UNIVERSITÀ DEGLI STUDI DI PADOVA
Department of Land, Environment, Agriculture and Forestry

Second Cycle Degree (MSc)
in Forest Science

Integrating visual stimuli in trapping protocols for longhorn beetles

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ACADEMIC YEAR 2023/2024

ABSTRACT

Longhorn beetles (Coleoptera, Cerambycidae) are among the most significant groups of invasive forest insects worldwide. Hidden within live plants and wood-packaging materials, these beetles can escape routine inspections at entry points become major pests in the invaded environment. Traps baited with pheromones and kairomones and set up in and around entry points are commonly used to improve chances of intercepting exotic species soon after their arrival. Factors able to affect longhorn beetle catches in traps have been extensively studied in the last years. Whether visual cues can be used to further increase trap attractiveness is instead still unclear. In a trapping study carried out in 2023 at 3 forest sites in northern Italy, we investigated the response of three target longhorn beetle species and a number of non-target species to baited traps characterized by five different visual stimuli, that are: i) black colour; ii) the colour of the stripes/bands present on the target species elytra; iii) black colour plus multiple reproductions of the colour and pattern of the stripes/bands present on the beetle elytra; iv) black colour plus multiple dots of the same colour of the stripes/bands present on the beetle elytra; v) black colour plus multiple pictures of the adult individual. Preliminary results clearly demonstrated a species-specific response of both the target and non-target longhorn beetles to the different visual stimuli, indicating that integrating them in baited traps can strongly improve trap efficacy.

SUMMARY

1.   INTRODUCTION

1.1   The family *Cerambycidae* and its main subfamilies

Cerambycidae, also known as longhorn beetles or longicorn beetles, are one of the most important families of Coleoptera worldwide, not only because their wide diversity, but also because of their economic impacts on various ecosystems. Around 36,300 cerambycid species are known to exist at present, which are distributed in more than 5,300 genera (Tavakilian, 2015). The family Cerambycidae includes eight subfamilies: Cerambycinae, Dorcasominae, Lamiinae, Lepturinae, Necydalinae, Parandrinae, Prioninae and Spondylidinae (Bouchard et al., 2011; Monné, 2012; Švácha and Lawrence, 2014). In this thesis, particular attention will be given to subfamilies Cerambycinae, Lamiinae and Lepturinae.

Cerambycinae is the second largest subfamily (11200 species and 1757 genera) and it's widely distributed in all biogeographic regions (Bouchard et al., 2011; Monné, 2012; Tavakilian, 2015), while the largest subfamily is represented by the Lamiinae, with more than 21,000 species in 2,964 genera. Lamiinae are also distributed worldwide, with greater diversity in the tropics and subtropics. Lepturinae have more than 1,500 species and 210 genera; this subfamily tends to be dominant in the Northern Hemisphere, with a few species in Afrotropical, Australian, Neotropical and Oriental regions (Monné and Monné, 2008; Švácha and Lawrence, 2014).
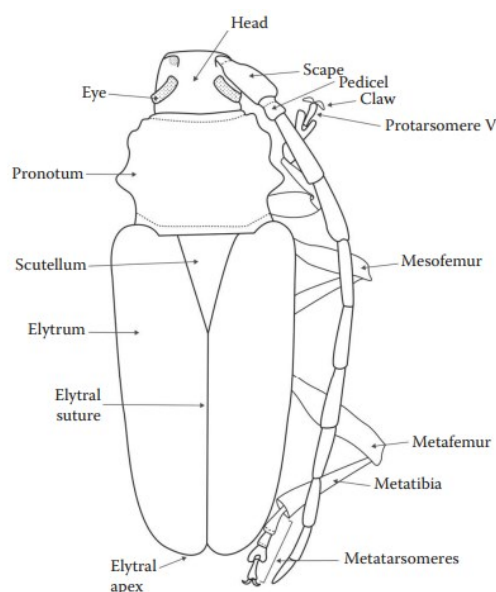
1.1.1   Longhorn beetle morphology

