) threshold. 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 (defined as when genetic plasticity opposes environmentally-induced variance) in development times has been shown in other insects, such as the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (e.g. Mitton & 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 that may be limited by finite moisture requirements and host defences (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 author is 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; Loerch & Cameron, 1983; Lyons & Jones, 2005; Haavik *et al.*, 2013; Orlova-Bienkowskaja & Bieńkowski, 2016). The pattern of smallest peristomal width at the highest temperatures in second instar larvae, although not significant, larger peristomal width at 20°C than 15 and 17.5°C, and largest peristomal width at the highest temperatures in fourth instar larvae, suggests a shifting thermal optimum (Atkinson, 1996), with early instar larvae attaining optimal growth at lower temperatures, and later instar larvae attaining optimal growth at higher temperatures (Figure 5). After hatching in late summer, optimal growth at low temperatures would allow early instar larvae to take advantage of cooler conditions in the early autumn. Conversely, fourth instar larvae, developing during the following summer, would be able to take advantage of warmer summer temperatures. This provides further support that the lower experimental temperatures chosen in this study, 15 and 17.5°C, although representative of summer temperatures within the beetle's range in England, are suboptimal for the development of *A. biguttatus;* the finding may, however, have been compounded by an effect of reduced food quality due to longer development times at these temperatures.

Conclusions

The results of the present study of *A. biguttatus*' development suggest its thermal requirements limit its UK distribution. The beetle's relatively long pupal and egg development times, at temperatures similar to current mean daily summer temperatures in the UK, appear to restrict the beetle to the warmer parts of England. The increasingly warmer summer temperatures expected under climate change may allow the beetle to spread to new areas of the UK. If *A. biguttatus* proves an essential component of AOD, the area affected by that disease syndrome is also expected to increase, and indeed, evidence of the beetle, and AOD, have been newly discovered in Wales (Denman *et al.,* 2016). The influence of climate change upon the *A. biguttatus* lifecycle may also allow the

beetle to become more damaging in its own right. Warmer summers may provide more frequent opportunities for the beetle to complete development, and additionally increase host availability, as more frequent stress events, such as droughts, impact oak trees (Lindner *et al.*, 2010; Netherer & Schopf, 2010). As host death, irrespective of AOD, is reportedly dependent on the density of larval galleries, with increasing abundance and opportunity, the beetle may become more harmful (Hartmann & Blank, 1992). The new insights into the beetle's life history found in the present study, including its thermal requirements, the presence of an obligatory diapause, and its adult lifespan and fecundity, are important for modelling the beetle's lifecycle and role in AOD, and how this may alter under a warming climate.

Paper 2*: Does a vigorous wound response inhibit the complete development of *Agrilus biguttatus* on trees with Acute Oak Decline?

Abstract

1. Although the larval galleries of the two spotted oak buprestid, *Agrilus biguttatus,* are almost always found in close proximity to the necrotic stem lesions characteristic of Acute Oak Decline (AOD), the exit holes of the adults are found on only approximately 30% of symptomatic trees. This study aimed to quantitatively assess the vigour of symptomatic trees with and without exit holes, in order to determine whether robust host resistance prevents the beetle from completing development on symptomatic trees.

2. Experiments were carried out at three sites monitored for AOD lesions and *A. biguttatus* exit holes, on trees within six symptomatic categories (two differing durations of AOD infection, and apparent remission, with and without exit holes) and asymptomatic control trees. As a proxy for the damage incurred by necrotic lesion formation and larval chewing, trees were mechanically wounded in late spring and early summer. Percentage wound closure was measured at four dates over two years.

3. After two years, wound closure was most efficient in asymptomatic trees, and least efficient in trees with long-term AOD symptoms. Within the remission and newly symptomatic categories, wound closure was less efficient when exit holes were present. These results suggest the irregular adult emergence observed on symptomatic trees is likely to be explained by a cessation of larval development on trees with robust host resistance, and robust wound response in particular.

4. Inconsistent results across the earlier measuring dates suggest other factors, including induced host defences, may influence new infections and inhibit beetle development. Wounding date and aspect also influenced percentage wound closure at all measuring dates.

5. Monitoring AOD-symptomatic trees' wound response may be a simple tool for managers to identify the symptomatic trees that are least likely to survive.

<u>Introduction</u>

The two spotted oak buprestid, *Agrilus biguttatus* Fabricius*,* is considered an important component of European oak decline, and damage by the insect has been increasingly reported since the 1990s (Moraal & Hilszczanski, 2000; Sallé *et al.*, 2014). In the UK, *A. biguttatus* is strongly associated with Acute Oak Decline (AOD), a newly-described syndrome that threatens English oaks, *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. (Denman *et al.*, 2014)*.* Decline occurs when trees, predisposed by factors such as genotype and poor soils, are weakened by inciting factors, such as drought or defoliation by insects or fungi, leaving them susceptible to attacks by secondary insects and / or pathogens (Manion, 1981). Physiologically, trees decline because of carbohydrate deficiency (McDowell *et al.,* 2008). Ring-porous, deciduous trees like oaks require particularly large carbon reserves for winter maintenance, the production of earlywood and spring growth, and as a buffer against stress (Barbaroux & Bréda, 2002; Sala *et al.*, 2012). Progressive, carbon-expensive responses to stress reduce carbon budgets over time, leaving trees unable to allocate sufficient resources to resistance (Führer, 1998; Bréda *et al.*, 2006). AOD is characterised by four symptoms on oak stems: fluid exudations; cracks between the bark plates; necrotic lesions beneath the bark; and the larval galleries of *A. biguttatus* (Thomas, 2008; Denman *et al.*, 2014). The specific, causal agents of AOD are under investigation, but trees are thought to be predisposed through reductions in carbon reserves due to environmental stress or poor soils, and several pathogenic bacterial species are thought to be responsible for the formation of bark necroses, potentially in association with *A. biguttatus*, although the causality of the beetle has yet to be shown (Brady *et al.*, 2010; Denman *et al.*, 2012; 2014; 2016)*.* In the UK, thousands of trees are currently affected by AOD, and on sites monitored for the syndrome, approximately 1.3% of affected trees are reported to die each year (Denman *et al.*, 2016). Many trees have also been shown to occlude bark lesions and larval *A. biguttatus* galleries with callus-like tissue, and enter a state of recovery or remission (Denman *et al.*, 2014).

Agrilus biguttatus is thought to be involved in AOD due to the co-occurrence of the syndrome and beetle at both the landscape-scale and on individual trees. Affected hosts, and the beetle, share a strikingly similar distribution, and, significantly, larval galleries are reportedly found adjacent to almost all bark lesions (Brown *et al.*, 2017). The beetle is not restricted to trees with AOD, and may colonise hosts without AOD symptom expression; necrotic lesions characteristic of AOD are not, however, readily found without adjacent beetle galleries (Denman *et al.*, 2014; N. Brown, pers. comm.; S. Denman, pers. comm.). Brown *et al.* (2014) summarised several possible roles and chronologies of *A. biguttatus* and pathogenic bacteria in AOD: 1. both the beetle and bacteria may be secondarily

colonising already-weakened trees; 2. the presence of the bacteria may attract the beetle, e.g., through volatile emissions from the lesions; 3. the beetle may facilitate bacterial colonisation, e.g. the bacteria may establish in host tissues damaged by larval galleries; 4. the beetle may vector the bacteria, either between trees or within trees, with both establishing simultaneously (Vansteenkiste *et al.*, 2004; Brown *et al.*, 2014; Vuts *et al.*, 2016).

Intriguingly, despite the high co-occurrence of AOD symptoms and *A. biguttatus* larval galleries, emergence holes have been found on only 30% of symptomatic trees (Brown *et al.*, 2017). The level of host decline required by the beetle to colonise a tree and complete development is not well understood. *Agrilus biguttatus* damages trees during the larval feeding stage, creating galleries, mostly at the cambial interface but additionally in the phloem and sapwood, cutting through tissues and damaging the host's vascular systems (Vansteenkiste *et al.*, 2004). As is characteristic of the Agrilinae, *A. biguttatus* larvae are thought to be restricted to weakened hosts, although the species has been described as "aggressive," and capable of killing off trees that would, in the absence of the beetle, probably recover (Thomas *et al.*, 2002; Evans *et al.*, 2007). Agrilinae only attack and kill healthy trees when introduced to naïve hosts: for example, in China, where it is native, the emerald ash borer, *A. planipennis,* is restricted to weakened and dying trees, yet it devastates healthy ash trees native to the US and Canada, where it was accidentally introduced, probably in the early 1990s (Herms & McCullough, 2014).

Potentially, all AOD-symptomatic trees have been sufficiently weakened to have attracted ovipositing females, and allow larval colonisation, but host resistance in most symptomatic trees may be too robust for larvae to complete development (Brown *et al.*, 2017). Quantitative measurements of host resistance would be required to test this hypothesis. On sites monitored for AOD symptoms, the vigour of trees has been assessed by classing crown condition, a proxy for tree health, on an ordinal scale, allowing coarse comparisons between the vigour of trees with and without AOD lesions and *A. biguttatus* exit holes. Trees with AOD symptoms generally show poorer canopy health: assessments at monitored sites found AOD symptoms more frequently, and in greater severity, on trees with thinned crowns, rather than on healthy crowns (Brown *et al.*, 2014). In a study of declining oaks in the Netherlands, *A. biguttatus* galleries were not found on trees with <25% canopy loss, and exit holes were not found on trees with <50% canopy loss, suggesting hosts susceptible to colonisation by *A. biguttatus* larvae were not necessarily weakened enough for complete development of the beetle (Hartmann & Kontzog, 1994; Vansteenkiste *et al.*, 2004). Finally, at AOD monitoring sites, over four years, stem bleeds and exit holes were found to occur sequentially: trees with AOD symptoms during the previous year were more likely to have emergence holes the following year, leading the

authors to suggest the exit holes appeared later in the syndrome, on trees with a greater degree of decline, than the characteristic AOD 'bleeds' (Brown *et al.*, 2017).

Measuring a tree's response to mechanical wounding may be a simple, quantitative way to assess the level of host resistance of trees with AOD. Rate of wound closure, through the formation of callus-like tissue, should be an indicator of a tree's vigour, is an important defence against AOD lesion development, and may protect trees against cambial borers such *A. biguttatus.* Efficient wound closure is necessary to restore vascular functionality, to inhibit fungal growth by excluding airflow, to prevent water loss, and to prevent infection by opportunistic bacteria and fungi (Neely, 1988; Pearce, 1996; Howe & Schaller, 2008). Wound closure has been found to be correlated with annual growth, an accepted indicator of host vigour, in a number of deciduous tree species (Neely *et al.,* 1988; Dobbertin, 2005; Armstrong *et al.,* 2007). Less efficient wound closure has also been shown in declined, than in healthy, deciduous trees (Wargo, 1977; Haavik & Stephen, 2011). Wound closure allows vigorous trees to wall off AOD damage: beneath the bark, trees have been observed to occlude lesions with callus-like tissue, and many trees are able to completely occlude lesions, a process involving compartmentalisation of damaged tissues, phellogen regeneration through growth of covering wound periderm and, ultimately, newly-differentiated vascular cambium and sapwood (Biggs *et al.,* 1984; Neely, 1988; Smith, 1988; Vansteenkiste *et al.*, 2004; Armstrong *et al.,* 2007). Trees with AOD have also been observed to close up *A. biguttatus* larval galleries (Denman *et al.*, 2014). Although relatively little is known about host resistance mechanisms against cambial borers in deciduous trees, and the repair of tissue damaged by larval galleries, it has been hypothesized that vigorous hosts may overgrow or crush slow-moving or inactive larvae (Dunn et al., 1990; Fierke & Stephen, 2008). Mimicking the damage inflicted by larval chewing, the rate of closure of mechanical wounds has been shown to be related to the success of colonisation by the larvae of two other wood and bark boring pests of oaks in Canada and the United States, *Agrilus bilineatus* Weber and *Enaphalodes rufulus* Haldeman (Dunn *et al.*, 1990; Fierke & Stephen, 2008).

The objectives of this study were: to quantitatively compare the current vigour of trees at different stages of AOD infection, by measuring their response to mechanical wounding; to determine whether host condition, as measured by wound response, explains the beetle's inability to complete its development on many AOD-symptomatic trees; to improve the understanding of wound closure as a defence against beetle larvae; and to evaluate the use of mechanical wound closure as a monitoring tool for comparing intra-site tree vigour.

Methods

Experiments were carried out at two established AOD monitoring plots within Hatchlands Park and Richmond Park, and at a third site where AOD was known to be present, Madingley Wood. Monitoring first began at the Hatchlands Park plot in 2009, and at the Richmond Park plot in 2010, and continued throughout the study period. See Table 1 for site details and descriptions. At Richmond Park and Hatchlands Park, annual monitoring, conducted between May and August, included counts of active AOD lesions below 3m and counts of *A. biguttatus* exit holes visible from ground-level on tree stems (up to approximately 3m). Tree canopies were also visually categorised on an ordinal scale by percent canopy loss, as compared to photographs in *FC Field Book 12: Assessment of Tree Condition (*Innes, 1990). The categories were: 1 = dead, 2 = 80-95% canopy loss, 3 = 35-75% canopy loss, 4 = 10-30% canopy loss, 5 = < 5% loss. See Brown *et al.* (2016) for further details of monitoring methodology at Hatchlands Park and Richmond Park. At Madingley Wood, the trees were surveyed in 2014 for active / recent lesions, old (occluded) lesions, and *A. biguttatus* exit holes.

Study trees were chosen in March 2015. Based on monitored history, trees from Hatchlands Park and Richmond Park were selected from seven categories, based on AOD symptom history and the absence / presence of *A. biguttatus* exit holes: long-term symptoms plus/minus exit holes, new symptoms plus/minus exit holes, remission plus/minus exit holes, and asymptomatic control. Long-term symptoms trees had at least one active lesion at the start of the monitoring period, and in 2014 or 2015; trees may have been in remission (e.g. no active lesions seen) for one or more years during the study. New symptoms trees developed at least one active lesion after monitoring began, and had active symptoms in 2014 or 2015. Remission trees had at least one active lesion during the monitoring period, but no active symptoms in 2014 or 2015. Where numbers were sufficient, the aim was to select seven trees in each symptomatic category, and fourteen asymptomatic trees (total = 56 trees per site). If possible, trees with very unusual form or signs of extensive root pathogen or other damage were excluded. Numbers of symptomatic trees, and those with beetle exit holes were, however, very low at Hatchlands Park and Madingley Wood, and nearly all symptomatic trees were included at these sites. To increase replicates at Hatchlands Park and Madingley Wood, extra trees in remission were selected. All trees were re-surveyed for active lesions and exit holes in April-May and October 2015, and categories were updated accordingly. See Table 2 for the final numbers and categories of study trees at each site. If trees died during the study, subsequent percentage wound closure was recorded as 0%.

Table 1. Description of the study sites at Richmond Park, Hatchlands Park, and Madingley Wood (Brown *et al.*, 2016, 2017; Natural England, 2017).

	Sheen Wood, Richmond Park (RP)	Hatchlands Park boundary belt (HP)	Madingley Wood (MW)
Location	Richmond Upon Thames, Greater London	Guildford, Surrey	Cambridge, Cambridgeshire
Latitude / Longitude	51.455636 / -0.27052781	51.263098 / -0.46337962	51.263098 / -0.46337962
Size of study area	3.4 ha	0.7 ha	13.8 ha
Approximate number of oaks within study area (DBH > 30cm)	150	140	310
Elevation (m a.s.l.)	20m	80m	60m
Description	Discrete *Quercus robur* plantation within urban parkland	Discrete *Quercus robur* shelter belt within parkland	Mixed-age stands of *Quercus robur* within ancient and secondary ash-maple woodland
Uniformity	Relatively uniform stand	Relatively uniform stand	Trees drawn from 5 compartments; variability in tree age and stand environment
AOD symptoms first recorded	1990	After 2001	2009-2010
Current monitoring began	2010	2009	2014
Level of foot traffic	High	Medium	Low
Other disturbance factors	Chronic Oak Decline; oak processionary moth; deer	Chronic Oak Decline	Chronic Oak Decline
Bacterial species *Brenneria goodwinii* and *Gibbsiella quercinecans* isolated at site?	yes	yes	yes

The canopies of the study trees were assessed in two ways in October 2015. 1. Trees were grouped according to the ARCHI protocol, in which trees are assigned to one of five classes in order to determine their future commercial viability ("Healthy", "Resilient", "Stressed", "Retrenching", "Irreversible Decline"), based on percent canopy loss and the architecture and abundance of epicormic growths (Drénou *et al.*, 2015). Of these categories, healthy and resilient trees and, potentially, stressed trees are considered to be commercially viable crop trees, whereas retrenching trees may survive but will not be good crop trees, and irreversibly declining trees are unlikely to survive. 2. Based on the protocol used in the AOD monitoring at HP and RP, trees were re-assessed on the ordinal scale criteria detailed above.

Table 2. Description of AOD and exit hole categories, and number of trees within each category, at each site.

AOD and exit hole category	Abbrev.	Hatchlands Park	Richmond Park	Madingley Wood
Long-term symptoms with exit holes	LS+	4	12	3
Long-term symptoms without exit holes	LS-	7	5	5
New symptoms with exit holes	NS+	2	5	-
New symptoms without exit holes	NS-	6	6	-
Remission with exit holes	R+	0	4	2
Remission without exit holes	R-	11	7	21
Asymptomatic of AOD or exit holes	C	14	14	15
Total number of study trees at each site		44	53	46

Trees were mechanically wounded at two time-points in 2015: first in spring (late April or early May), and second in summer (June). At each time-point, trees were wounded on the north and south faces. Wounds were made through the bark and phloem to the sapwood with a 1cm diameter arch punch and wooden mallet, and the sapwood interface was scored with a pocket knife. It was sometimes difficult to judge the location of the sapwood interface, and some wounds breached the sapwood by up to approximately one cm. To prevent between-tree bacterial spread through wounding, arch punches were thoroughly cleaned with 70% IMS after each use, and separate arch punches were used to wound asymptomatic and symptomatic trees.