Figure 1 General morphology of cerambycid adult; dorsal view (Source: *Cerambycidae of the world*, Q. Wiang, CRC Press, 2017, p.9)
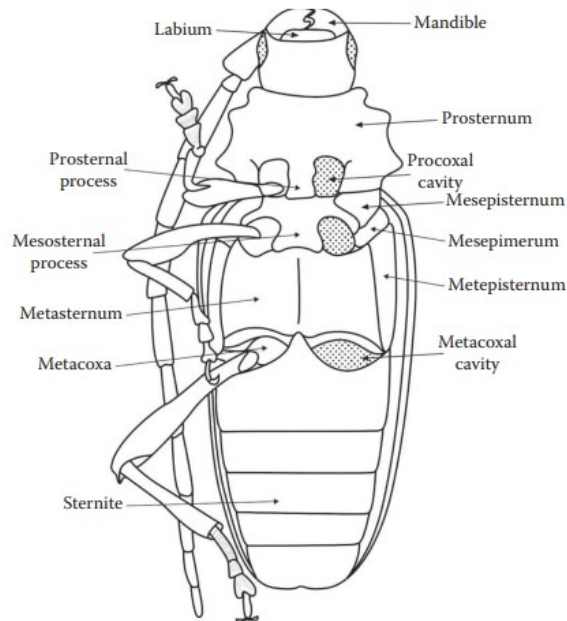
Figure 2 General morphology of cerambycid adult; ventral view (Source: *Cerambycidae of the world*, Q. Wiang, CRC Press, 2017, p.9)

Information about morphology of Cerambycidae is extracted from Ślipiński and Escalona (2013) and from Švácha and Lawrence (2014). Generally, adult body length varies from less than 2 mm to more than 170 mm. General aspect of adults varies depending on the subfamilies but also among species within the same subfamily. Colours vary too, in particular in relation to species ecological habits: for example, Cerambycinae includes both nocturnal dark coloured species and diurnal bright coloured ones. Antennae are filiform, elongate and 11-segmented, usually inserted on tubercles. The way of insertion of antennae can vary in according to the subfamily: before or between the eyes in Lepturinae, high and more or less embraced by eyes in Cerambycinae and Lamiinae (Fig. 5), or near the base of mandibles in other subfamilies. Some diurnal species of Lamiinae present fewer antennomeres, while 12 antennomeres are present in a few Cerambycinae. Antennal structure results to be similar between sexes in the subfamily Lepturinae, and very dissimilar in Cerambycinae and Lamiinae. Eyes tend to be emarginated and can be more or less faceted; in Cerambycinae, eyes are more specialized. Abdomen is covered by elytra and has five visible sternites, with fifth sternite entire. In Cerambycinae subfamily elytra are not abbreviated, while they appear narrowed posteriorly and rarely abbreviated in Lepturinae (Figs. 3 and 4).

Figure 3 Example of cerambycine adult (*Coccoderus sexmaculatus*), where it is possible to observe how elytra are not abbreviated at the end (Source: *Cerambycidae of the world*, Q. Wiang, CRC Press, 2017, p.41)

Figure 4 Example of lepturine adult (*Strangalia melanura*), where it is possible to observe elytra narrowed at the end (Source: *Cerambycidae of the world*, Q. Wiang, CRC Press, 2017, p.57)

In Lepturinae and Cerambycinae, head is produced anteriorly and can form a short to quite long muzzle (Figs. 5A and 5B). Differently, in Lamiinae the head tends to be more vertical or retracted (Fig. 5C).
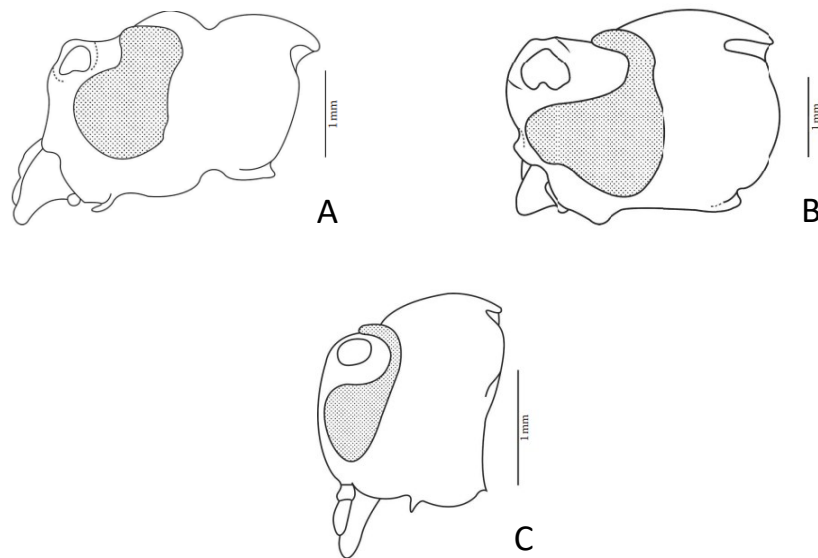

Figure 5 Lateral view of head in Lepturinae; in 5A there's a representation of head in Lepturinae, in 5B a representation of head in Cerambycinae and in 5C in Lamiinae (Source: *Cerambycidae of the world*, Q. Wiang, CRC Press, 2017, pp. 11-12)

Legs are mostly cursorial (modified for running) and usually moderately long in most long-horn beetles; they're shorter in Lamiinae where, also, fore legs appear to be enlarged in a few species, particularly males.

1.1.2 Longhorn beetle feeding behaviour

With regards to feeding habits, general trends at subfamily level can be observed, even though not many studies have been conducted so far. All Lamiinae and Lepturinae feed as adults, while in the Cerambycinae subfamily some adults feed and others don't (Duffy, 1953). It's possible to say that nearly all cerambycids are phytophagous during both larval and adult age; an interesting exception is represented by cerambycids of genus *Elytroleptus*, where adults mimic and prey on adult lycid beetles (Eisner et al., 2008; Grzymala and Miller, 2013). Some cerambycids are monophagous and feed only on a single species or genus of plants, others prefer several genera within a single family, while some are highly polyphagous (Craighead, 1923; Duffy, 1953; Linsley, 1959,1961). According to Butovitsch (1939), adult cerambycids mainly feed on flowers, bark, foliage, cones, fruit, roots and also fungi: for example, lepturine, cerambycine and a few lamiine feed on pollen and nectar (Duffy, 1953; Linsley, 1959); only lamiines eat bark and stems (Duffy, 1953); cerambycines and lamiines

can also feed on fruits (Duffy, 1953). Type of food consumed by adults has an influence on their longevity and fecundity (Smith et al., 2002; Hajek and Kalb, 2007). Studies have shown that many cerambycids don't feed during adult stage and reproduce soon after emergence (Iwabuchi, 1982). On the other side, there are also species that feed after emergence, like Lamiinae, and they need food for one to three weeks before being sexually mature, a period of time called "maturation feeding" (Alya and Hain, 1985; Hanks, 1999; Edwards, 1961; Slipinski and Escalona, 2013).

Larvae feed on many different plant species, plant parts and tissues, but also on hosts in various conditions, from living to dead and decaying plants, or on hosts in a particular state of decay which harbour some fungi which make wood palatable and help providing essential nutrients (Hanks, 1999; Saint-Germain et al., 2010; Filipiak and Weiner, 2014). In general, larvae of most cerambycid species develop in woody plants. Despite most species show preferences for oviposition towards hosts in particular conditions, this relationship may be modified when the insect encounters new hosts. Since larvae are usually legless, they're not able to move among hosts, so choice of host plant totally depends on the mother: because of this, females have to oviposit in the appropriate parts of plants of the right species and which are in good conditions to allow larvae development (Švácha and Lawrence, 2014). Sometimes, larvae can compensate for poor host choice made by their mother by migrating to tissues of higher quality as they're feeding within host plant (Saint-Germain et al., 2010).

The high importance attributed to larval stage is due to the fact that, worldwide, in many cerambycid species larvae are economically relevant pests on herbaceous plants. Similarly, information on larval hosts is best known for those species which have an economic impact in some regions of the world.

1.1.3   Longhorn beetle phenology and mating behaviour

Species which are native to temperate regions are univoltine (one generation per year) or semivoltine (more than one year is needed to complete a generation) and adults are active for a few weeks; however, voltinism may vary with latitude. An example is *Neoclytus acuminatus*, which is univoltine in north-central United States and trivoltine in southern US (Hanks et al., 2014). Tropical and subtropical species, on the other side, can be active for longer periods, thus leading to overlapping generations (Shah and Vora, 1976; Qian, 1985; Sontakke, 2002).