To assess wound closure over time, the wounds were photographed on six dates: June, August, and October 2015, April and August 2016, and April 2017. Digital photographs were taken using a mobile phone camera with flash. Initially, a laminated paper grid was pinned to the sapwood interface to standardise scale, but this was felt to be unreliable, and the scale was discarded for the last set of observations. Photographs from four measuring dates, August 2015, April and August 2016, and April 2017, were analysed in ImageJ (Rasband, 2012). The area covered by callus-like tissue and the area of sapwood still exposed were measured, and percentage wound closure was defined as the area covered, divided by the sum of the area covered and the area still exposed.

See Appendix B, Figure B.3, for photographs of the experimental set-up.

Statistical analysis

All analyses were carried out in R (R Core Team, 2016).

Due to a low number of replicates in some categories, data from all three sites were pooled. Data were analysed using "Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)," or "glmerMod" models, in R package lme4; a binomial error structure and logit link function (appropriate for proportional data), and the "bobyqa" optimizer, were specified (Bates *et al.,* 2014). The response was percentage wound closure. Data from each measuring date (August 2015, April 2016, August 2016, and April 2017) were analysed separately. The fixed effects were time-point of wounding (spring or summer), aspect (north or south), and AOD and exit hole category (factor with seven levels). To account for potential variance in resistance due to tree age (Jenkins & Pallardy, 1995), estimated age from the increment cores (Paper 3), scaled using the scale function in R, was also included as a fixed effect. Interactions between the fixed effects were not included. To account for site- and stand-level differences, a nested random effect of compartment within site was fitted. The best fit models were selected according to Akaike's Information Criteria (AIC). Having determined the best model, the significance of the fixed effects was determined based on the significance of the F statistic from the type II tests within the anova function in R. Post hoc tests using the R package lsmeans were conducted on the best fit model, to estimate differences within significant factors, correcting for multiple comparisons using Bonferroni adjustments to the p-value (Lenth, 2015). Further post hoc tests were performed to test the specific question of whether percentage wound closure differed with the presence or absence of beetle exit holes.

Results

AOD and exit hole category was a highly significant predictor of percentage wound closure at all measuring dates (Table 3). Contrasts between exit hole treatments were also highly significant at all measuring dates (z-ratio on 08/2015, 04/2016, 08/2016, 04/2017 = -4.8, 4.8, 8.6, 10.9, respectively; at all dates, $p < 0.0001$). Post hoc testing showed that by the final measuring date, there was a clear reduction in percentage wound closure rate from asymptomatic and remission trees, to new symptoms, and to long-term symptoms, with reductions for exit holes within the remission and newly symptomatic categories (Figure 1). At the second and third measuring dates, percentage closure was consistently lowest in the long-term symptoms categories, while the relative rankings of the newly symptomatic, remission, and control categories varied (Figure 1).

Table 3. Significance of the fixed effects in the percentage wound closure models: AOD and exit hole category, estimated tree age, time-point of wounding, and aspect of wounding.

	AOD and exit hole category				Age			
Date	**F**	**Df**	***p***	**sig.**	**F**	**Df**	***p***	**sig.**
Aug-15	16.9	6	<0.0001	***	822.0	1	<0.0001	***
Apr-16	188.3	6	<0.0001	***	328.9	1	<0.0001	***
Aug-16	298.7	6	<0.0001	***	592.3	1	<0.0001	***
Apr-17	500.5	6	<0.0001	***	309.1	1	<0.0001	***
	Time-point				**Aspect**			
Date	**F**	**Df**	***p***	**sig.**	**F**	**Df**	***p***	**sig.**
Aug-15	341.2	1	<0.0001	***	57.4	1	< 0.0001	***
Apr-16	2730.3	1	<0.0001	***	14.3	1	< 0.0001	***
Aug-16	1870.0	1	<0.0001	***	128.5	1	< 0.0001	***
Apr-17	305.8	1	<0.0001	***	22.0	1	< 0.0001	***
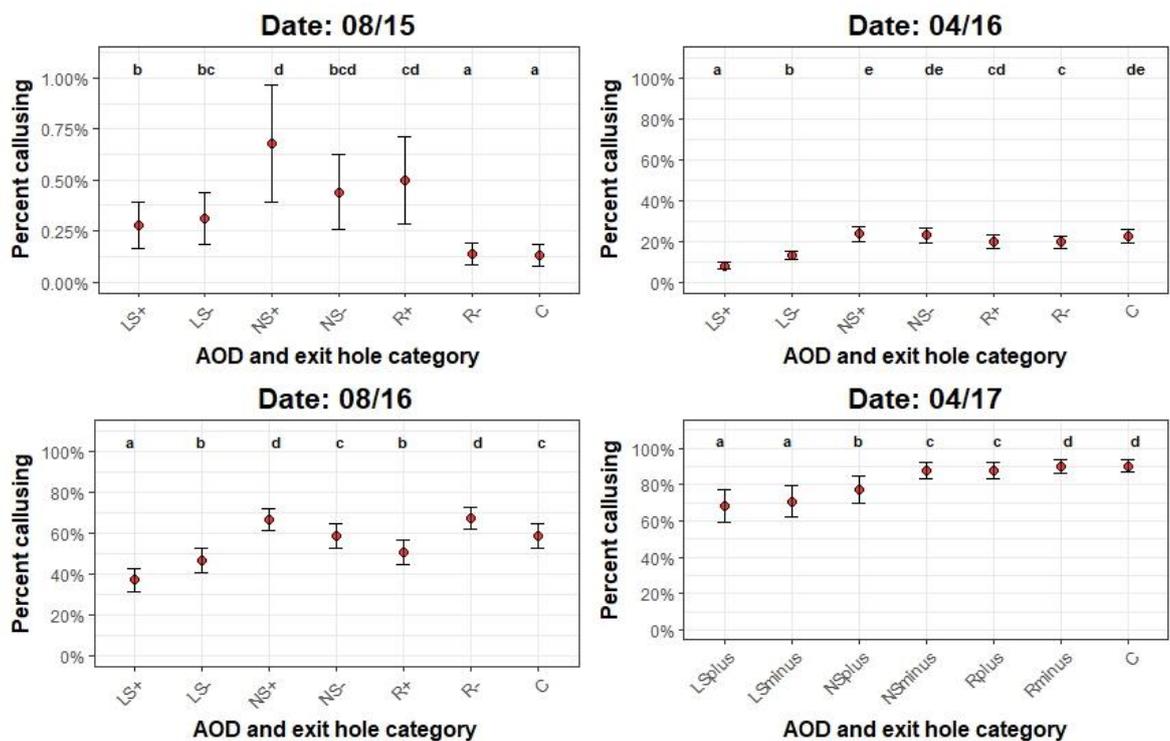
Figure 1. The model-predicted percentage wound closure at each measuring date (mean ± SE). LS+ / - = long-term symptoms plus / minus exit holes, NS+ / - = new symptoms plus/minus exit holes, R+ / - = remission plus/minus exit holes, C = asymptomatic control. Note: for clarity, the scale on the 08/15 figure differs from the scales on the figures representing the later dates.

Time-point of wounding (spring or summer) strongly influenced wound closure at all measuring dates (Table 3, Table 4). Wounds made in the spring had a higher percentage of closure than wounds made in the summer, even when recorded one or two years after wounding. Aspect of wounding (north or south) also affected rate of wound closure at all measuring dates, with more complete closure of wounds made on the southerly faces of

trees (Table 3, Table 5). Increasing estimated tree age also negatively affected the rate of wound closure at all measuring dates (the general linear mixed model estimates for the effects of scaled age (± SE) on August 2015, April 2016, August 2016, and April 2017, respectively, were: -1.86 ± 0.06, -0.33 ± 0.02, -0.36 ± 0.01, and -0.32 ± 0.02; significance may be found in Table 3).

Very little wound closure was recorded by August 2015 (< 1% in all categories (Figure 1)). Percentage wound closure increased sharply when measured in April 2016, and again in August 2016; by April 2017, mean predicted percentage wound closure across the AOD and exit hole categories ranged from 68% to 90% (Figure 1).

A total of 10 trees at Richmond Park and Hatchlands Park showed 0% wound closure by April 2017. The ARCHI assessment, named for its emphasis on tree architecture, classed all but one of the these trees as irreversibly declining, and the trees' percent canopy loss was within category 2, or 80-95% loss, for 6 trees, and category 3, or 35-75% loss, for four trees (See Table 6; percentage canopy assessments were very similar and are not presented) (Drénou *et al.*, 2015). These included one tree at Hatchlands Park, and three trees at Richmond Park, that had died, or were monolithed (all branches and crown removed) or felled because they appeared to be dying and presented a health and safety threat. At Madingley Wood, all trees had at least some wound closure by April 2017, and no trees died by 2017.

Table 4. Predicted % wound closure (mean ± SE) at the four measuring dates, by time-point of wounding.

Date	time-point	predicted mean % wound closure	SE
Aug-15	2	0.04%	0.0%
	1	2.44%	0.9%
Apr-16	2	10.20%	1.8%
	1	28.84%	4.0%
Aug-16	2	43.90%	5.9%
	1	66.40%	5.4%
Apr-17	2	79.50%	6.8%
	1	86.49%	4.9%

Table 5. Predicted % wound closure (mean ± SE) at the four measuring dates, by aspect of wounding.

Date	aspect	Predicted mean % wound closure	SE
Aug-15	N	0.23%	0.1%
	S	0.40%	0.2%
Apr-16	N	17.07%	2.8%
	S	18.27%	2.9%
Aug-16	N	52.75%	6.0%
	S	58.06%	5.9%
Apr-17	N	82.52%	6.0%
	S	84.02%	5.6%

Table 6. Number of trees within each ARCHI crown assessment category: H = Healthy, R = Resilient, S = Stressed, Ret = Retrenching, ID = Irreversible decline (Drénou *et al.,* 2015). See Table 2 for AOD / exit hole category details.

AOD / exit hole category	Crown condition (ARCHI)				
	H	R	S	Ret	ID
LS+	0	4	5	0	10
LS-	0	8	5	0	4
NS+	0	5	1	0	3
NS-	2	11	13	3	4
R+	3	9	6	0	1
R-	5	5	7	0	1
C	8	11	6	0	3

Discussion

In this study, AOD symptom category was strongly associated with the percentage closure of mechanical wounds. The trees were wounded in April/May and June 2015. The results from the final measuring date, April 2017, showed a clear reduction in percentage wound closure across the categories from asymptomatic (most rapid wound closure), to remission, although closure in remission trees without exit holes was not significantly different from asymptomatic trees, to new symptoms, to long-term symptoms (slowest closure rate) (Figure 1). Over the two years of the study, trees that had exhibited AOD symptoms over a number of years had consistently slower wound closure, as compared to asymptomatic trees, newly symptomatic trees, and trees in remission. Less efficient wound closure in declining trees is consistent with the results of other studies (Wargo, 1977; Haavik & Stephen, 2011). These results imply that trees with AOD are less vigorous than trees without AOD, and that the vigour of symptomatic trees is related to the duration of the symptomatic period. Host defensive responses to AOD and *A. biguttatus,* particularly in the form of physical responses to tissue damage by AOD lesions and *A. biguttatus* feeding galleries, would require considerable carbon expenditure, and long term infection by AOD would be expected to diminish trees' carbon budgets and ability to respond to additional stress (Haavik *et al.*, 2015). In addition, if predisposing stress is a

prerequisite for trees to develop AOD, the carbon reserves of affected trees would have already been depleted through responding to environmental or other stress, and their ability to respond to additional stress should be reduced (Führer, 1998; McDowell *et al.,* 2008; Thomas, 2008; Denman *et al.*, 2014).

Trees asymptomatic of AOD did not consistently exhibit more efficient wound closure than trees in remission or trees with new bleeds across the measuring dates (Figure 1), which suggests that other factors besides host vigour may also be important predictors of new AOD infection. At the first measuring date, mean predicted percentage wound closure in the asymptomatic category was lower than in all categories except remission without exit holes. At the second and third measuring dates, newly symptomatic trees with exit holes exhibited faster wound closure than asymptomatic trees. These trees had not developed AOD symptoms, even though at various points in this study they had less efficient wound closure than the newly symptomatic trees, which implies that they were less vigorous. The canopy assessments also suggest many of the asymptomatic trees were lacking in vigour: 30% were classed as either stressed, or in irreversible decline, yet had not developed AOD (Table 6). Wound closure is only one indicator of defensive ability, and many other constitutive and induced chemical and physical defences are likely to be important in resistance against AOD. Location within the stand, and relative distance to symptomatic trees, may be important: infected trees have been shown to occur in clusters, and trees with bleeds tend to occur in close proximity to trees with *A. biguttatus* exit holes (Brown *et al.*, 2016; Brown *et al.*, 2017).

The newly symptomatic trees may have initially exhibited more rapid wound closure because their defensive wound responses were already "primed" by larval feeding and bacterial damage to tissues (Conrath, 2009). Infection by pathogens and, also, damage by feeding insects may stimulate defensive responses to both pathogens and insects, including faster growth of wound periderm, both in the area of wounding and in other parts of the plant (Conrath, 2009; Eyles, 2010). At the first measuring date, wound closure was lowest in the asymptomatic trees and in the trees in remission without exit holes, which may indicate induced defences to wounding were already active in the other categories. Wound closure was relatively rapid in the newly symptomatic trees, only dropping below the asymptomatic and remission trees at the final measuring date (Figure 1); this pattern may be due to primed defences at the start of the study and, initially, relatively high vigour, with the drop-off by the end of the study the result of continuous, expensive allocation of carbohydrate resources in defence against AOD and *A. biguttatus* depleting carbohydrate reserves (Haavik *et al.,* 2015).

The association between exit hole category and percentage wound closure suggests the irregular emergence of adults on symptomatic trees may be explained by a cessation of larval development on more vigorous hosts. Exit hole category was significantly related to percentage wound closure at all measuring dates, and by the final measuring date, exit holes were related to reduced wound closure within the remission and newly symptomatic categories, suggesting the beetles were only able to complete development on the least vigorous hosts within these categories (Table 3, Figure 1). These results provide strong evidence that the beetles require low host resistance, and, particularly, low efficiency of wound closure in order to complete development on, and emerge from a tree with AOD. Although larval feeding damages host tissues, and in high density is likely to further reduce host vigour (Hartmann & Blank, 1992), in this study it is unlikely that the less efficient wound closure in trees with exit holes may be explained by tissue damage resulting from the successful development of the beetles. Larvae are present on almost all symptomatic trees, but do not regularly emerge as adults (Brown *et al.*, 2017). In other studies, the beetle has also been shown to require significantly weakened hosts / host tissues for survival of its larvae, as measured by canopy condition (Hartmann & Kontzog, 1994; Vansteenkiste *et al.*, 2004) and as observed in the laboratory, where larvae were unable to establish on logs from healthy oak trees, even ten days after cutting (Reed *et al.,* in press). Trees in remission, but without exit holes, had high mean percentage wound closure at the last two measuring dates, which strengthens the case for host resistance inhibiting larval development, since these trees had successfully occluded AOD lesions without developing exit holes. These results also support the body of evidence suggesting females may oviposit onto, and larvae may colonise trees that have host defences that remain too strong for complete development (Hartmann & Kontzog, 1994; Vansteenkiste *et al.*, 2004; Brown *et al.,* 2017).

The results of this and several other studies, including two that employed similar methodology (Dunn *et al.,* 1990; Fierke & Stephen, 2008), support a defensive role of rapid wound closure against phloem-mining beetle larvae (Miller *et al.,* 1991; Haavik & Stephen, 2011). How wound closure would be employed defensively remains unclear. Wound closure, which operates relatively slowly, seems unlikely to be effective against neonatal colonisation, or against feeding larvae, which move rapidly through host tissue. Efficient deployment of callus-like tissue may, however, be able to damage quiescent larvae, whether overwintering, or moulting, when they are particularly fragile (Dunn *et al.,* 1990; Fierke & Stephen, 2008, Brown *et al.,* 2014; pers. obs.). Overwintering *A. biguttatus* larvae may be susceptible in the early spring, when trees are building early wood but temperatures remain below the lower threshold of larval development of 11.9°C (Fierke & Stephen, 2008; Reed *et al.*, in press).

Close examination of the rankings of the exit hole categories shows that, at the earlier measuring dates, the reducing effect of exit hole presence on wound closure rate was inconsistent (Figure 1), which suggests other factors besides host resistance may be preventing the beetle from completing its lifecycle on at least some symptomatic trees. The newly symptomatic trees with exit holes exhibited surprisingly rapid wound closure, particularly compared to the control and remission trees without exit holes, in April and August 2016. This again may point to a "priming" of the trees' wound responses (Conrath, 2009). More surprising is the inability of beetles to complete development on the long-term symptoms trees without exit holes, which had been symptomatic for at least 5-6 years and consistently exhibited slow wound closure, including as compared to other trees with exit holes in the newly symptomatic and remission categories. This suggests that other factors besides efficient wound closure and host resistance prevent complete *A. biguttatus* development on some trees. It is possible that exit holes high in the canopy were not seen during monitoring, but most emergence holes have been shown to occur below 7m (Habermann & Preller, 2003). One explanation is that on most trees, the beetles may only be able to complete development on dying host tissues. On AOD monitoring sites, the majority of beetles have been shown to emerge from only a few, severely declined trees (Brown *et al.*, 2017). Exit hole numbers on many trees are low, and perhaps a few individuals are taking advantage of limited time-periods in which sections of stems of trees that are themselves not that declined briefly become severely compromised, e.g. areas adjacent to lesions.