As Linsley (1959) observed, many cerambycid adults mate on larval host plant, suggesting that that a strong association exists between finding host plant and finding mates. Males usually call possible partners, by using aggregation pheromones, from larval host plant or from that of adults (Landolt and Phillips, 1997); because male adults arrive first on host plant, it's important they're able to locate host without pheromones; similarly, female adults must find hosts where oviposition can

happen, independently of males and pheromones: the hypothesis formulated is that plant volatiles may be responsible for mediating location of host plants in nocturnal species (Beeson, 1930), while diurnal species seem to rely on visual cues. An interesting fact is that cerambycids seem to be sensitive to bark beetle pheromones: this ability is probably adaptive, since it serves to guide longhorn beetles towards hosts already weakened, where colonization can happen and where larvae can feed opportunistically on bark beetle larvae (Schoeller et al., 2012). In Lamiinae, males are able to detect females over short distances (about few centimetres) and, for diurnal species, this is possible thanks to visual signals that adult insects exploit to find the partner (for example, in *Anoplophora chinensis*). Ultimate recognition of females by males is performed by touching them with antennae, mouthparts or tarsi, since species- and sex-specific contact pheromones are present in the cuticular wax layer of females; also, females can release non-volatile compounds that serve as chemical trail to guide males towards them.

As mentioned earlier, larval stages of Cerambycids are phytophagous (Linsley, 1959; Hanks, 1999; Slipinski and Escalona, 2013), with possible events of predation in case of encounters.

1.2   Longhorn beetles as exotic species

Longhorn beetles have seen their importance increasing over recent years because of the high number of species introduced outside their native range. Some of them have been objective of very expensive eradication campaigns that have been carried out after introduction, and others caused extensive economic and ecological damages in the invaded ecosystems. For example, eradication of *Anoplophora glabripennis* in the United States costed more than 537 million of dollars between 1996 and 2013 (Ronda Santos, USDA-APHIS, personal communication February 2014).

With "invasive", related to a species, we intend the condition in which a species is non-native to the ecosystem considered and has the potential to cause an economic or environmental damage (Definitions Subcommittee of the Invasive Species Advisory Committee, approved April 27, 2006). A similar definition is given by FAO (UN Food and Agriculture Organization) and quoted by EPPO (European and Mediterranean Plant Protection organization): according to this definition, a "quarantine pest" is intended as "pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled" (ISPM 5, FAO).

Some of the main factors that contribute to increase the importance of longhorn beetles as invaders are: i) the numerous and diverse pathways along which they can move, ii) their potential for

causing damages, and iii) the difficulties in detecting juvenile stages during routine inspections carried out at entry sites. Woody products which are actually infested may have small signs of infestation, or not have them at all: for this reason, visual inspections alone are not enough and additional methods are needed.

1.2.1 Introduction pathways

The main pathway through which longhorn beetles are introduced in non-native regions worldwide is represented by movements of plants for planting, according to Liebhold et al. (2012) more or less 70% of damaging forest pests and pathogens established in the United States, in the period between 1860 and 2006, mostly entered on imported plants. An important example of invasive longhorn beetle introduced with plants is *Anoplophora chinensis* (Fig. 6), also known as citrus longhorn beetle, originating in Asia. *Anoplophora chinensis* is a damaging pest of many deciduous trees, especially of fruit trees. Its first discovering outside Asia was in 2000, during an outbreak happening in Lombardy, in northern Italy, and since that moment many other outbreaks have been discovered in Italy and Europe (FAO, 2014). Another example of invasive longhorn beetle associated with trading of plants is *Oemona hirta* (Fig. 7), the lemon tree borer, native to New Zealand. Its first detection was in the United Kingdom once in 1983 and in two nurseries in 2010; it's not known to be established elsewhere (EPPO, 2013).

Figure 6 Adult of *Anoplophora chinensis* (Source: https://www4.ti.ch/dfe/de/servizio-fitosanitario/organismi/tarlo-asiatico-delle-radici-anoplophora-chinensis)

Figure 7 Adult of *Oemona hirta* (Source: https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.37124)

The second important pathway of introduction for invasive longhorn beetles is represented by movement and use of wood packaging materials (Fig. 8): usually, they're made of raw wood of poor quality and sometimes not treated enough to remove or kill pests which may be already present in the material itself (IPPC, 2009). A link seems to exist between heavy commodities and interceptions of longhorn beetles: for such heavy materials, thickness of wood required to support them is much higher and this wood is then more likely to host insects and, because of its dimensions, it's more difficult to treat it with heat or fumigants. Another reason for this relationship between heavy commodities and longhorn beetle interceptions is that wood is of a very low quality, as mentioned before, so quite often it can harbour a higher quantity of larvae (John Morgan, Forestry Commission, UK, personal communication). When pests were identified to genus and/or species level, half of them were represented by *Anoplophora glabripennis* (57 interceptions out of 181). The second most intercepted species was *Apriona germari* (Fig. 11) (Linsley et al., 2010). According to EPPO, *A. germari* represents a major risk to Mediterranean area, southeast Europe, northern Turkey and oceanic lands of southwest Europe (EPPO 2014). In the period between 1984 and 2008, about 3500 cerambycid interceptions related to wood products and packaging were found at U.S. points of entry. Among them, one of the most intercepted genus was *Xylotrechus* (Haack et al., 2014).

Figure 8 Pallet infested by wood borer beetles; the holes on the wood are visible signs of infestation (Source: Biebl, S. and Querner, P., 2020, photo by P. Querner)

The third introduction pathway worthy to be mentioned is the one related to finished wood products. Normally, risks associated with finished products are low because of the processing operations which take place during production. Risk is reduced also because these woody products are less likely to be stored outside, so emerging pests have less possibilities to reach host plants, and because products are quickly dispersed, rather being stored together in one location, so founder population will be reduced. For all these reasons, longhorn beetles intercepted in association with finished wood products are rare. However, in recent years (in the period between 2000 and 2010) many interceptions of longhorn beetles have been related to manufactured wood products, since they are not "controlled goods" in the EU (Cocquempot and Lindelow, 2010). Among the species detected there are *Chlorophorus annularis* and *Trichoferus campestris*. An interesting case is represented by a third species, the brown fir longhorn beetle, also known as *Callidielum villosulum* (Fig. 9). This species is native to southeastern China and Taiwan (Cocquempot and Mifsud, 2013). In April 2013 this longhorn beetle was found in Malta on wooden commodities imported from China and arrived via Italy. In 2012 the same organism was found in Canada on logs with bark attached that were used as bases for artificial Christmas trees (Burleigh, 2013).

Figure 9 Adult of *Callidielum villosulum* (Source: https://www.biolib.cz/en/image/id319868/, photo by S. Valley)

Fourth and last pathway of introduction for longhorn beetles in non-native areas is related to timber not used for packaging. In the period between 1998 and 2013, on EUROPHYT 73 interceptions of Cerambycidae were recorded in association with woody products or bark. EUROPHYT is a phytosanitary databases with recordings of plant pests and pathogens provided by member states and Switzerland for European Union. The most present species in these interceptions was *Monochamus* spp. For example, *M. galloprovincialis* (Fig. 10) was found in Turkey, in 2011, on industrial wood imported by Ukraine (Bozkurt et al., 2013).

Figure 10 Adult of *M. galloprovincialis* (Source: https://plantwiseplusknowledgebank.org/doi/10.1079/PWKB.Species.34722)

Between 2002 and 2005 a survey was performed by Ostrauskas and Tamutis (2012) on temporary site for storing timber and wood coming from Russia into Lithuania by using baited multi-funnel traps: at the end, seventeen species of longhorn beetles, all native to Europe, were found. In a similar way, four species of Cerambycidae were detected in northern Sweden on timber coming from Siberia: these species were *Tetropium gabrieli*, *Tetropium aquilonium*, *M. urrosovi* and *Acanthocinus griseus*; however, none of these species was considered as invasive, since they're native, but rare, in Sweden itself (Lundberg and Petersson, 1997).

Understanding how pest longhorn beetles can travel and their routes is an important step in the assessment of potential risks for the importing country.

Irrespective of the introduction pathway, the prevention of longhorn beetle establishment through efficient early-detection represent a fundamental part of surveillance programs in any country around the world (Haack et al., 1997).

1.3 Baited traps as key tool for early detection

As trades increase continuously worldwide, risks of expansion of native species and of introduction non-native insect species outside of their natural range increase as well (Brockerhoff and Liebhold, 2017). As mentioned before, longhorn beetles are usually moved in association with ornamental plants and/or woody materials at larval stage (Wu et al., 2017; Meurisse et al., 2019), making their visual detection complicated. At the same time longhorn beetles are able to cause significant damages both at ecological and economic level (Haack, 2017) and eradication campaigns are usually very costly (Haack et al., 2010; Faccoli and Gatto, 2015). This leads to the need of having effective tools for early detection of these insects to increase the chance of intercepting introduced species before they can actually establish in the new area: this may lead to a significant reduction, or even prevention, in damages caused by insects and in total costs incurred.