Trees may, however, have severe AOD symptoms over many years, and may even die, without the appearance of beetle exit holes, and on these trees, other constitutive or induced defences or attributes such as defensive chemicals, bark moisture, or host microsite conditions must be more important than wound closure (Mattson & Haack, 1987; Brown *et al.*, 2014, 2017; pers. obs.). Indeed, in this study, of the four trees that were monolithed, felled, or died, exit holes were only observed on two. Perhaps some trees have prohibitively high chemical defences that inhibit larval development, even in severely declined tissues. Chemical defences employed against phloem-mining beetles by deciduous hosts such as oaks are likely to include induced proteins which may be toxic or have antifeedant properties (Eyles *et al.,* 2010), or bark phenolics; for example, studies on the resistance of ash trees to *Agrilus planipennis* Fairmaire have hinted at a role for induced bark lignans, which have been shown to be associated with reduced growth and development in other insects (Chakraborty *et al.,* 2014; Villari *et al.,* 2015). Phloem borers such as *A. biguttatus* are thought to have finite moisture requirements, with excessively high or low moisture levels inhibiting the colonisation of neonates and subsequent development, for example by drowning or desiccation, respectively (Hanks *et al.,* 2005; Sallé *et al.,* 2014; Brown *et al.,* 2017; Reed *et al.,* in press). Under-bark temperatures may

also be important: the beetle has been shown to have high thermal requirements, and the UK is on the northern edge of its suitable thermal range (Brown *et al.,* 2014). On some trees, microsite conditions may be too cool for complete development in some years (Reed *et al.*, in press). The development time of other *Agrilus* species has been shown to vary with host condition, as well as temperature, and unfavourable host tissue nutritive properties and unsuitable temperatures may interact to inhibit development (Muilenburg & Herms, 2012; Villari *et al.,* 2015).

Date of wounding influenced percentage wound closure for up to two years after wounding (Tables 3, 4). Very little wound closure was observed before the second measuring date, a year after the spring wounds were made; mature oak trees may require a considerable amount of time to cover over even small wounds (Neely, 1988). Wounds made in late April / early May healed more rapidly than wounds made in June. The influence of season on the rate of wound closure, particularly of spring wounds healing more rapidly than summer wounds, is consistent with the results of other studies, and has implications for the timings chosen in similar studies (Marshall, 1931; Neely, 1988). Fluctuating wound response may also influence the severity of damage inflicted by AOD and *A. biguttatus* in different seasons. Although the mechanisms behind lesion formation remain under investigation, reduced host wound responses in the summer could presumably facilitate both larval feeding, and bacterial tissue degradation. At sites monitored for AOD, stem bleeds were more frequently observed in the summer, and this may be, in part, because lesions are more likely to form when the wound response is less efficient (Brown *et al.,* 2017).

Aspect of wounding also significantly influenced percentage wound closure: southerly wounds closed more quickly than northerly wounds (Tables 3, 5). This may be due to higher temperatures accelerating tissue growth. Where the canopy is open enough to allow sunlight onto deciduous tree stems, the southerly faces are warmer (Vermunt *et al.,* 2012; K Reed, unpublished data). The effect size of aspect on percentage wound closure was relatively small (Tables 3, 5). *Agrilus biguttatus* is a thermophilic insect, and the benefit of the warmer temperatures for the larvae probably outweighs the slightly more vigorous wound response on southerly faces (Reed *et al.,* in press). Increasing estimated tree age negatively influenced percentage wound closure at all measuring dates (Table 3), reflective of reduced host defences in older trees (Jenkins & Pallardy, 1995).

As a tool to quantify and compare the levels of decline of trees within different categories of AOD infection, measuring the response to mechanical wounding was relatively non-destructive, simple and effective. At the three later measuring dates, the long-term symptomatic trees, with and without exit holes, exhibited consistently slower percentage

wound closure than all of the other categories. If wounding and monitoring of AOD-symptomatic trees with unknown symptom histories were undertaken, after one or two years, the trees most likely to survive AOD (with sufficient carbohydrate reserves to be able to mount vigorous defences) would be expected to have a higher percentage wound closure than those trees in which reserves had been exhausted. Drawbacks to the method include (1) a tendency for the wounds to exude fluid, particularly when the sapwood is breached, which is unsightly; (2) the possibility that wounds may act as an entry-point for pathogens; and (3) potential damage to the timber, particularly in young crop trees (Shigo, 1977). It may be possible to limit pathogen entry through the application of antimicrobial substances, and to limit timber damage by employing a smaller arch punch, and progressively removing thin bark sections to ensure the sapwood is not breached. Although additional experimental study would be required to quantitatively evaluate mechanical wounding as a monitoring tool to predict tree death, this study suggests it has the potential to be a useful tool, particularly when trees are grown ornamentally, and when combined with canopy assessments. Four trees had died by the time of writing, of which none had exhibited any wound closure, and all were classed as irreversibly declining in the ARCHI assessment, and were classed as having 80-95% canopy loss (Drénou *et al.*, 2015). Interestingly, across the sites, 26 trees in total, or 18%, were classified as irreversibly declining (Table 4). Although these trees are not expected to recover, their deaths may not occur for some time; oaks have been shown to take many years, and even decades, to die (Pedersen, 1998; Drobyshev *et al.,* 2007; Haavik *et al.,* 2008).

Conclusions

By showing that trees at differing stages of known infection by AOD and *A. biguttatus* colonisation exhibit different rates of wound closure, this study improves the understanding of oaks' resistance mechanisms against attacks by pathogens and phloem-mining beetle larvae. The relatively high levels of wound closure in newly symptomatic trees, a year to eighteen months after wounding, may be indicative of a priming effect of host resistance to larval activity and / or AOD lesion damage. Artificial priming of host defences may provide a useful avenue in management against AOD. Monitoring the closure of mechanical bark wounds may be a relatively non-destructive and simple additional survey tool for managers to identify trees with very low host defences, and, along with other tree health assessments such as canopy condition, could be usefully employed in decision-making for operations such as thinning. Finally, if the beetle is shown to be involved in the formation of AOD lesions, an additional tool to identify trees in which host resistance is lowest and, thus, that are more likely to be suitable for complete development by the beetle, may aid in the prevention of new AOD infections, as the

removal of these trees may help to reduce the numbers of emerging *A. biguttatus* adults (Moraal & Hilszczanski, 2000).

Acknowledgements

Paper 3*: Reduced stem growth predisposes oaks to Acute Oak Decline at three woodlands in England

Abstract

1. Acute Oak Decline currently affects thousands of English oaks, *Quercus robur* and *Quercus petraea.* The syndrome is thought to be caused by multiple, interacting bacterial species, and may also include the two spotted oak buprestid *Agrilus biguttatus.* To investigate the nature of predisposition in AOD, and to examine growth trends after AOD onset, the stem growth of asymptomatic control trees, and trees across a range of AOD categories (long-term symptoms, new symptoms, and remission), with and without *Agrilus biguttatus* exit holes, was analysed.

2. Throughout the lifespans of the trees, as represented in the stem growth record, mean growth was lower in trees with long-term AOD symptoms than in asymptomatic trees, at all sites, despite differing site histories reflected in site-wide growth patterns. Trees appeared to be predisposed to AOD by inherently poorer growth, potentially due to inter-tree competition or genotypic or microsite differences.

3. There was no evidence of additional, recent growth divergences between asymptomatic and symptomatic trees before AOD onset, reinforcing the finding that long-term, rather than recent stress, rendered the trees susceptible to the syndrome.

4. While long-term growth trends were not related to the absence / presence of *A. biguttatus* exit holes, analysis of recent growth trends suggests trees that experienced recent declines were less likely to recover when exit holes were present.

5. Surprisingly, there was no evidence of growth divergences after the onset of AOD symptoms. Site-level influences may have masked recent divergences, or trees may not exhibit sharply declining growth until just before death.

Introduction

Oak decline has been a subject of great concern in Europe and the UK for at least 100 years (Falck, 1918; Day, 1927). Tree decline has been defined as a combination of three conditions which interact chronologically: first, over the long-term, "predisposing" factors lower the tree's resilience to stress; second, abiotic and / or biotic "inciting" factors stress the tree; and third, attacks by "contributing" secondary insects or pathogens decrease the vigour of the tree (Manion, 1981; Houston, 1987; Waring, 1987). Acute Oak Decline (AOD), a specific type of oak decline which is characterised by stem bleeding and necrotic inner bark lesions, was recently described in the UK, and now affects thousands of English oaks (*Quercus robur* L. and *Quercus petraea* (Matt.) Liebl.), of which 1.3% die each year (Denman *et al.,* 2014, 2016). Predisposition and inciting stress are thought to render trees susceptible to attack by multiple, interacting bacterial species that are able to degrade bark tissues, potentially in combination with the two spotted oak buprestid *Agrilus biguttatus* Fabricius (Denman *et al.,* 2012, 2014, 2016; Brady *et al.,* 2010, 2017). AOD does not necessarily result in tree mortality; approximately 40% of trees are able to isolate lesions and larval galleries with callus-like tissue and enter a state of recovery or remission, although it is not known whether they continue to harbour the bacteria and risk again becoming symptomatic (Denman *et al.,* 2014; Brown *et al.,* 2017).

Although *A. biguttatus* is strongly associated with AOD, the relative chronologies of the beetle and bacteria in AOD, and the degree of host weakening required for colonisation by both agents, have not yet been determined (Brown *et al.,* 2014; Denman *et al.,* 2014). In the UK, the beetle and the syndrome share a similar distribution, restricted to south-central England, with a northerly limit of Manchester, and a first reported discovery of AOD symptoms and beetle galleries in Newport, Wales (Brown *et al.,* 2014; Denman *et al.,* 2016). Although the beetle also may occur on trees without AOD symptoms, at monitored sites, there is a strong co-occurrence between beetle exit holes and AOD lesions, and larval galleries are almost always found adjacent to lesions (Brown *et al.,* 2017). Both the beetle and the bacteria may be secondary colonisers of very stressed trees; they may arrive simultaneously, and their interaction may allow them to colonise only slightly weakened trees; the beetle may be coincidental, taking advantage of trees already weakened by bacterial lesions; or, conversely, the bacteria may colonise areas of host tissue damaged by larval feeding (Hartmann & Blank, 1992; Vansteenkiste *et al.,* 2004; Denman *et al.,* 2014; Brown *et al.,* 2014, 2016).

Predisposition is a prerequisite for tree declines, including AOD (Manion, 1981). Long-term "predisposing" factors increase susceptibility, and trees decline when prolonged, repeated, or severe stresses due to abiotic and biotic "inciting" factors reduce their carbon

budgets, and leave them unable to mount adequate defences against secondary pests and pathogens (Führer, 1998; Haavik et al., 2015). Tree age may be an important predisposing factor at the stand scale or in individual trees: older trees may suffer more severe disease and higher mortality (Führer, 1998). Poor management, e.g. overstocking, may reduce stand resilience to stress because of intense competition for resources (Waring, 1987; Sonessen, 2010). At the individual tree scale, the resilience of individual genotypes may vary, as will the level of stress due to inter-tree competition (Manion, 1981; Waring, 1987).

Environmental factors may also predispose trees to AOD at the stand scale. A predisposition model linking environmental variables to AOD found the syndrome occurs at warm sites with low rainfall (Brown et al., 2018, in prep.). Agrilus biguttatus is thermophilic, and, in the UK, is likely to be limited to sites with warm temperatures; if the beetle is essential to AOD, the syndrome will be limited to the areas suitable for the beetle (Brown et al., 2014; Reed et al., in press). Drought is thought to be the major inciting factor in oak decline throughout the world (Thomas, 2008; Scharnweber et al., 2011; Sallé et al 2014; Haavik et al 2015), and warm temperatures exacerbate water loss and increase carbon consumption in times of water scarcity (Allen et al., 2010). In the UK, trees are more likely to be limited by water scarcity than excess (Pilcher & Gray, 1982). There is also some evidence that stands affected by AOD tend to be on poorly drained soils, and these trees may lack deep roots that allow them to withstand unusually severe droughts (Hilsczanski & Sierpinski, 2007; Denman et al., 2014). Droughts and unusually warm conditions, particularly in the growing season, are likely to increase in frequency and severity in the UK under a changing climate (Stocker, 2014). The predisposition model also found AOD prevalence was linked to high levels of nitrogen and base cation deposition, and low levels of sulphur deposition (Brown et al., 2018). Anthropogenically-induced nitrogen deposition may exacerbate soil chemical imbalances, and, by leading to nutritional imbalances in trees, decrease tree resilience to drought, frost, and defoliating insects (Thomas et al., 2002).

Evidence of the relative chronologies of the beetle and the bacteria from sites monitored for AOD is inconclusive. Exit holes were more likely to appear on trees that had AOD symptoms in the previous year, or which had been symptomatic for a number of years, but the lifecycle of the beetle is likely to take at least two years in the UK, and it has been suggested that eggs may be laid on trees unsuitable for complete larval development (Vansteenkiste et al., 2004; Brown et al., 2017; Reed et al., in press). Thus external indications (exit holes) may not be apparent, although the beetles may be present in a tree.

The limited evidence base on the chronology of AOD suggests predisposition occurs before AOD and colonisation by *A. biguttatus,* and *A. biguttatus* exit holes occur later in the syndrome. At monitored sites, bleeds were more common, and more numerous, on trees with poorer crowns (Brown *et al.,* 2017). New exit holes were more likely to appear on trees observed to be bleeding in the previous year, or over several years, suggesting further weakening was necessary for complete development by the beetle (Brown *et al.,* 2017). Paper 2, within this thesis, which examined the response of trees with and without AOD and *A. biguttatus* exit holes to mechanical wounding, suggested a general reduction in vitality from (1) asymptomatic control trees, to (2) those in remission, to (3) newly bleeding trees, and finally (4) trees continuously bleeding for a number of years. Vigour was further reduced when *A. biguttatus* exit holes were present. In Belgium and Germany, *A. biguttatus's* larval galleries were only found on trees with >25% canopy loss, and exit holes on trees with >50% loss, suggesting one threshold of predisposition enables larval establishment, and a further threshold enables completion of the lifecycle (Hartmann & Kontzog, 1994; Vansteenkiste *et al.,* 2004).

Dendrochronology, or the systematic cross-dating of tree rings, may be used to study forest disturbances that influence tree growth (Fritts & Swetnam, 1989), and may therefore be employed to look for evidence of predisposition to AOD. Tree stem growth is thought to be a useful indicator of vitality because the growth of leaves, roots and buds, and allocations to carbohydrate storage, take priority over stem growth. At times when stress reduces a tree's carbon budget, its less important processes should be reduced first (Waring, 1987; Dobbertin, 2005). Predisposition may be present in the stem growth record as reduced growth: many studies of oaks have shown lower mean stem growth in the years before decline onset or tree death, as compared to the growth of healthy trees (Hartmann & Blank, 1992; Dwyer *et al.,* 1995; Amorini *et al.,* 1996; Pedersen, 1998; Drobyshev *et al.,* 2007a, b; Andersson *et al.,* 2011). Several studies have also shown greater variability in the ring widths of declining oaks, suggesting predisposed trees have decreased stability in response to climatic stress (McDowell *et al.*, 2008; Levanič *et al.*, 2011). Dendrochronology has also been used to show differences in stem growth between oak trees with different levels of colonisation by the stem-boring beetles *Enaphalodes rufulus* Haldeman and *Agrilus bilineatus* Weber (Dunn *et al.,* 1990; Haavik *et al.,* 2008). The effects of AOD upon radial growth have not previously been explored.

This study aims to examine the long-term and recent stem growth records of trees with and without AOD symptoms, in order to determine whether symptomatic trees are predisposed to AOD by reduced or declining growth. By comparing recent growth trends in trees at various stages of decline by AOD, including those in remission or recovery, this study aims to determine the impact of AOD on recent growth. Finally, by comparing the

growth of symptomatic trees with and without the exit holes of adult *A. biguttatus,* the relationship of the beetle with observed stem growth patterns is analysed.

Methods

Study trees were selected from woodlands at three sites (see Table 1 for details). Two of the sites, Hatchlands Park and Richmond Park, had been monitored for active AOD lesions and *A. biguttatus* exit holes since 2009 and 2010, respectively. Monitoring included annual counts of active AOD lesions and *A. biguttatus* exit holes visible from ground level, or up to approximately 3m. Both sites have had a long-term AOD presence: symptoms were first recorded at Hatchlands Park around 2001, and around 1990 at Richmond Park (Gibbs & Greig, 1997; Brown *et al.,* 2016). At Madingley Wood, where AOD symptoms were seen in 2009-2010 (K. Russell, pers. comm.), all oaks were surveyed in 2014 for occluded, active and recent AOD lesions, and *A. biguttatus* exit holes.

Trees were assigned to one of four AOD categories according to their history of lesion symptoms: long-term symptoms, new symptoms, remission, and asymptomatic control (= four level AOD category), and further sub-categorised according to the presence/absence of *A. biguttatus* exit holes: long-term symptoms plus/minus exit holes, new symptoms plus/minus exit holes, remission plus/minus exit holes, and asymptomatic control = seven level AOD and exit hole category). See Table 2 for category details and abbreviations. Trees with long-term symptoms trees had at least one active lesion at the start of the monitoring period. New symptoms trees developed at least one active lesion after monitoring began. Long-term and new symptoms trees had active symptoms in 2014 or 2015, although they may have been in remission (e.g. no active lesions seen) for one or more years during the study. Remission trees had at least one active lesion during the monitoring period, and no active symptoms in 2014 or 2015, or were in remission for at least two years. At Madingley Wood, due to insufficient monitoring history, all trees with active lesions were assigned to the long-term symptoms categories. At each site, where present, up to seven trees were selected in each of the symptomatic categories, along with 14 asymptomatic control trees. Where possible, trees with potentially confounding conditions such as unusual growth, extensive damage, or symptoms of infection by root pathogens were not selected.

Table 1. Description of the study sites at Richmond Park, Hatchlands Park, and Madingley Wood (Brown *et al.*, 2016, 2017; Natural England, 2017).

	Sheen Wood, Richmond Park (RP)	Hatchlands Park boundary belt (HP)	Madingley Wood (MW)
Location	Richmond Upon Thames, Greater London	Guildford, Surrey	Cambridge, Cambridgeshire
Latitude / Longitude	51.455636 / -0.27052781	51.263098 / -0.46337962	51.263098 / -0.46337962
Size of study area	3.4 ha	0.7 ha	13.8 ha
Approximate number of oaks within study area (DBH > 30cm)	150	140	310
Elevation (m a.s.l.)	20m	80m	60m
Description	Discrete *Quercus robur* plantation within urban parkland	Discrete *Quercus robur* shelter belt within parkland	Mixed-age stands of *Quercus robur* within ancient and secondary ash-maple woodland
Uniformity	Relatively uniform stand	Relatively uniform stand	Trees drawn from 5 compartments; variability in tree age and stand environment
AOD symptoms first recorded	1990	After 2001	2009-2010
Current monitoring began	2010	2009	2014
Level of foot traffic	High	Medium	Low
Other disturbance factors	Chronic Oak Decline; oak processionary moth; deer	Chronic Oak Decline	Chronic Oak Decline
Bacterial species *Brenneria goodwinii* and *Gibbsiella quercinecans* isolated at site?	yes	yes	yes

Replicates within all categories were low. At all three sites, nearly all possible trees within the long-term and new symptoms categories were chosen, and, in addition, seven extra trees within the remission categories were chosen at Madingley Wood and Hatchlands Park to increase replicates of symptomatic trees. Where a choice of trees was available, asymptomatic trees were selected by proximity to the symptomatic trees, and similarity in age. Due to the preferences of site managers, the largest asymptomatic trees were avoided. All trees were surveyed again for active bleeds and exit holes in April – May and October 2015, and categories were updated according to these data. See Brown et al (2016) for additional details of the monitoring carried out Hatchlands Park and Richmond Park. Diameter at breast height (DBH) was measured for each tree. Finally, tree crowns were assessed, and assigned to one of four crown position categories: dominant (extending above the mean stand canopy); codominant (similar to the mean stand

canopy); subdominant (upper portion of the canopy extending into the mean stand canopy and the rest below); and suppressed (below the mean stand canopy).

Table 2. Description of AOD and *Agrilus biguttatus* exit hole categories, and numbers of trees within the categories, at each site.

Four-level AOD category	Seven-level AOD category	Abrev.	Hatchlands Park	Richmond Park	Madingley Wood
Long-term symptoms	Long-term symptoms with exit holes	LS+	4	12	3
	Long-term symptoms without exit holes	LS-	7	5	5
New symptoms	New symptoms with exit holes	NS+	2	5	-
	New symptoms without exit holes	NS-	6	6	-
Remission	Remission with exit holes	R+	0	4	2
	Remission without exit holes	R-	11	7	21
Asymptomatic control	Asymptomatic of AOD or exit holes	C	14	14	15

Increment cores were collected in May 2015 using 5.15mm Haglof Increment borers, 400mm in length. Two cores were taken from each tree at breast height (1.3m), preferentially from the east and west aspects, except where the tree was leaning, on a slope, or adjacent to a footpath, in which case aspects were chosen to minimise these influences (e.g. cores were taken perpendicular to a lean). If deformation was present at breast height, cores were taken up to 50cm below or above breast height. Active and healed-over AOD lesions were avoided. To minimise the risk of between-tree spread of AOD bacteria, increment borers were thoroughly cleaned with 70% IMS after each use.

Cores were transported to the laboratory in labelled paper straws, and dried between sheets of corrugated cardboard. One core per tree was prepared for measurement, with the east or north cores chosen preferentially. Cores were mounted on wooden blocks and carefully sanded using progressively finer papers, up to 1000 grit, with a Makita Orbital Sander. Cores were then scanned at 4,800 dpi using an Epson Perfection 4990 photo scanner. Ring widths (RWs) were measured to 0.01mm in CooRecorder 7.8 (Larsson, 2014). Incomplete rings from the current year were not measured. If the first core was damaged, or its most recent rings were found to comprise of irregular callus-like tissue which had overgrown AOD lesions or *A. biguttatus* larval galleries, the second core was prepared. Second cores were also used when the first core proved difficult to resolve due to sections comprising very narrow rings consisting of poorly-demarcated early-wood growth. RW series were cross-dated using a combination of visual assessments and comparisons of correlation coefficients (a measure of the covariance between the

normalised, overlapping portion of the chronologies) and T-scores (based on the correlation coefficient, but weighted to account for the length of the overlapping segment) in C-Dendro 7.8 (Larsson, 2014). To determine and verify the year of each annual ring (Fritts & Swetnam, 1989), trees within individual sites were cross-dated with each other first. Mean chronologies from the three study sites were then compared to each other, and to published ring-width series from 18 sites in England.

See Appendix B, Figure B.4, for photographs of the experimental set-up.

Statistical Analysis

All analyses were carried out in R (R Core Team, 2016).

Analysis of long-term growth trends

There was a clear indication, from initial analysis, of an increase in variance with increasing ring width at all three sites. As such, the ring widths (RWs) were natural-log transformed, which improved the homoscedasticity of the data. Data were combined across the three sites, and year was centred based on the range across all individual trees (the year 1960 was used as the zero point, as this fell within the time series of all trees); this aided model fitting and reduced correlation between slopes/intercepts.

The first stage of the analysis was to detrend the data. Given that ring widths had been natural-log transformed, any linear model applied to the transformed data is equivalent to applying a (negative) exponential; this is a common technique for detrending RW data. However, to ensure full detrending, a range of models were applied to the transformed RWs to allow for any further long-term growth trends. These included a simple linear model (i.e. transformed RW~year(zeroed)) plus a range of polynomial models with an increasing number of turning points (e.g. year + year^2 + year^3 etc.). Biologically, these turning points were likely to coincide with growth releases caused by events resulting in a mass mortality of trees, including active management (thinning), or climatic events such as wind-throw (Cook, 1987); as such, the model included site-specific fixed effects to account for these changes. Mixed effects models in the nlme package in R (Pinheiro *et al.,* 2017) were applied to the dataset to allow for compartment-level and tree-level effects within individual sites. For compartments, random intercepts were included only, as further random effects applied to year were highly correlated (>0.95) with the intercept term. For individual trees, random effects were included in line with the fixed effects polynomial. The best fit model for detrending the data was determined using Akaike's Information Criteria (AIC) applied to the maximum likelihood fits.

Having detrended the data, the best fit model was then analysed for temporal autocorrelation. A range of corARMA (autoregressive moving average) models were applied to the model in the nlme package in R (Pinheiro *et al.,* 2017), to account for any correlation with previous time points (AR regresses the current RW with its previous n values; MA models the error term of the current RW based on previous n error terms). Analysis of the (partial) autocorrelation function applied to the model fit indicated that up to two previous years needed to be included within the corARMA structure. All combinations of coARMA structure (up to 2 time points) were applied and the best fit model was determined using Akaike's Information Criteria (AIC) applied to the maximum likelihood fits.

Having determined the appropriate autocorrelation structure, the potential effects of the four-level AOD category were included within the best fit model, allowing main effects and interactions with year. The data were also fitted allowing for the seven level AOD and exit hole category. The best fit model was determined using Akaike's Information Criteria (AIC) applied to the maximum likelihood fits. The significance of the fixed effects were determined based on the Chi-squared test statistics from the analysis of deviance, using the car package in R (Fox & Weisberg, 2011).

Any effects on mean growth predicted by the best fit models should be reflected in the current size of the trees, which will also be influenced by tree age. Although ring width counts per core should act as a proxy for age, cores did not necessarily include all of the radius of each tree, due to the length of the increment borer being shorter than the radius on very large trees, or the presence of heart-rot degrading the ends of cores. Therefore, the age of each tree was estimated by dividing its core's RW count by the percentage of the radius (DBH/2) made up by its core's RW sum. An ANOVA model was fitted in R to test for corrected DBH differences between the AOD categories. The response was DBH/estimated age, log-transformed, and the explanatory variables were the four level AOD category and woodland. The interactions between woodland and four level AOD category were not included in the model. The significances of the fixed effects were determined based on the F statistics from the analysis of variance, using the car package in R (Fox & Weisberg, 2011). Post hoc tests were conducted in the lsmeans package of R, correcting for multiple comparisons using Bonferroni adjustments to the p value (Lenth, 2015). The assessments of crown position (dominant, co-dominant, sub-dominant, supressed) should also reflect any effects on mean growth predicted by the linear models. A G-test in the R package DescTools was used to determine whether the trees in the four-level AOD category were evenly distributed within the four crown position categories (Signorell, 2016).

Analysis of recent growth trends

Having determined the best fit model, the same model was re-run but restricting the data sets to time points up to 1990 only (i.e. to exclude any recent AOD effects). This model was then used to forecast RW up to 2014 for each individual tree, to compare with the actual RW data. Differences were calculated as ln(actual 2014 RW/1990 forecast 2014 RW), with the fixed effects of beetle exit holes, four level AOD category and woodland (plus interactions) and compartment within woodland as a random effect. The significances of the fixed effects were determined based on the Wald Chi-square test statistics from the analysis of deviance, using the car package in R (Fox & Weisberg, 2011).

Further analyses of recent growth trends were carried out using a change-point Excel model developed by researchers at Swansea University (M. Gagan, unpublished). Adapted from the non-parametric change-point test (Siegal & Castellan, 1988), the model analyses the last 100 years of stem growth and determines the year of the most recent significant increment growth decline, if present. A positive slope from the year of decline, if present, indicates a recovery in growth. To assess for growth increases over the most recent ten and fifteen years, the model also assesses the respective slopes, whether positive or negative. The change-point methodology applied to the raw data generated a number of binary responses (growth decline, growth recovery since decline, increasing growth in the last ten years, increasing growth in the last fifteen years). These responses were tested statistically using a generalised linear mixed effects model (GLiMM) with binomial errors and logit link function in the lme4 package of R (Bates *et al.,* 2014). The combination of four-level AOD category, seven-level AOD and exit hole category, and absence/presence of exit holes were all tested separately to determine whether these were significant drivers of differences in the binary responses; in all cases, compartment nested within woodland was included as the random portion of the model. The significances of the fixed effects were determined based on the Wald chi-square test statistics from the analysis of deviance, using the car package in R (Fox & Weisberg, 2011). Post hoc tests were conducted in the lsmeans package of R on the best fit model to estimate differences within significant factors, correcting for multiple comparisons using Bonferroni adjustments to the p value (Lenth, 2015).

The change-point methodology also generated a response to indicate the last year when a significant decline was identified. The number of years since this decline (i.e. 2014 minus the last year of decline) was used as the response, and tested statistically using a generalised linear mixed effects model (GLiMM) with Gamma errors and reciprocal link function (appropriate for this time-to-event analysis), in the lme4 package of R (Bates *et*

al., 2014). The combination of four-level AOD category, seven-level AOD and exit hole category, and absence/presence of exit holes were all tested separately to determine whether these were significant drivers of differences in time since last decline. The significance of the fixed effects were determined based on the Wald chi-square test statistics from the analysis of deviance, using the car package in R. Post hoc tests were conducted on the best fit model to estimate differences within significant factors, correcting for multiple comparisons using Bonferroni adjustments to the p value (Lenth, 2015).

Results

Analysis of long-term growth trends

Table 3 shows the correlations between the three site chronologies and several recent published chronologies. See Appendix A, Table A1 for summary data on the cores and ring widths by site and four-level AOD category.

The best fit model required site-level fixed effects up to a quartic polynomial (i.e. 3 turning points) (df = 32, AIC = 18444.8, L.Ratio = 1078.4, $p < 0.0001$). The AIC values dropped rapidly up to this level of polynomial, before moderately increasing, indicating that the model starts to become inefficient beyond this point (see Appendix A, Figure A1).

Table 3. Correlation coefficients (CorrC) and T-scores (TT) calculated in C-Dendro (Larsson, 2014), between several recent reference chronologies and the three study woodlands (Bridge & Winchester, 2000; Arnold *et al.,* 2005)

Site	Hatchlands Park		Richmond Park		Madingley Wood		First and last year	
	CorrC	TT	CorrC	TT	CorrC	TT	start	end
Gutteridge Wood, Hillingdon, Greater London	0.41	5.9	0.60	9.7	0.51	7.8	1815	1994
Hatch Park, Ashford, Kent	0.32	4.6	0.49	7.5	0.51	8.1	1819	2003
Lullingstone Park, Eynsford, Kent	0.39	5.5	0.49	7.4	0.46	6.8	1829	2003
Old Park Wood, Hillingdon, Greater London	0.47	7.4	0.59	9.5	0.44	7.1	1786	1994
Scotney Castle, Tunbridge Wells, Kent	0.53	8.9	0.52	8.2	0.34	5.3	1752	2003
Madingley Wood, Cambridge, Cambridgeshire	0.32	4.9	0.63	11.3	-	-	1797	2014
Sheen Wood, Richmond Park, Richmond Upon Thames, Greater London	0.45	6.9	-	-	-	-	1821	2014
Hatchlands Park, Guildford, Surrey	-	-	-	-	-	-	1776	2014

Analysis of the (partial) autocorrelation function applied to the model fit are shown in Appendix A, Figure A2 (note the significant correlations for two time lags, indicating that up to two previous years needed to be included within the corARMA structure). AIC applied to the maximum likelihood fits of the different corARMA combinations indicated that a (2,1) corARMA structure best explained the data (df = 35, AIC = 12992.3, L.ratio = 717.4, $p < 0.0001$).

The inclusion of the four-level AOD category within the model improved the fit when included as an intercept term only. The seven-level AOD and exit hole category model was a poorer fit, and including exit holes as a separate factor (with and without the four AOD types) did not improve fit and was not a significant factor ($\chi^2 = 1.67$, df = 1, n(trees) = 143, $p = 0.20$). Analysis of deviance on the best fit model indicated that the effect of the four-level AOD category was highly significant ($\chi^2 = 11.88$, df = 3, n(trees) = 143, $p = 0.008$), with post hoc tests indicating that asymptomatic control trees grew significantly better (or had larger ln(RW)) throughout the life time of the oak trees versus trees with long-term symptoms. This pattern was evident at all three sites, despite different site-level growth patterns which are probably reflective of silvicultural management (Figure 1). There was no difference in variation in RWs, as calculated by the root mean square error for each tree ($F_{3,137} = 0.34$, $p = 0.80$). Appendix A, Figure A3 visualises the conversion of the raw ring width data into the best fit linear models, for each individual tree, within the four AOD categories and across the three woodlands. Here, the effect of individual woodland has been removed from the models.
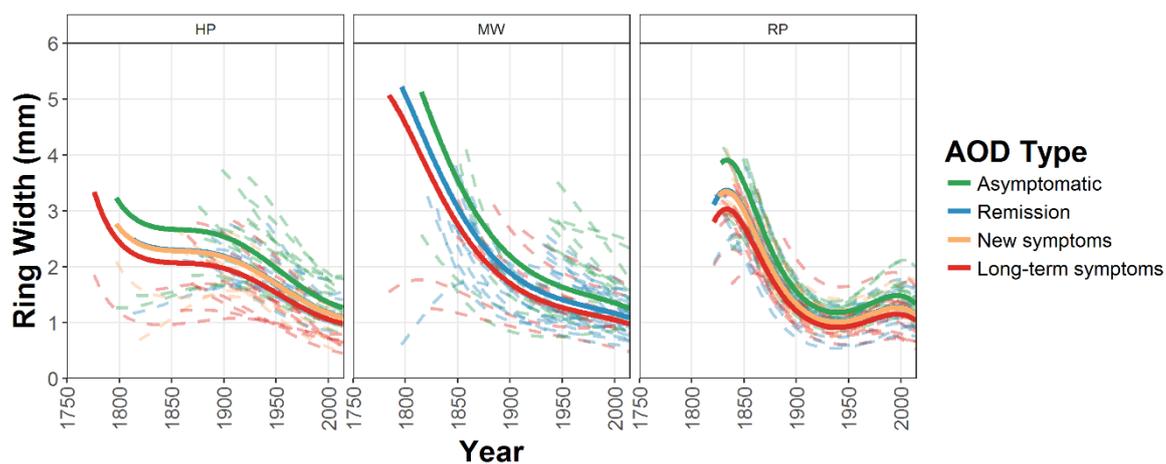
Figure 1. Best fit model predictions applied to each site and AOD type (solid lines indicate site-level best fit models, dashed lines indicate tree-level best fit models. At Madingley Wood, due to a shorter monitoring history, all symptomatic tress were assigned to the long-term symptoms categories).

When DBH was corrected for age, as estimated from the core RW counts and percentage of the tree's radius covered by the core, there was a significant effect of the four-level AOD type on tree size (F = 4.8, df = 3, $p = 0.003$), but not of woodland (F = 1.6, df = 2, $p =$

0.20). Post hoc tests showed the same pattern as the best fit models: after the age correction, trees with long-term symptoms were smaller than asymptomatic control trees, and trees with new symptoms and in remission were intermediate, but not significantly different from either (least-square mean +- SE for long-term symptoms, new symptoms, remission, asymptomatic control = 0.32 ± 0.01, 0.33 ± 0.02, 0.35 ± 0.01, 0.39 ± 0.02, respectively). The G-test also showed that the trees were not equally distributed within crown position categories (G = 20.4, X^2 df = 9, p = 0.02) (Table 4).

Table 4. Numbers of trees by four-level AOD category and crown position category.

	Long-term symptoms	New symptoms	Remission	Asymptomatic control
Dominant	6	3	11	6
Co-dominant	13	11	23	31
Sub-dominant	10	3	9	6
Suppressed	7	2	2	0

Analysis of recent growth trends

Figure 2 shows the comparison between the actual RWs and those predicted using the projected mixed effects model limited to data from 1990 and earlier. Statistical analysis (based on 2014) showed a significant difference in projected versus actual data by woodland (χ^2 = 8.42, df = 2, n(trees) = 143, p = 0.015), but not by four level AOD category (χ^2 = 4.23, df = 3, n(trees) = 143, p = 0.238) or exit hole category (χ^2 = 0.40, df = 1, n(trees) = 143, p = 0.528). In general the projected model over-predicts data for Hatchlands Park, irrespective of AOD type, with the other two woodlands being consistently estimated.