Among the tools already available for detecting longhorn beetles, traps baited with pheromones and kairomones placed at and nearby entry points result to be one of the most used and effective approach in intercepting longhorn beetles (Eyre and Haack, 2017). They are cheap and easy to use, and they benefit from the newest discoveries regarding longhorn beetle chemical ecology (Hanks and Millar, 2016; Millar and Hanks, 2017) and factors that improve trapping efficacy (Allison and Redak, 2017). For many species, sex and aggregation-sex pheromones have already been discovered (Millar and Hanks, 2017); similarly, the effects linked to type of traps (Graham et al., 2012) and to their design (Allison et al., 2014), traps' coating (Graham and Poland, 2012) and position of traps considering height above ground (Flaherty et al., 2019; Ulyshen and Sheehan, 2019; Miller et al., 2020) and distance from edge of forests (Allison et al, 2019) have been described. Thus, species-specific and generic protocols s for surveillance of longhorn beetles have been described and are available (Poland and Rassati, 2019).

On the contrary, the understanding of whether the effectiveness of traps in capturing longhorn beetles can be improved by exploiting trap colours or other visual stimuli is less exploited. Normally, traps used for surveillance and interception of cerambycids are black (Brockerhoff et al., 2006; Bashford, 2008; Rassati et al., 2015; Canadian Food Inspection Agency, 2017): this happens because black traps can resemble the aspect, in terms of shape and colour, of host plant bark. However, it's known that visual stimuli are important cues that are normally exploited by longhorn beetles to locate not only feeding substrate, but also to spot mates (Monnè et al., 2017). For this reason, the exploitation of different trap colours to attract longhorn beetles has been recently explored as a potential way to increase trap attractiveness. Nonetheless, most of the studies carried out in the past were limited to

part of colour spectrum or to single longhorn beetle species (Braman et al., 2023; Campbell and Han-ula, 2007; Shipman, 2011; Skvarla and Holland, 2011; Skvarla and Dowling, 2017; Rassati et al., 2019; Imrei et al., 2014; Toshova et al., 2016; Kerr et al., 2017). Cavaletto et al. (2020), instead, recently carried out a study targeting a wide range of species. More specifically, they tested whether trap colour could affect species richness and abundance (respectively, number of species and number of individuals per species): in particular, comparisons between trap colours were made following two criteria. The first one based on taxonomy (intended as family, subfamily and species level), since a study published before demonstrated how colour is able to differently influence longhorn beetles at subfamily and species level (Rassati et al., 2019); the second criterion considered was based on bio-logical bases, so flower-visiting species were expected to prefer traps with flower colour, while non flower-visiting species were thought to choose black traps. Results showed that, both at family and subfamily level, trap colour was able to affect significantly both richness and abundance of captured longhorn beetles. Going more in depth, then, an influence of trap colour on species richness of flower-visiting and non-flower-visiting beetles was highlighted, despite effects were quite the opposite: flower-visiting species preferred traps coloured similarly to flowers more, while, on the other side, black traps were chosen more by species not living on flowers. Similarly, abundance was affected in the same way in both these two groups of species.

So, as it was said at the beginning, despite surveillance programs usually rely on traps baited with hormones and host volatiles nearby entry points, and traps are almost exclusively black, it's been demonstrated how colours in traps have an important role in attracting longhorn beetles, with a dif-ferent sensitivity at subfamily and species level, considering both species richness and abundance: for example, Lepturinae, which are mostly diurnal and flower-visitor species (Monnè et al., 2017), had a preference for flower-associated colours; on the contrary, Lamiinae comprehend mostly noc-turnal and crepuscular species which don't live on flowers (Monnè et al, 2017): results demonstrated that species belonging to this subfamily were more attracted to dark colours of traps; finally, Ceram-bycinae include both diurnal and nocturnal/crepuscular species and, with this study, no clear pattern of choice was discovered. With the research carried out by Cavaletto et al., it was demonstrated that longhorn beetles are able to distinguish colours and this ability is then used to identify both feeding substrates and mates (Johnson et al, 2019). What is still unknown, however, is whether other visual stimuli beside colour can be used to further increase the attractiveness. For example, the reproduction on traps of certain visual cue present on beetle elytra might lead to important improvements, but this remains to be tested.