According to the change-point analysis, 72% of the trees experienced a statistically significant growth decline at some point in the last 100 years, and 56% of these subsequently experienced a growth recovery, or had positive slopes from the year of decline to the present. The date of onset of the most recent growth decline ranged from 1932 to 2009, with 1986 the average year, and 2003 the most frequent (Figure 3).
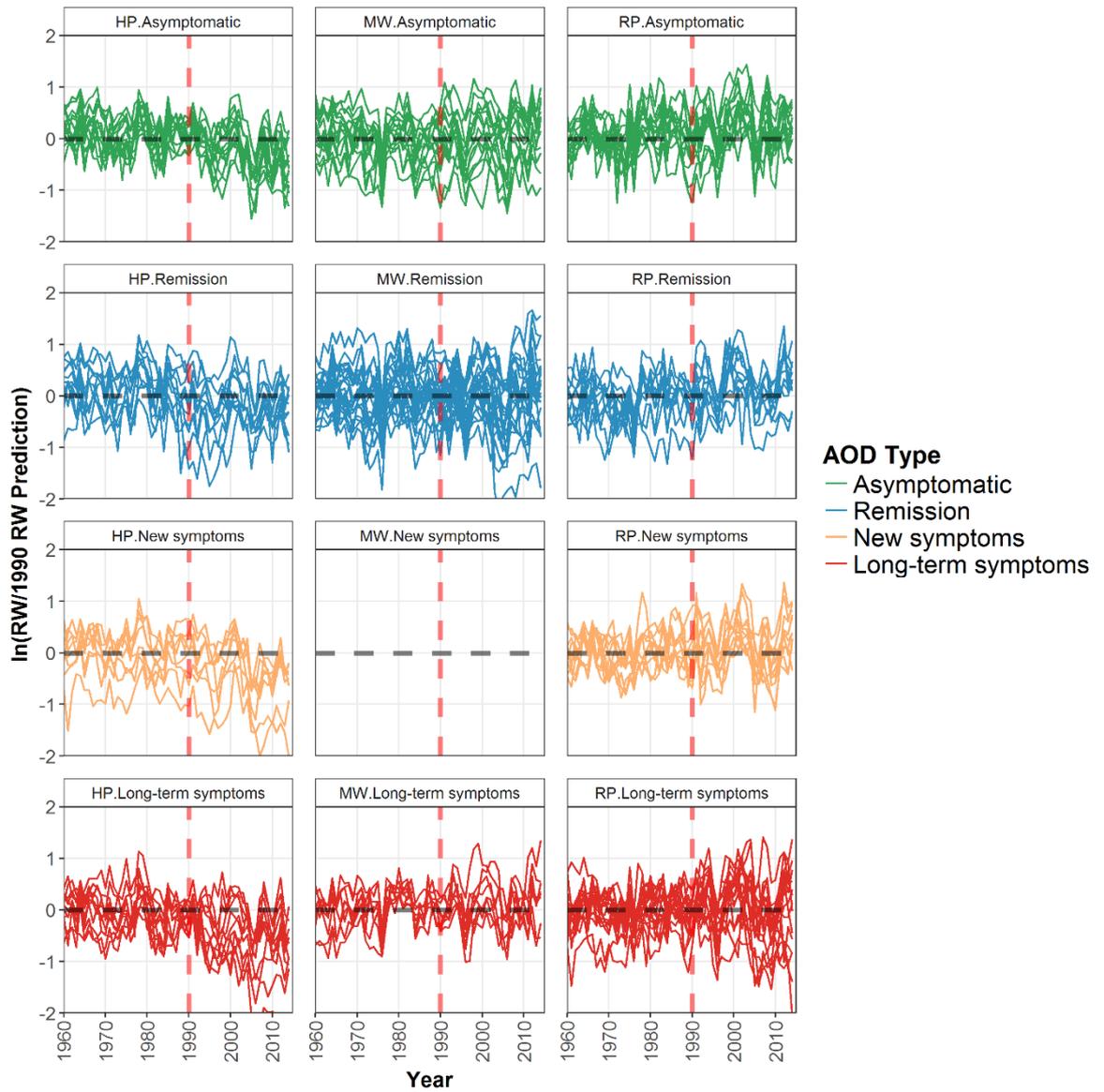
Figure 2. Comparison between the projected vs actual ring width data from 1990 (comparison is defined as ln(actual RW / forecast RW)), by site and AOD category. Whereas actual and forecasted trends at Madingley Wood (MW) and Richmond Park (RP) are similar, all categories at Hatchlands Park (HP) are underestimated by the model projections, indicating lower than expected growth at the site.
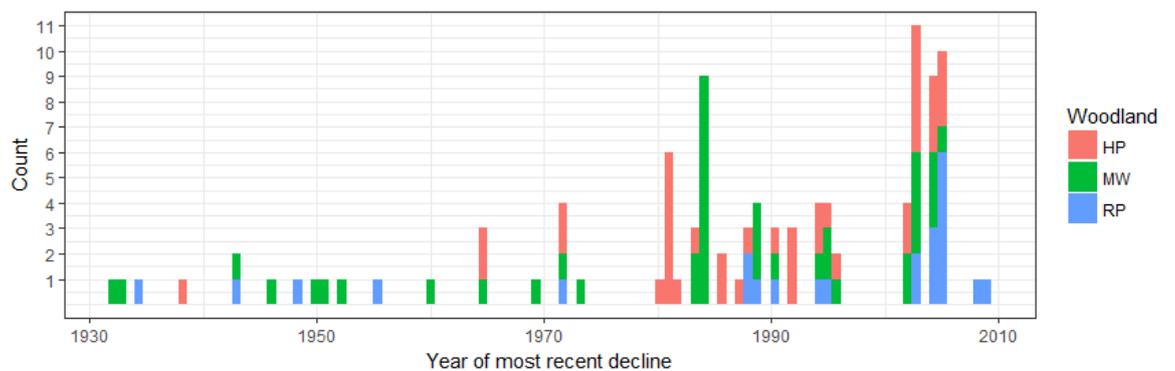
Figure 3. Histogram of years of initiation of most recent decline, if present; the colours represent data from the three woodlands (Hatchlands Park = pink, Madingley Wood = green, Richmond Park = blue).

Whereas 84% of trees had increasing slopes, indicating increasing growth, in the last 10 years, only 38% experienced increasing growth in the last 15 years. The results of the fifteen statistical analyses of the change-point data are provided in Table 5. There were two significant results in the analyses of the change-point data. . Post hoc tests showed that if trees experienced a decline, for "growth recovery since decline", the absence/presence of exit holes was a significant factor, with trees without exit holes present nearly twice as likely to recover as trees with exit holes present (Figure 4, Table 5). There was also a significant result of exit hole absence / presence on years since the most recent decline: post hoc tests showed that if trees experienced a decline, the last decline was more recent in trees with exit holes (Figure 5, Table 5).

Table 5. Results of the change-point analyses of recent growth, by AOD and *Agrilus biguttatus* exit hole category.

| Response	Category	χ^2	Df	*p*	Sig.
Experienced a growth decline in the last 100 years	AOD and exit holes (7 levels)	3.86	6	0.696	
	AOD only (4 levels)	1.89	3	0.595	
	Exit holes only (2 levels)	0.01	1	0.928	
Recovery since growth decline (if present)	AOD and exit holes (7 levels)	6.69	6	0.350	
	AOD only (4 levels)	4.82	3	0.185	
	Exit holes only (2 levels)	4.12	1	0.042	*
Increasing growth in last ten years	AOD and exit holes (7 levels)	6.48	6	0.371	
	AOD only (4 levels)	5.28	3	0.153	
	Exit holes only (2 levels)	0.02	1	0.885	
Increasing growth in last fifteen years	AOD and exit holes (7 levels)	2.42	6	0.878	
	AOD only (4 levels)	2.59	3	0.459	
	Exit holes only (2 levels)	0.05	1	0.821	
Years since most recent growth decline	AOD and exit holes (7 levels)	11.55	6	0.073	
	AOD only (4 levels)	7.07	3	0.070	
	Exit holes only (2 levels)	4.77	1	0.029	*
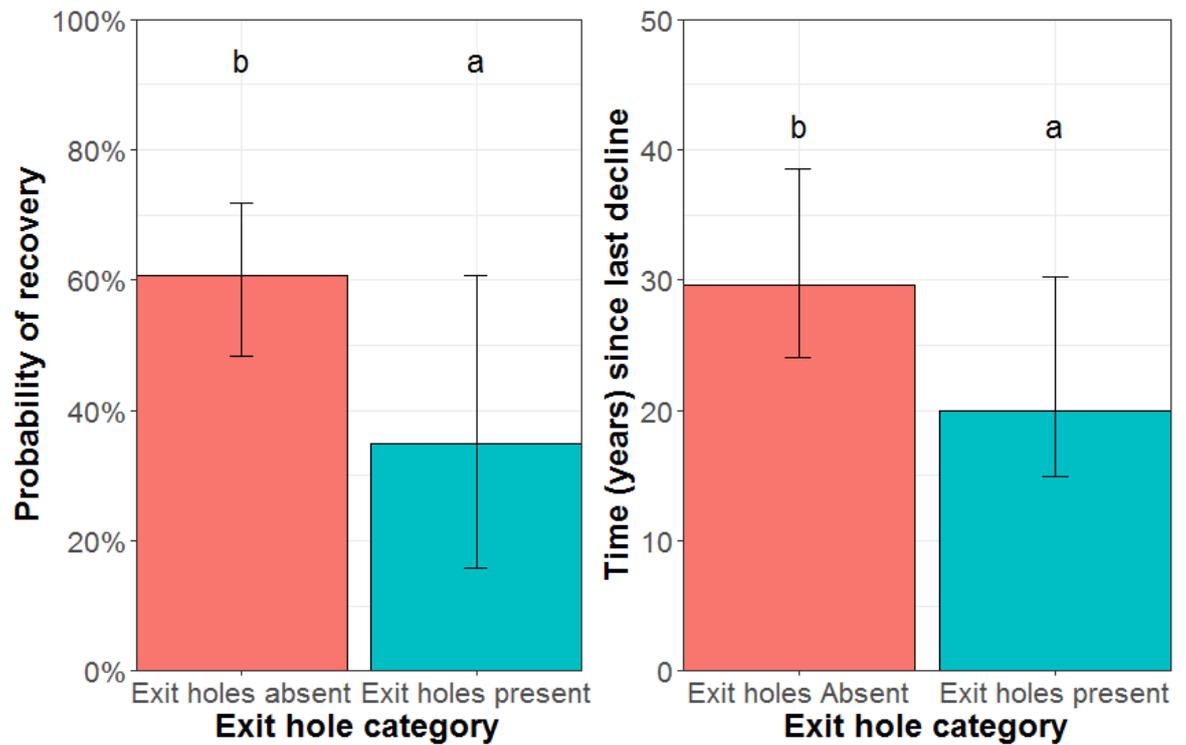
Figure 4 (L). Probability of recovery in stem growth increment since the initiation of the most recent stem growth decline, if present, in trees with and without *Agrilus biguttatus* exit holes. The data presented are the model-predicted mean and 95% confidence limits. The letters indicate a significant difference in mean probability (b > a).

Figure 5 (R). Time, in years, since the initiation of the most recent stem growth increment decline, if present, for trees with and without *Agrilus biguttatus* exit holes. The data presented are the model-predicted mean and 95% confidence limits. The letters indicate a significant difference in mean probability (b > a).

<u>Discussion</u>

The application of mixed effects models to the growth of trees with and without AOD symptoms at the three woodlands analysed in this study indicated significant effects of woodland and AOD category, but not *A. biguttatus* exit holes, on stem growth. There were site-level differences in the shape of the growth curves, as described by the fitting of different turning points at each site (Figure 1). These turning points are most likely reflective of different management strategies and timings at the different sites, and probably reveal the growth releases exhibited by the remaining trees after thinning (Cook, 1987). Despite these differences, at all three sites, from the beginning of the stem growth record to the date of coring, the mean stem growth of trees with symptoms from the outset of monitoring (long-term symptoms) was lower than that of the asymptomatic control trees. This relatively reduced growth suggests the trees that were affected by AOD symptoms for a number of years were predisposed to the syndrome by underlying issues. Trees that only developed AOD symptoms in the last few years (new symptoms), and trees that were able to successfully occlude lesions and enter a state of remission or recovery, had mean stem growth that was in between that of the asymptomatic controls and long-term symptomatic trees, but was not significantly different from either.

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Neither including the seven-level AOD and *A. biguttatus* exit hole categories, nor including the absence / presence of exit holes, improved the fit of the linear mixed effects models. This indicates that long-term growth patterns are not good predictors of whether or not the beetle is able to complete development on trees with AOD. The long-term growth of the trees, well represented by the four AOD categories, could not be subdivided into exit hole absence / presence, suggesting the beetles are not simply completing development on trees with the poorest long-term growth within each category.

The persistent growth differences between long-term symptomatic and asymptomatic control trees suggest competition for resources may have predisposed the trees to AOD. When DBH was corrected for age as estimated from the tree core data, long-term symptomatic trees were smaller than asymptomatic control trees, and newly symptomatic and remission trees were intermediate. These results suggest these trees may have been predisposed to AOD by being poor competitors in over-stocked, dense stands. Examination of the crown dominance assessments also indicated that the distribution of trees within the AOD categories was unlikely to have arisen independently (Table 4). This uneven distribution was not driven by study tree selection; replicates were limited, and the cores taken are an accurate representation of the populations of trees at each site. Rather, all of the suppressed trees at the sites had active symptoms at one time during the study.

Inter-tree competition for resources such as light and water strongly affects the growth of individual trees, and, for example, was found to account for more than 50% of the variation in growth in Norway Spruce in one study (Bigler & Bugmann, 2003). Genotypic or microsite differences may have resulted in poorer initial growth in some trees that continued under dense management regimes. Suppressed trees may have anatomical differences and lower carbohydrate reserves that increase their susceptibility to stress (Waring, 1987). Several other studies have found that smaller, more slowly-growing trees are prone to decline and attack by secondary pests and pathogens. In a study of dieback of Atlantic oaks, the only trees that died were those predisposed by prolonged, intense competition (Rozas & Garciá-González, 2012). The spruce beetle *Dendroctonus rufipennis* Kirby was found to attack slowly-growing white spruce trees *Picea glauca* (Moench) Voss, stressed by higher stocking rates (Hard, 1985). Conversely, other studies have found that the trees that declined and died originally grew more quickly, suggesting that initially-smaller survivors had allocated more resources to stress tolerance (e.g. root growth rather than stem growth) early on (Haavik *et al.*, 2008; Levanič *et al.*, 2011).

In this study, the mixed effect models and the model forecasts from 1990 provide very little evidence of recently-induced predisposition in the form of growth divergences. Before

the onset of symptoms, the growth of AOD-symptomatic trees did not appear to drop below that of asymptomatic trees, and nor did the growth of trees with *A. biguttatus* exit holes appear to drop below that of trees without exit holes. A growth divergence after severe climatic conditions, such as drought, in declining vs asymptomatic trees, has frequently been observed in other studies of declining oaks, often decades before tree death (Dwyer *et al.,* 1995; Pedersen, 1998; Drobyshev *et al.,* 2007b; Andersson *et al.,* 2011). AOD symptoms were first recorded in 1990, 2001, and 2009-2010, respectively, at Richmond Park, Hatchlands Park, and Madingley Wood. Recent divergences between the growth of asymptomatic and symptomatic trees, before AOD onset, may suggest AOD occurred after individual trees exhibited varying resilience to short-term, inciting factors such as climatic (e.g. drought) stress. Figure 1, modelling the actual data, does indicate that a reduction in growth occurred at Richmond Park just before 2000, and a difference between the actual and the projected data from 1990 onwards was evidence of poorer than expected growth at Hatchlands Park (Figure 2); these growth reductions may be due to climatic stress such as drought, or other site-wide stresses such as management. Asymptomatic trees as well as symptomatic trees, however, seemed to be affected at both sites, and thus there is no evidence of a reduction in growth in the symptomatic, as compared to the asymptomatic categories. This provides further support that differences in long-term vigour, rather than varying responses to recent stress events, predisposed trees to AOD.

The change-point analysis also provides little support for recent growth divergences in the four level AOD category, but, in contrast with the mixed effect models, seems to show a relationship between the absence / presence of exit holes and recent growth patterns. In total, 72% of trees experienced a growth decline at some point in the last 100 years. Similarly to the findings of the linear models, Figure 3 shows that many trees initiated most recent declines between 1980 and the late 1990s, responding to climatic or other site-wide stress. As neither year of most recent decline, nor presence/absence of a decline, however, was related to four or seven-level AOD category (Table 5), there is no indication of variance in resilience to stress between the symptomatic and asymptomatic trees. After declining, 58% of trees' growth from the year of decline to the present was positive, indicating growth was increasing since the decline. While neither AOD category (four or seven levels) influenced the likelihood of returning to positive growth after the most recent growth decline, trees with exit holes were less likely to have increasing growth after declining (Table 5, Figure 4). The most recent decline episodes in trees with exit holes also started more recently (20 years ago) than trees without exit holes (30 years ago), if a decline occurred (Figure 5), whereas the year of most recent decline was unrelated to the four or the seven-level AOD / exit hole categories. In contrast with the influence of long-term trends on AOD incidence, and independent of AOD status, recent

trends seem to be more important to the likelihood of the beetle completing development on trees. The presence of exit holes seems to indicate a lower likelihood of recovering positive growth, but whether the beetle is preventing host recovery, or whether a lack of recovery made the trees more suitable to beetle development, is unclear. Similarly, beetle exit holes are associated with trees that started to decline more recently, but whether they were influential in the decline of the trees, or coincidental, is difficult to determine.

Surprisingly, neither the change-point data nor the model forecasting show evidence of a divergence between the growth of asymptomatic and symptomatic trees, or trees in remission, after AOD symptom onset and beetle colonisation. The change-point analysis shows a number of trees initiated growth declines from 2000-2010 (Figure 3), but declines occurred across AOD categories, implicating a site-wide factor such as climatic stress, rather than AOD (Table 5). The change-point analysis also shows that 84% of trees experienced increasing growth in the last ten years, and these trees were evenly distributed between the seven AOD and exit hole categories. At least some trees with AOD seemed to continue to accumulate good growth despite their symptoms. Perhaps a number of the symptomatic trees benefited from a reduction in competition due to the death of some trees, through growth releases, counteracting growth reductions due to AOD on other trees. Another explanation is that site-level effects on growth were more important than individual growth variations, and masked the declining growth of some individual trees. In the best-fit models, site-specific turning points were required at the woodlands studied, and in the models of projected growth after 1990, a site-wide decrease in actual vs projected growth at Hatchlands Park was detected, irrespective of AOD category. In addition, the change-point analysis shows that nearly a third of trees had not recovered good growth after recent declines, irrespective of AOD category: trees within all AOD categories, including the asymptomatic controls, were declining, potentially due to causes distinct from AOD, such as root pathogens (Denman & Webber, 2009), again potentially masking individual tree declines due to AOD symptoms. Growth declines in the symptomatic trees, due to AOD and beetle damage, may also be masked by the fact that only trees living at the start of the study were sampled, and many of the worst-affected trees may have already died, or may have been removed as part of woodland management. The time period after the current AOD symptom onset or recovery may have been too short for growth differences to be apparent in the linear models or the change-point analyses. It is also possible trees with AOD do not exhibit sharply reduced stem growth until the years just prior to tree death, whether due to AOD or other factors. Although several of the study trees died after sampling, the number of replicates was too low for a meaningful examination of pre-death stem growth patterns to be made.

The finding in this study that AOD affects trees with underlying issues, rather than recent growth declines, is not consistent with a dendrochronological study of trees with AOD-like symptoms in northern Germany, in which trees within all growth classes were affected by decline, and the growth of symptomatic trees diverged from that of asymptomatic trees several years before the onset of severe stem lesions and colonisation by *A. biguttatus* (Hartmann & Blank, 1992). In the decade before symptom onset, the trees affected by decline in northern Germany were subjected to extensive crown defoliation, severe droughts and severe late frosts, and the trees less able to tolerate these stresses did not recover good growth and were subsequently colonised by *A. biguttatus* and showed AOD-like symptoms (Hartmann & Blank, 1992). The time period before the current episode of AOD was not characterised by intense stress events such as severe defoliation, although major droughts did occur in 1990-1992 and 1995-1997, and several less important droughts have followed (Gibbs & Greig, 1997; Marsh *et al.,* 2007; Denman *et al.,* 2014). Although testing for AOD bacteria on trees with similar symptoms in Europe has not been undertaken, it is likely that in the UK, AOD could also be expected to develop on trees weakened by abiotic and biotic inciting factors, as well as trees predisposed by underlying conditions. Comparisons between healthy oaks, and those with AOD-like symptoms, in the 1990s found differing evidence of predisposition in the stem growth record at different sites, from similar growth, to divergences in growth from several years, and up to 20 years before symptom onset (Gibbs & Greig, 1997).

Conclusions

At the three studied woodlands, despite differing site histories, the linear mixed models showed that trees with long-term AOD symptoms were predisposed to the condition by consistently poorer stem growth throughout the stem growth record. The change-point analysis of recent growth trends suggests an influence of beetle exit hole presence / absence on recent growth trends, particularly in the likelihood of returning to positive growth after the most recent growth decline. The best-fit models of stem growth at Hatchlands Park and Richmond Park (Figure 1) reveal stand-wide growth releases which were probably related to thinning. Although opening up stands by removing the most suppressed trees may reduce the number of trees predisposed to long-term AOD infection, and increase the resources available to the remaining trees, the role of *A. biguttatus* in AOD would need to be better established before a management recommendation of thinning out suppressed trees could be made, because opening up stands may increase beetle developmental success by raising under-bark temperatures (Reed *et al.,* in press). The lack of divergence in symptomatic vs asymptomatic tree growth after AOD onset was surprising, and may be due to site-specific conditions influencing growth more than AOD. Examination of stem growth across a wider range of AOD sites would help to determine whether the growth patterns observed at these woodlands are typical, or whether at some sites AOD may also affect, for example, recently stressed trees with previously high mean growth. An examination of the pre-death stem growth of a number of dead AOD-symptomatic trees might also clarify the patterns that lead to ultimate tree mortality.

Integrative discussion of the paper findings

Summary of the thesis aims and objectives

Agrilus biguttatus is considered to be an important factor in European oak decline, and is linked to AOD in the UK, although its role in the syndrome is not well understood. Although AOD is a serious threat to UK oaks, many gaps in our knowledge about the syndrome remain, particularly concerning the role of the beetle. This thesis aimed to address three of these evidence gaps. First, in the UK, the beetle and AOD share a markedly similar distribution, restricted to south and central England. *Agrilus biguttatus* is strongly associated with AOD, and if it is shown to be an essential component of the syndrome, AOD will also be limited to the beetle's geographic range. Although temperature probably restricts the beetle to its current distribution, its life history and thermal requirements are not well understood; climate change may also affect the geographic area suitable for beetle development. A better understanding of the beetle's thermal requirements is particularly important to assess the risks of the beetle's current and future distributions under climate change, and the potential impacts these may have on AOD. In order to explain the beetle's current distribution, data were collected on the beetle's life history, and the role of temperature in its development was experimentally investigated. Second, although the larval galleries of the beetle are nearly always found adjacent to AOD lesions, the exit holes of the adults are only found on some trees. Although all symptomatic trees may be attractive for oviposition, host resistance may prevent the beetles from completing development on some trees. In order to improve the understanding of the impact of host vigour on beetle developmental success, the wound response of a range of trees in various stages of decline by AOD was measured. The use of mechanical wounding as a management tool for selecting the most vigorous trees was also evaluated. Third, although trees are assumed to be predisposed to AOD, the vigour of trees before AOD onset and colonisation by *A. biguttatus* has not been measured. The stem growth of trees at various stages of decline by AOD (long-term symptoms, new symptoms, and remission) was analysed, in order to determine the role of predisposition in AOD. The impact of AOD on recent growth was also assessed, as was the relationship between the stem growth of trees with and without *A. biguttatus* exit holes.

Paper 1: The lifecycle of *Agrilus biguttatus*: the role of temperature in its development and distribution, and implications for Acute Oak Decline

Summary of findings on the thermal requirements and life history of the beetle

The calculated thermal requirements indicate that the beetle is likely to be restricted to its current distribution in England by its lower threshold temperatures. In particular, the lower threshold of pupae (to adult emergence), 15.1°C, is very similar to mean early summer air temperatures within its core range in England (UK Climate, 2016). The beetle must therefore rely on warm, sunny days, and, probably, sun-warmed tree stems, to be able to pupate and emerge early enough for it to reproduce, and for its eggs to hatch, before autumn temperatures become too cool for egg development (Régnière, 2009). The observed, protracted mean date of initial oviposition, 28 days after emergence, would further reduce the available temporal and thermal window. The thermal requirements of the beetle may contribute to the relatively low rate of lifecycle completion on trees with AOD: whereas larval galleries are nearly always present, exit holes are found on approximately 30% of trees with AOD (Brown *et al.,* 2017). The beetle seems to have an obligatory prepupal diapause at all temperatures studied, which is consistent with a related species *A. planipennis* (Duan *et al.*, 2013; Liang & Fei, 2014), and this probably serves to synchronise and regulate the lifecycle.

As well as the developmental work, detailed observations were made of all life stages of the beetle. The sex ratio of emerging adults varied by site and by year. Many females were able to live for two months or more, and laid multiple batches of eggs. Egg batches often hatched over several days or a week, which may be a bet-hedging strategy to hedge against variation in weather (Hopper, 1999). *Agrilus biguttatus* developed through four larval instars, which is in line with other species within the genus (Cote & Allen, 1980; Loerch & Cameron, 1983; Lyons & Jones, 2005; Haavik *et al.,* 2013; Orlova-Bienkowskaja & Bieńkowski, 2016). The study also found evidence that the optimal developmental temperature is lower in early-instar larvae than in late-instar larvae, suggesting that the larvae are adapted to attain optimal growth at seasonally-appropriate temperatures (Atkinson, 1996). Anecdotal observations of larval ecology suggest the larvae are, at least in part, limited by finite moisture requirements.

Strengths and weaknesses of the collected data and culturing techniques

An important achievement of this study was the development of novel methods to culture each life stage of the beetle. Significant efforts, including extensive trial and error, and collaboration with international scientists working on similar species in the United States,

were required to develop the culturing methods employed in this study. Not only had this species never been cultured in the laboratory, as far as the author is aware, the only other species within the genus to have been cultured from egg to adult in the laboratory is *A. planipennis* (Duan *et al.,* 2011; Lopez & Hoddle, 2014; J. P. Lelito, pers. comm.). The data generated on the species' thermal requirements will allow detailed modelling of the beetle's lifecycle and voltinism to be undertaken across the geographical range of the UK, which can then be used to predict its distribution under current climatic conditions and under the future temperatures forecast by climate change models (Wainhouse *et al.,* 2014).

Although the method of culturing larvae on logs was successful for the purposes of this study, there were numerous drawbacks to this technique. The log method was employed because the larvae are fragile, prone to desiccation, and easily damaged through handling. It was, however, not possible to continuously track the growth of individual larvae; they could only be observed when the logs were dissected, and many were killed during dissections. Late instar larvae often moved towards the lower portion of the logs as they stood in water, and drowned in the saturated tissues. Many logs deteriorated and desiccated too quickly for the larvae to develop. Although efforts were made to standardise food quality by standing the logs in water, monitoring tissue quality, and discarding data from logs in which tissue condition had visibly deteriorated, differences in food quality may still have influenced the developmental results, particularly at the lower temperatures where these development periods were longer.

Additional weaknesses, associated with spatial constraints, include a lack of incubator replication, and limited number of replicates, particularly at the later developmental stages. Due to long larval development times and a limited number of incubators, it was not possible to incorporate multiple incubator replicates at each temperature treatment, which would have eliminated the potential effects of individual incubators, although differing effects are considered to be unlikely. It was also necessary to conduct the experiments at 20°C in a growth room, which had differing light and humidity levels from the incubators. Indeed, humidity levels were measured to be different in all of the incubators, although this is not considered likely to have affected the results. Although as many logs were employed as the limited available space would permit, the high level of mortality in the larval stage meant that there were very limited replicates in the pre-pupal and pupal stages, and all remaining individuals had to be incorporated into the pre-pupal (to adult emergence) analysis, despite the repeated measurements on some of these individuals. The limited replication meant that the modelling did not allow for random effects to account for these repeated measurements.

Paper 2: Does a vigorous wound response inhibit the complete development of Agrilus biguttatus on trees with Acute Oak Decline?

Summary of findings on host vigour in relation to AOD and A. biguttatus

Percentage wound closure was strongly related to AOD category and the presence / absence of exit holes in this study. By the final measuring date, two years after the initial wounds were made, wound closure was most efficient in asymptomatic control trees, suggesting these trees were the most vigorous, and was progressively less efficient in remission, newly symptomatic, and long-term symptomatic trees, suggesting these trees were least vigorous. Within the remission and newly symptomatic categories, when exit holes were present, wound closure was less efficient. The findings provide further evidence that *A. biguttatus* females may be attracted to oviposit on, and larvae may initially colonise, trees in which host defences are too strong for complete development of the beetle (Hartmann & Kontzog, 1994; Vansteenkiste *et al.*, 2004; Brown *et al.,* 2017). These results also support the theory that efficient wound closure operates as a defence against phloem-mining beetles such as *A. biguttatus,* potentially damaging fragile, quiescent larvae in early spring or during moulting (Dunn *et al.,* 1990; Miller *et al.,* 1991; Fierke & Stephen, 2008; Haavik & Stephen*,* 2011)*.*

Initially slow closure rates in the asymptomatic trees suggest other factors than host vigour – at least as measured by wound closure efficiency – may also influence the probability of new AOD infections; for example, the proximity to other symptomatic trees may be important (Brown *et al.,* 2017). The effect of less efficient wound closure when exit holes were present was also inconsistent over time, signifying that other factors besides host resistance, such as microclimatic temperatures or tissue nutritive qualities, may also inhibit beetle development (Muilenburg & Herms, 2012; Villari *et al.,* 2015). Relatively high closure rates in the newly symptomatic categories at the earlier measuring dates also hint at a role of "priming" of host defences in the presence of AOD bacteria and beetle larvae (Conrath, 2009). Wounds made in late spring healed more rapidly than wounds made in summer; a less efficient wound response in summer may, in part, explain a higher frequency of new AOD lesion formation in the summer months (Brown *et al.,* 2017).

Strengths and weaknesses of the collected data and experimental techniques

In order to develop appropriate management strategies for AOD, a more complete understanding of the syndrome is required, and particularly of the role of *A. biguttatus* in AOD. The methods employed in this study enabled a quantitative comparison of host vigour across a range of trees at varying stages of AOD infection, and with and without

the exit holes of *A. biguttatus.* This enabled an investigation into host defences employed against the beetle, in the context of AOD. The inclusion of data from multiple measuring dates put the final results in context, and shed light on the complexities underlying AOD infection and host resistance to both AOD and *A. biguttatus* larvae. Of particular interest was the relatively rapid wound closure observed in the newly symptomatic categories, which is suggestive of a defensive "priming" effect (Conrath, 2009).

The results of this study also suggest that monitoring of mechanical wound closure may be a relatively simple way to identify trees with very low host resistance. After one year of monitoring, trees with long-term AOD symptoms had consistently poorer percentage wound closure than the other categories. Site-wide monitoring of mechanical bark wounds could be a useful tool to preferentially identify and protect vigorous trees, even if AOD symptoms are present, and to select the poorest trees for stand thinning. The technique may be most useful in distinguishing between the most and least vigorous symptomatic trees, and to employ it in this manner eliminates the need to wound trees without active AOD symptoms.

Although the clear results from the final measuring date were sufficient to address the aims and objectives of the study, wound response is only one measure of host defences, and the inclusion of an additional factor may have allowed for a more in-depth analysis of host resistance mechanisms. For example, high moisture levels may inhibit successful colonisation of neonate larvae, and too low or too high moisture inhibiting larval development (Hanks *et al.,* 2005; Sallé *et al.,* 2014; Brown *et al.,* 2017; Reed *et al.,* in press). Experimental results have been mixed: whereas one study found no difference in oak bark moisture between *Enaphalodes rufulus* infestation classes (Fierke & Stephen 2008), neonate *Phoracantha semipunctata* were unable to colonise eucalyptus logs with high bark moisture contents (Hanks *et al.,* 2005). In this study, moisture levels were measured in the cores of bark that were removed at the first wounding time-point. A bark moisture content analysis, using the same model structure as the wound recovery data (see Paper 2), found a significant effect of seven-level AOD category (X^2 = 17.5, df = 6, *p* = 0.008). Tree age was almost significant and was left in the model (X^2 = 2.86, df = 1, *p* = 0.09), but aspect was not (X^2 = 1.10, df = 1, *p* = 0.29). Post-hoc tests incorporating the seven-level AOD category found moisture in trees with long-term symptoms with exit holes was lower than in asymptomatic control trees or trees in remission but with exit holes (Figure 1). The experiment was felt to be unreliable, because, after removal, cores were cut into sections and moisture may have been squeezed out, and therefore was not presented in the paper.
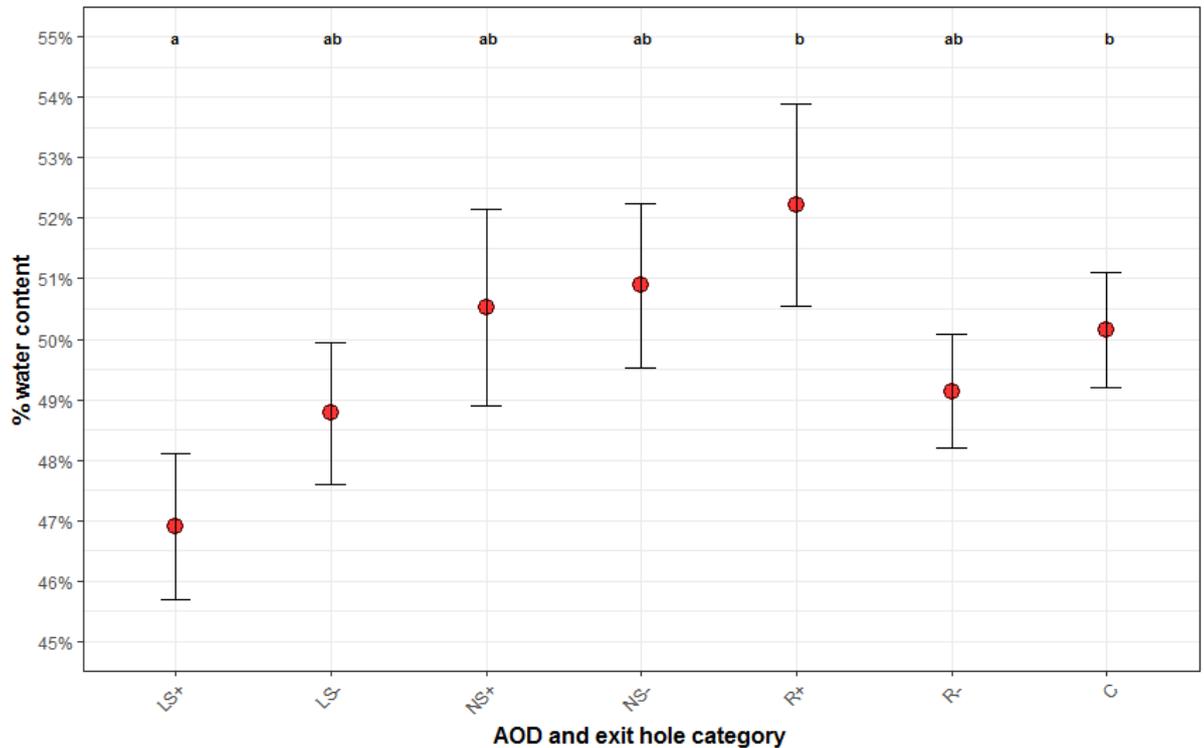
Figure 1. Boxplot showing the model-predicted % moisture content and SE by AOD and exit hole category (LS+/- = long-term symptoms with/without *Agrilus biguttatus* exit holes; NS+/- = new symptoms with/without exit holes; R+/- = remission with/without exit holes; C = asymptomatic control. Letters indicate significant differences (a > b, ab not significantly different from a or b).

Physiologically, trees decline because of depleted carbohydrate reserves (Führer, 1998; Bréda *et al.,* 2006), and therefore a comparison of carbohydrate levels may also have added an interesting component to the study. A relationship has been shown between reduced carbohydrate levels and colonisation by *Agrilus bilineatus:* trees with lower winter root starch reserves were more likely to be colonised by the beetle in one study, and in a second study, colonised trees had lower sucrose and total carbohydrate concentrations in the xylem (Dunn *et al.,* 1987, 1990). In the present study, carbohydrate levels were measured in the cores of bark removed at the first wounding time-point. Unfortunately, the chemistry used was inconsistent across samples, and the analysis was not felt to be reliable.

Other weaknesses relate to the constraints of the available study sites. The long-term monitoring, carried out by Nathan Brown, at Richmond Park and Hatchlands Park provided valuable information over several years on these trees. The oaks at these sites and at Madingley Wood did, however, have other health issues: many seemed to be declining due to conditions separate from AOD, for example due to colonisation by root pathogens, and while the worst-affected trees were not selected for study, it is possible that some of the study trees, including the asymptomatic control trees, had additional, confounding health issues. This may, in part, explain why the rate of wound closure in asymptomatic control trees was lower than expected at the earlier measuring dates. The

limited number of trees also resulted in low replicates within each category at the three woodlands, and although the modelling accounts for this imbalance, the inclusion of more trees within the newly symptomatic categories may have strengthened the patterns observed in this study.

There were several drawbacks to the use of mechanical wounding in this study. The wounds were unsightly, and many "bled" in a way that resembled the fluid exudations from AOD lesions, particularly when the sapwood was breached. The wounds may have also provided an entry-point for pathogens, despite precautions that were taken to prevent between-tree infection. Testing of the exuded fluid for the characteristic AOD bacteria yielded some positive results across all categories, including in asymptomatic trees, which was surprising; the trees may already have harboured the bacteria, or the wounds may have been secondarily colonised by the bacteria. Nearly all wounds had dried by the end of the study period, and all final tests of fluid from still-moist wounds at Madingley Wood were negative. Wounding that breaches the sapwood may result in permanent defects to the timber (Shigo *et al.,* 1977), particularly in young trees, and therefore this technique may be more suitable for use on amenity trees than high-value timber trees, or on trees with AOD lesions, where the amount of wounding created by the arch punches is likely to be negligible compared to the tissue degradation within the lesions.

Paper 3: Reduced stem growth predisposes oaks to Acute Oak Decline at three woodlands in England

Summary of findings on the long-term and recent stem growth trends of trees with AOD

The best-fit linear mixed effects models included up to three turning points at each site, reflective of differing site histories and management. Across the sites, despite the different site growth patterns, AOD category was a significant predictor of mean ring width throughout the stem growth record. Post hoc tests showed trees with long-term symptoms had consistently poorer mean growth than asymptomatic control trees; trees with new symptoms and trees in remission were intermediate. This finding was also reflected in the size of the trees, which, when corrected for estimated tree age, reflected the same pattern. The trees with long-term symptoms appeared to be predisposed to AOD by inherently poorer growth, potentially due to persistent competition because of over-stocking, or due to genotypic or microsite differences (Manion, 1981; Waring, 1987; Bigler & Bugmann, 2003).

The long-term stem growth of the trees was not related to the absence or presence of *A. biguttatus* exit holes, suggesting the beetles are not simply more successful on the

poorest growing trees within each category. Trees without *A. biguttatus* exit holes were nearly twice as likely to have returned to positive growth, although it is unclear whether the beetles are influencing tree recovery, or simply more successful on continuously-declining trees.

Neither the examination of the recent growth trends through model projections, nor the change-point analysis, found evidence of additional, recent growth divergences between symptomatic and asymptomatic trees, which may be indicative of lower resilience to recent inciting factors resulting in recent predisposition, and which have been observed in many dendrochronological studies of tree decline (Hartmann & Blank, 1992; Dwyer *et al.,* 1995; Pedersen, 1998; Drobyshev *et al.,* 2007b; Andersson *et al.,* 2011).

Surprisingly, neither the model forecasting nor the change-point analysis showed evidence of growth divergences in the AOD categories, including trees in remission, after AOD symptom onset and beetle colonisation. Site-level influences may exert a stronger influence on stem growth than AOD, and may include favourable climatic conditions or growth releases caused by reduced competition for resources due to some trees dying (Cook, 1987), or, conversely, climatic stress. It is also possible that the time period after AOD onset or recovery was too short for growth differences to be apparent in the analyses, or that trees do not exhibit sharply declining growth until just before death.

Strengths and weaknesses of the collected data and modelling techniques

Although predisposition was assumed to be a prerequisite of AOD, the long-term vigour of trees with AOD had not been previously assessed. This study found strong evidence of long-term predisposition in trees that went on to develop AOD symptoms over a number of years (e.g. symptoms at the start and end of monitoring): these trees exhibited poorer growth throughout their lifespans (Rozas & Garciá-González, 2012). In contrast, the exit holes of *A. biguttatus* were related to recent growth trends only, although whether they exerted an influence on growth, or were more successful due to host stress is unclear. This finding has implications for AOD management. Taken on its own, consideration should be given to felling trees with long-term AOD symptoms, as these are likely to have inherently poorer stem growth. As part of this study, a robust linear growth model was developed that directly incorporates a categorical component, and that may enable analysis of growth patterns at other AOD sites. With slight alterations, the model may also be adapted to different study questions.

In order to determine whether the growth patterns observed in this study are consistent in AOD epidemiology, it would be helpful to expand this study to incorporate more sites, and

particularly those where a good number of healthy trees may be found. Underlying issues affecting the resilience of the asymptomatic control trees may have masked any subtle, recent growth divergences before AOD onset, a pattern which was expected due to the findings of a study of similar decline in northern Germany (Hartmann & Blank, 1992). The lack of further impact of AOD on the recent stem growth of the trees was also surprising, given the severity of vascular damage due to necrotic lesions and larval galleries on many trees (Denman *et al.,* 2014). The limited number of trees available at the sites also meant that it was not possible to compare trees within the same crown position classes, which could clarify whether recent growth divergences have taken place.

Although a useful tool to examine recent growth trends, it may have been preferable to tailor the change-point analysis to better answer the objectives of this study. For example, the model determines whether trees have recovered after a growth decline by examining the slope of the growth since the decline initiation. In AOD, to determine the nature of remission, for example, it may be more meaningful to determine whether mean growth has returned to its initial level after declining (McDowell *et al.,* 2008). To better understand the growth declines exhibited by the majority of trees in this study, and, potentially, determine the cause of decline, it may be important to determine the date of individual trees' first episode of growth decline, as well as most recent growth declines. Even if trees recover after a reduction in vigour due to stress, resilience to future stress may be reduced, and trees may decline for decades as a result of an initial, severe stress event (Pedersen, 1998). Analysis of recent declines in individual symptomatic trees could also have incorporated more of the available data. For example, including the precise year of symptom onset and counts of AOD lesions and beetle exit holes within models may have allowed a closer examination of the syndrome chronology, and the impacts of varying symptom severity on trees. More replicates would be required to make the fullest use of the available data.

Integration of the findings on *A. biguttatus'* development, host vigour, and stem growth, and implications for management

The overarching aim of this thesis was to improve the understanding of how predisposing, inciting, and contributing factors interact to cause AOD, with particular attention to *A. biguttatus,* to inform future management of the syndrome. Data were collected that address gaps in knowledge about the UK distribution of the beetle, the role of host resistance in beetle larval success, and the nature of predisposition in AOD and susceptibility to the beetle. Integrating the findings from the three studies contributes to the available evidence about AOD, and also highlights future research needs.

The thermal requirements calculated in Paper 1 suggest the beetle may be restricted, even within its current UK geographic range, to warmer microsites, such as sun-warmed tree stems (Vermunt *et al.,* 2012), or may rely on warm summer temperatures to maintain populations. Preliminary modelling of the developmental data collected in Paper 1, with air temperatures from several sites in England, suggests that the beetle should be unlikely to complete development at ambient temperatures, even within its core range in south / central England. Air temperatures are a coarse approximation of under-bark conditions; other studies, and a limited pilot study conducted at Madingley Wood, Cambridge, and Alice Holt, Surrey, have shown that open-grown, sunny tree stems may reach considerably higher temperatures than corresponding air temperatures, which may shorten development times (Vermunt *et al.,* 2012; K. Reed, unpublished). England is at the northern edge of the beetle's European distribution (Brown *et al.,* 2014), and it is possible that the beetle struggles to complete development on many trees, in part due to its thermal requirements. This theory is consistent with anecdotal observations: even at sites where AOD and *A. biguttatus* have been present for many years, exit holes are infrequently found, with only one or two trees showing high numbers of exit holes, and these are often observed on the south sides of trees (Brown *et al.,* 2017; pers. obs.). Accurate modelling of the geographic area thermally suitable for beetle development will need to incorporate collected, or modelled, under-bark temperature data.

The integration of the findings on the development of *A. biguttatus* in Paper 1, and the findings on host resistance in Paper 2, illustrate the complexity inherent in modelling its development and thermally-suitable geographic range. The findings of Paper 2 suggest the vigorous condition of many AOD-symptomatic hosts, even those attractive to females for oviposition, restricts larval success (Hartmann & Kontzog, 1994, Vansteenkiste *et al.*, 2004; Brown *et al.,* 2017). The beetle's thermal requirements may, in part, explain the relatively low percentage of AOD-infected trees with beetle exit holes, and a frequently observed lack of exit holes on severely declined or even dead trees (Denman *et al.,* 2014; Brown *et al.,* 2017). Because host vigour strongly influences larval survival, development time is also likely to be influenced by host condition (Muilenberg & Herms, 2012; Villari *et al.,* 2015). The beetle may be able to complete development on more vigorous hosts in warmer locations. Drought stressed plant tissues may also be significantly warmer, due to stomatal closure (Mattson & Haack, 1987). Severe droughts may allow development on trees which otherwise would be thermally unsuitable, e.g. marginal geographic areas, or within stands with a high degree of canopy closure. Lifecycle modelling of the beetle will need to incorporate an understanding of the potential interactions between host vigour and development time.

The use of the same study sites and trees permits direct comparison between the findings of the wound response (Paper 2) and dendrochronology (Paper 3) studies. The average rate of wound closure across the four wounds on each tree at the final measuring date (two years after they were made), and the mean average ring width, were positively correlated, both over the entire core (Pearson's rho = 0.29, t = 3.53, df = 140, p < 0.001) and in the last 10 years (Pearson's rho = 0.36, t = 4.59, df = 140, p < 0.001). A correlation between stem growth and wound periderm growth is consistent with the results of other studies, although the two processes are under different regulation, and their respective growth rates may differ (Neely, 1988; Dunn et al 1990). AOD category was strongly linked to percentage wound closure and stem growth in both studies. In contrast, the presence of *A. biguttatus* exit holes was strongly correlated with a reduced host wound response, but not with mean stem growth, with the only relationships between exit hole absence/presence and stem growth evident in some of the change-point analyses. Trees allocate carbohydrate resources preferentially to the growth of new foliage and roots, and then to storage, stem growth, and the production of defensive compounds (Waring, 1987). If resources are limited, trees prioritise the most vital processes (Dobbertin, 2005). Early wood, which comprises approximately 30% of total annual stem growth (Barbaroux & Breda, 2002) (and potentially more in stressed trees), is vital to ring-porous trees like oaks. In these studies, trees may have prioritised maintaining stem growth over efficiently closing wounds, and thus wound closure may be a better indicator of current vigour and carbohydrate resources than recent stem growth trends (Dunn *et al.,* 1990). The findings in the wound closure study suggest that trees with exit holes have lower vigour, and therefore presumably lower carbohydrate reserves and lower resilience to stress. As this is not evident in the stem growth trends, there may be a lag between the manifestation of stress in wound closure and growth trends, where mean ring widths are not affected until later in the syndrome.

The pattern of predisposition observed in Paper 3 suggests it may be useful to develop management strategies directly targeting the causal agents of AOD, when these are identified. Recent growth divergences between healthy and symptomatic trees, in the years before symptom onset, would imply that varying resilience to inciting factors, such as droughts, had left some trees very weakened and, therefore, highly susceptible to secondary pests and pathogens (Pedersen, 1998; Haavik *et al.,* 2015). In such a case, management specifically targeting the causal agents of AOD – the bacteria, or the beetle if it is essential to AOD – may not be particularly useful, and management should aim to improve stand-wide resilience, e.g. by improving adverse stand water relations or nutrient imbalances, or thinning overstocked stands. Growth divergences were not, however, apparent in this study, and therefore, management efforts against the specific causal agents, when they are determined, may be useful, as well as removing the most

susceptible trees, which may be facilitated by monitoring wound responses. The observed patterns are based on the stem growth at only three sites, and testing of stem growth at additional sites is important to determine if the observed patterns are typical.

The integration of the developmental findings in Paper 1, the findings of the role of host vigour in beetle developmental success in Paper 2, and the dendrochronological work in Paper 3, highlight the importance of experimentally determining the role of *A. biguttatus* in the syndrome before the recommendation of management strategies. The trees predisposed to long-term AOD symptoms are also the slowest growing (Paper 3). If the beetle vectors the bacteria between trees, an effective management strategy may be thinning out the most declined symptomatic trees, with the highest chance of successful beetle development in any numbers, which should decrease the likelihood of emerging adults spreading bacteria to asymptomatic trees. Trees would need to be cut before spring, and the sapwood destroyed, to prevent fully developed prepupae from pupating and emerging from trees after felling (reviewed in Brown *et al.,* 2014). In densely planted stands, felling some trees may also increase the vigour of the remaining trees by decreasing inter-tree competition (Paper 3). Conversely, conducting thinning operations may worsen the symptoms of trees that already have AOD if the beetle is involved in the formation of new lesions on symptomatic trees. Thinning would create canopy gaps, increasing sunlight penetration and stem temperatures (Vermunt *et al.,* 2012). Increased temperatures may increase larval development rates and survival, and as larvae have been experimentally implicated in spreading the AOD bacteria within trees, this may increase the number of lesions on trees where beetle larvae are already present (Denman *et al.,* in press). The stem temperature increase could be countered to some extent by planting shady understorey species (Wachtendorf, 1955). Regardless of the role of the beetle in AOD, management to increase trees' resilience to stress, and increase their vigour, should help to limit AOD infections and mitigate the impacts of AOD (Brown *et al.,* 2014, 2018).

Conclusions and future research needs

A better understanding of the essentiality of both *A. biguttatus* and the consistently isolated bacterial species to AOD, and their interaction within the syndrome, is necessary to inform management efforts. Numerous groups within a multi-disciplinary research consortium are examining many aspects of the syndrome, from the mechanisms of attraction of the beetle to declining trees, to environmental sources of the bacteria, to within and between-tree bacterial spread and the mechanisms of lesion formation.

Further funding is currently being sought to continue work on the beetle's development in the UK, by developing a predictive model to estimate the geographic range thermally suitable for *A. biguttatus'* development. This work would incorporate under-bark temperature monitoring, which should closely represent the conditions experienced by all life stages of the beetle, measured at thermally marginal and unsuitable sites, as well as sites within the beetle's core range. Under-bark temperature data would be combined with local air temperatures and projected temperatures under a range of future climate change scenarios, to determine the likelihood that the range of the beetle will increase, and how this may impact AOD. An understanding of how host vigour affects developmental success and development time would also need to be integrated into future lifecycle models.

To determine whether the pattern of predisposition observed in this study is typical, it will be important to analyse growth trends at a wider range of sites, and particularly at sites with healthy control trees. Funding is being sought to expand the dendrochronological study to incorporate additional sites. Examining the growth of recently dead trees may also help to clarify the growth patterns that lead from AOD onset to host death.

The findings on predisposition in the dendrochronological study suggest it may be useful to manage the causal agents of AOD, when they are known. Potential management techniques against the putative causal agents of AOD – the bacterial species, and the beetle – must be trialled to determine their success. Management to experimentally manipulate populations of *A. biguttatus* must be sensitive to the native status of the beetle, and to other insects associated with oak host trees. Silvicultural management that may reduce under-bark temperatures, such as under-planting, may have a significant impact on beetle populations. Techniques to exclude the beetle from individual trees with AOD could also be trialled, and by determining whether exclusion increases the likelihood of remission, could also answer a key question about the role of the beetle in the syndrome.

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Table A1. Ring count and ring width summary data by site and four-level AOD category (LS = long-term symptoms, NS = new symptoms, R = remission, C = asymptomatic control)

	Category	Hatchlands Park	Madingley Wood	Richmond Park
Average number of rings	LS	147	160	175
	NS	152	-	170
	R	126	138	166
	C	129	108	160
Maximum number of rings	LS	239	231	194
	NS	218	-	189
	R	202	219	194
	C	218	200	187
Minimum number of rings	LS	106	103	127
	NS	111	-	145
	R	74	76	118
	C	91	63	65
Average ring width (mm) (± SD)	LS	1.5 ± 0.15	1.8 ± 0.19	1.6 ± 0.07
	NS	1.7 ± 0.13	-	1.7 ± 0.10
	R	2.0 ± 0.13	1.8 ± 0.08	1.5 ± 0.10
	C	2.2 ± 0.17	2.1 ± 0.23	1.8 ± 0.09
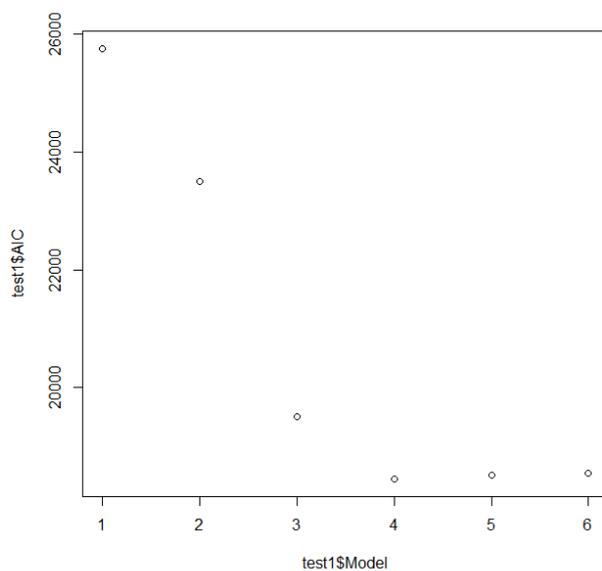
Figure A1. Change in AIC with increasing number of turning points (1 = linear (no turning points); 6 = sextic(5 turning points)).

Series residuals(model1qrcr4r, retype = "normalized")
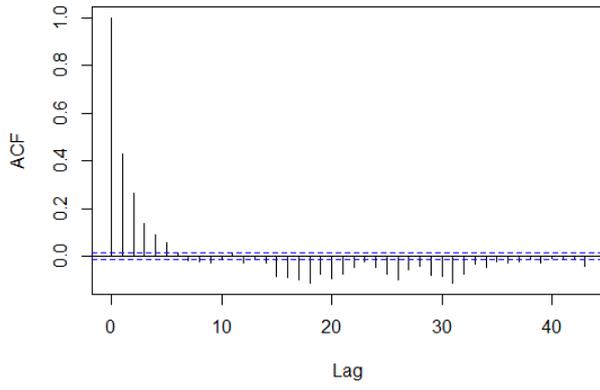
**Series residuals(model1qrcr4r, retype = "normalized")**
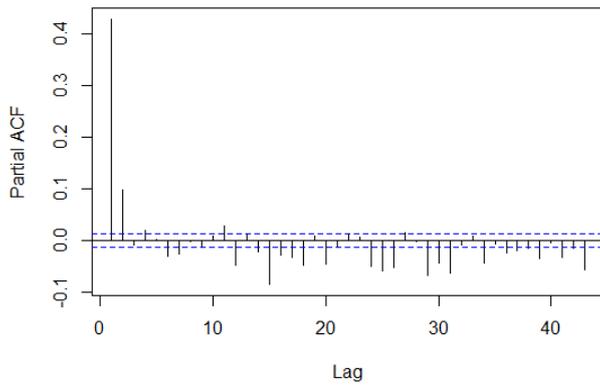
Figure A2. Autocorrelation results of the model residuals. Results within the dotted lines are significantly autocorrelated ($p < 0.05$).
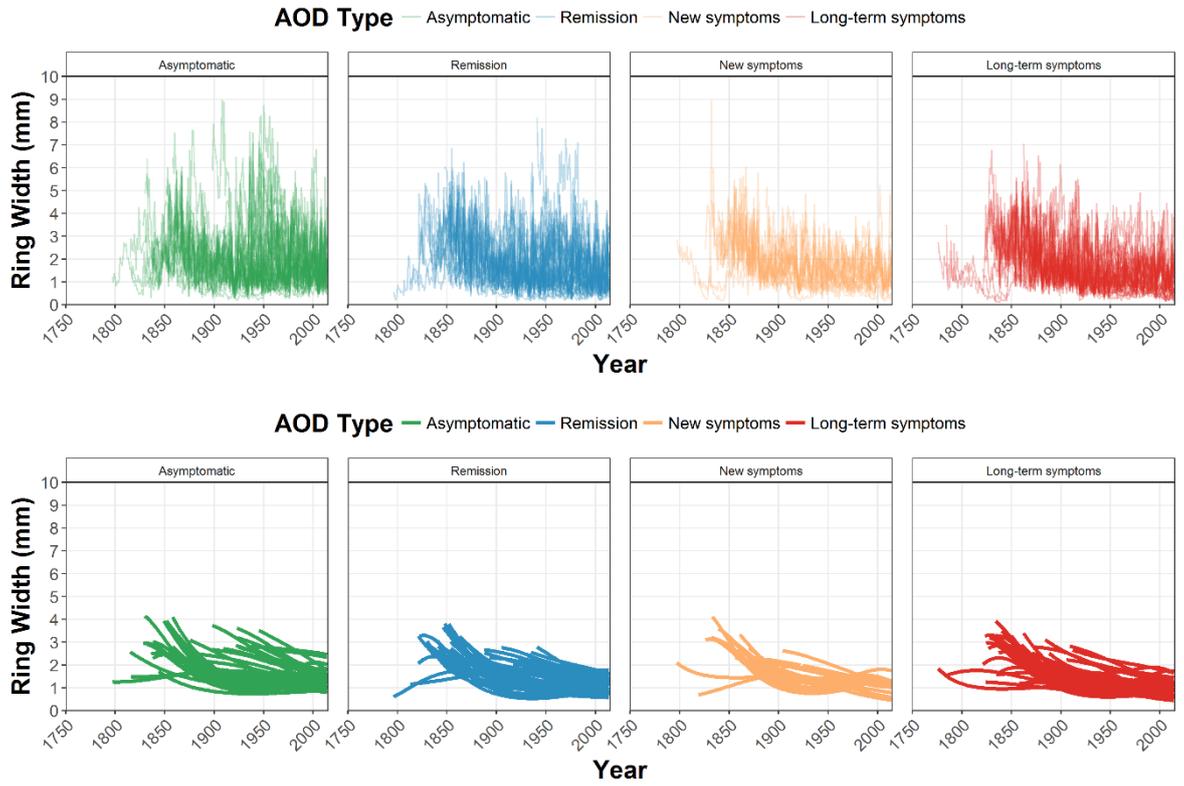
Figure A3. Raw ring widths of each individual tree (above) and best fit model predictions applied to each individual tree (below) by AOD category and across the three sites.
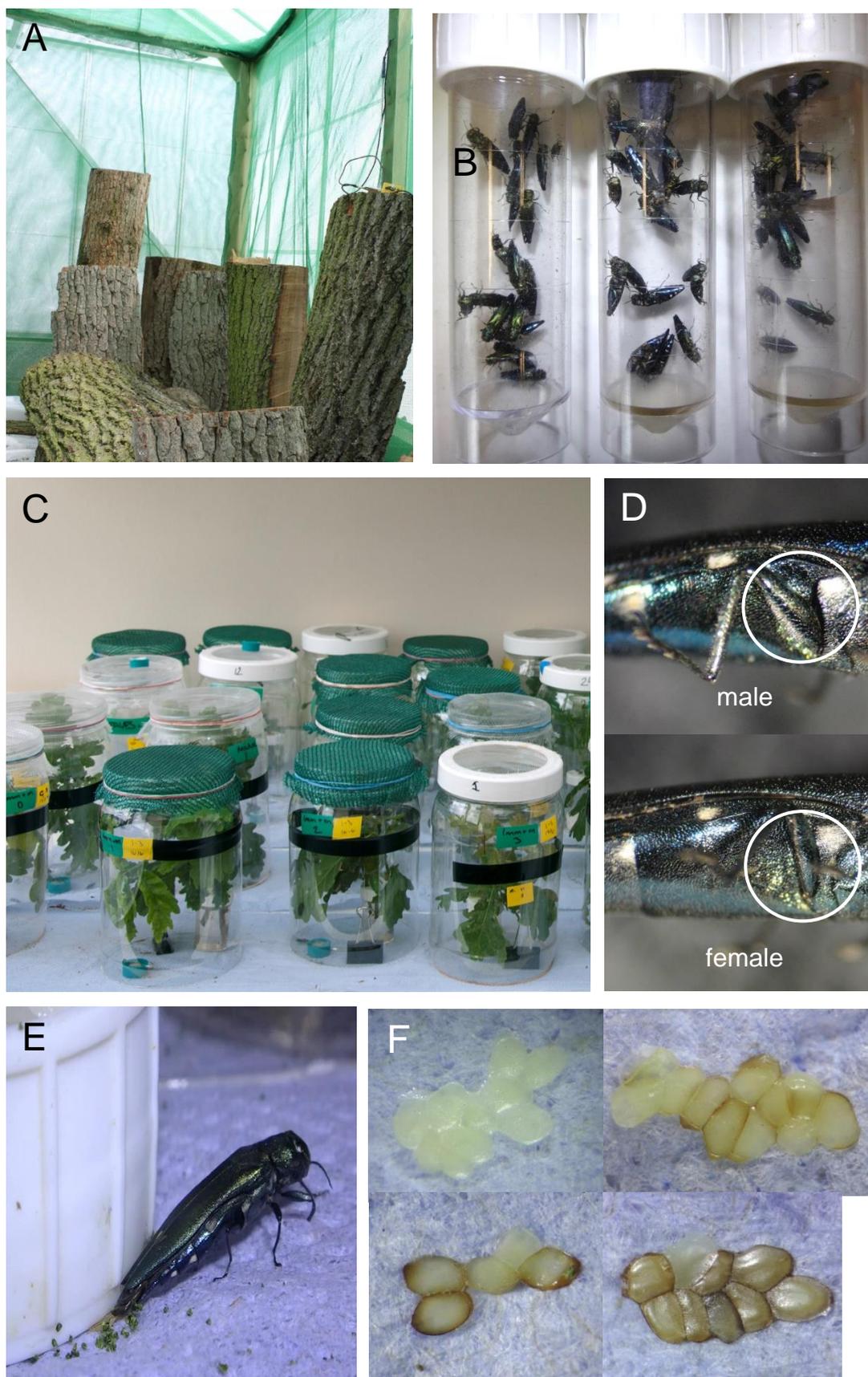
Figure B.1. Photographs of the experimental set-up for collecting and culturing adult *Agrilus biguttatus* in Paper 1. A: slabs of bark and sapwood within an emergence cage; B: adults collected from an emergence cage; C. jars containing adults, food (leaves) and water (20% sugar solution); D. ventral surface of the hind femur of *Agrilus biguttatus* adults, the character used to determine sex: with long setae (male, above), and without long setae (female, below); E. female ovipositing underneath a water container; F. eggs laid on blue paper towel, at different stages of development.
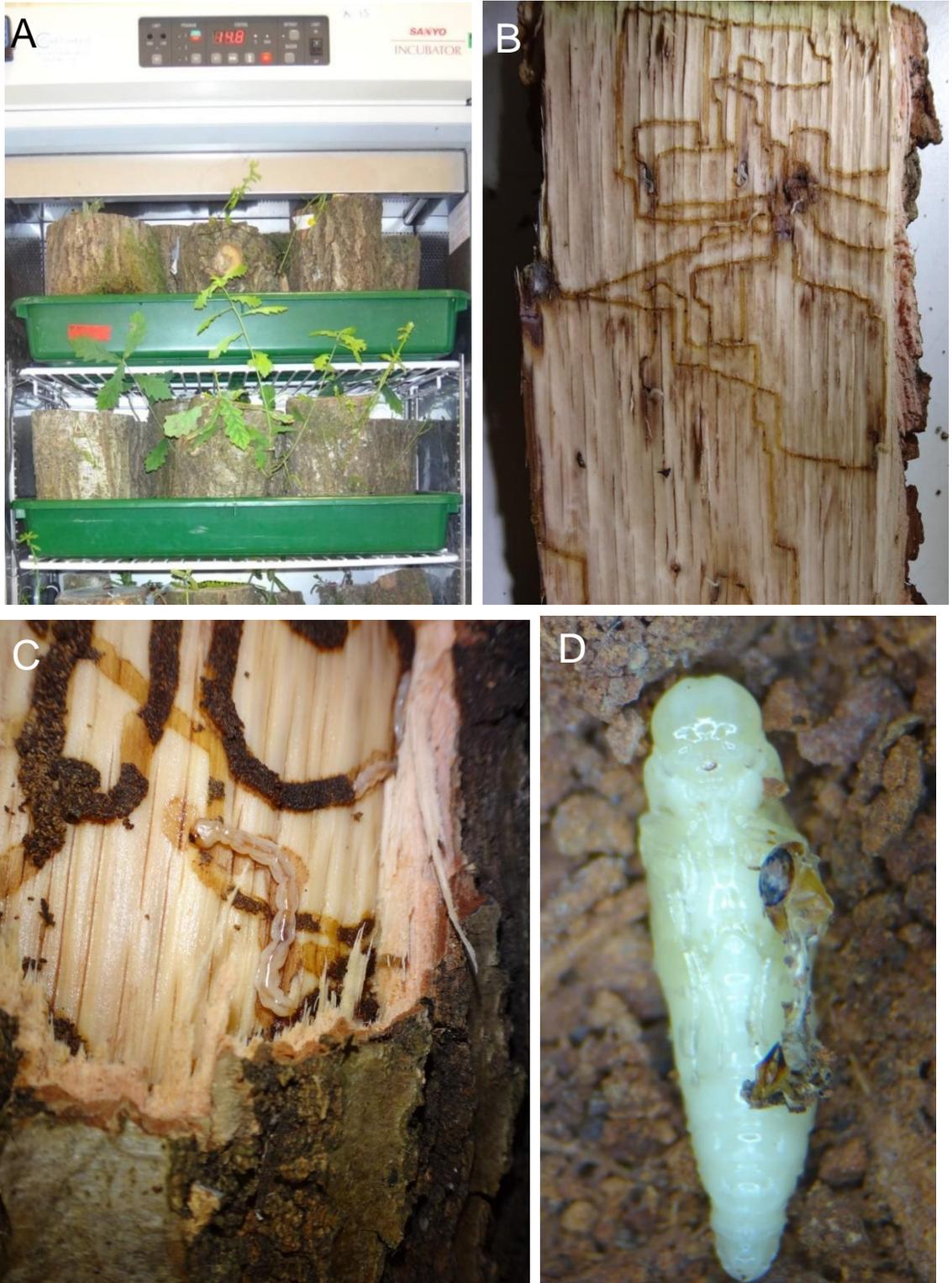
Figure B.2. Photographs of the experimental set-up for culturing *Agrilus biguttatus* larvae and pupae in Paper 1. A: Logs, with larvae developing in the inner bark tissues, standing in trays of water within an incubator; B: excised panel of phloem tissue, with moulting, early instar larvae and larval galleries; C. partially dissected log, exposing two late instar larvae; D. exposed, monitored pupa on moist ground bark.

Figure B.3. Photographs of the experimental technique of mechanically wounding oaks employed in Paper 2, and of wounds with varying levels of % closure. A: mechanical wounding with arch punch; B: fresh wound; C: 0% wound closure; D: 72% wound closure; E: 95% wound closure; F: 100% wound closure.
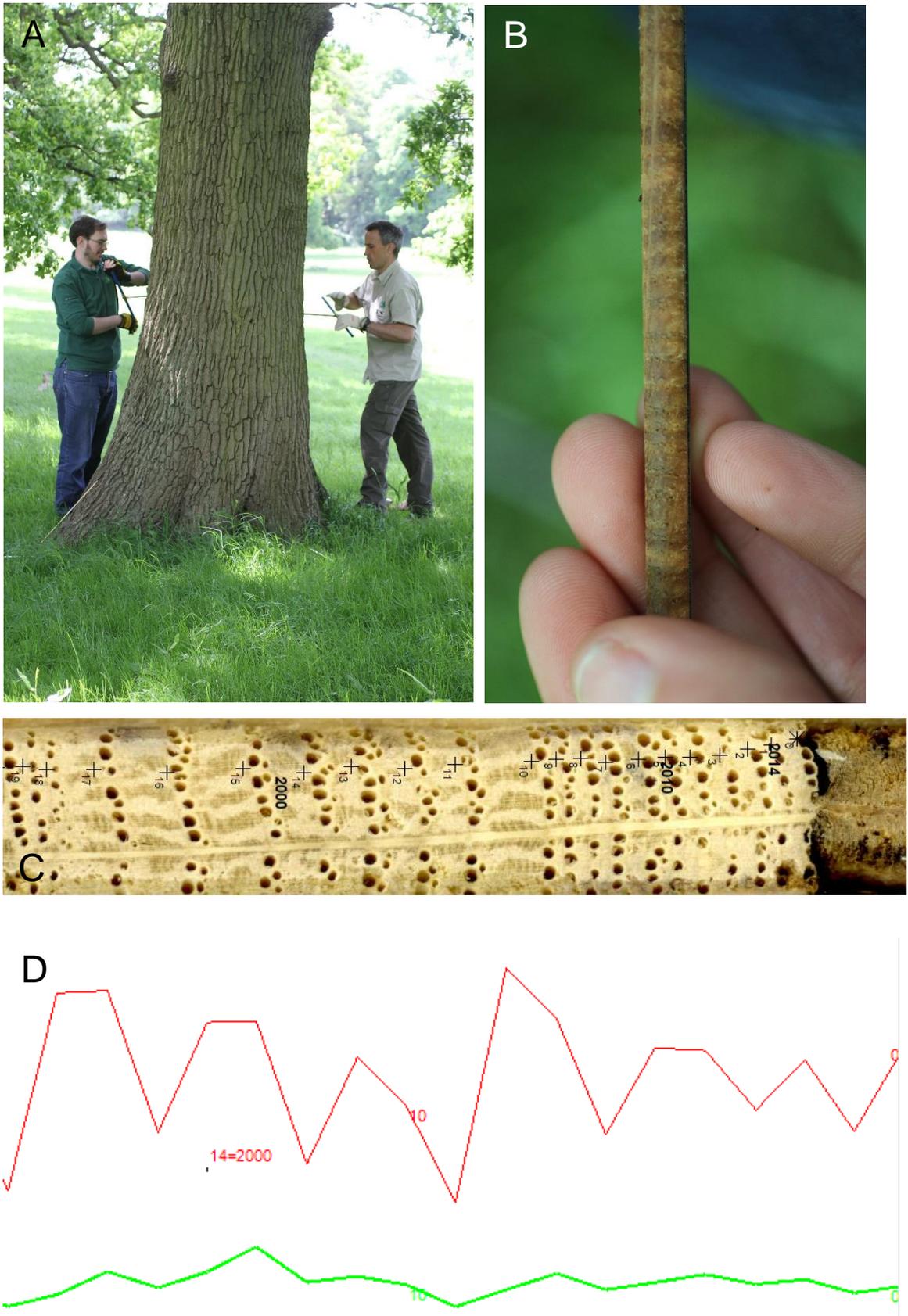
Figure B.4. Photographs of the dendrochronological study. A: cutting increment cores; B: freshly collected core; C: section of scanned core, including the outer bark (right), with the rings numbered in CooRecorder; D: corresponding visualisation of the ring widths in CDendro (red = normalised ring widths, green = raw ring widths)