2.   OBJECTIVES OF THE WORK

In this thesis, the aim was to test the use of particular and innovative traps baited with pheromones and integrated with visual stimuli for improving trapping protocols for three main target species: *Neoclytus acuminatus*, *Xylotrechus antilope* and *Xylotrechus stebbingi*. Together with them, other non-target species belonging to the subfamilies of Lepturinae, Cerambycinae and Lamiinae were taken into consideration. The main purpose was to understand if abundance of beetles in traps may be improved by integrating species-specific visual stimuli on traps. Visual stimuli included: i) black background, ii) yellow background, iii) black background with yellow dots, iv) black background with silhouette of species' elytra coloured in yellow, v) black background with real image of target species.

3. MATERIALS AND METHODS

3.1 Study areas

The first two locations where the study has been conducted are Teolo and Baone, two municipalities in the province of Padova and belonging to the Regional Park of Euganean Hills, in Veneto (Figs. 11 and 12). In particular, Teolo is located on the northern side of the Park, while Baone is located on the southern part.

The Regional Park of Euganean Hills extends for about 18,694 hectares of land and it was created in 1989 with the aim of protecting this area from a geomorphological, faunistic and cultural point of view. This Park is inhabited since Palaeolithic era and it's characterized by a volcanic substrate, formed about 35 million of years ago. A considerably high number of plant species can be found in Euganean Hills: geology of soil, shape of terrain (responsible for creating different microclimates and biotopes) and isolation from other mountain ranges are the main reasons why Euganean Hills can boast such a big range of flora. Here, it's possible to find species used to live in mountain areas existing side by side with species adapted to hot climates: it's possible to find Oak (*Quercus pubescens*) mixed with European hop hornbeam (*Ostrya carpinifolia*) and Ash (*Fraxinus ornus*), chestnut stands (*Castanea sativa*) which host beech trees (*Fagus sylvatica*), but also black locust tree forests (*Robinia pseudoacacia*) and pseudo-Mediterranean scrubs made of shrubs, most of which evergreen, like holm oak (*Quercus ilex*), broom (*Spartium junceum*) and wild asparagus (*Asparagus acutifolius*).

A part of the Park, in particular 13,699 hectares (~73%), represents a site of Natura 2000 Network, as some priority habitats and animal species at European level are host in this zone (Dir. 92/43 and 09/147). Considering priority habitats and flora, it's possible to find "*semi-natural dry grasslands and scrubland facies on calcareous substrates*" which host flowering wild orchids, also known as dry grasslands; "*Rupicolous calcare-ous or basophilic grasslands of the Alysso-Sedion albi*"; "Pannonian woods with *Quercus pubescens* (white oak)". (Source: https://www.parcocollieuganei.com/Eindex.php)
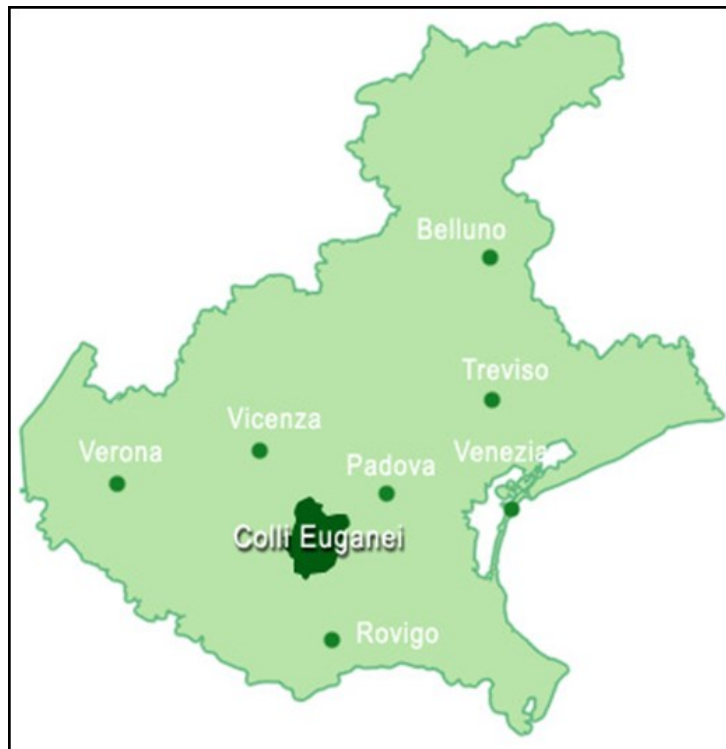
Figure 11 Map of Regional Park of Euganean Hills in Veneto (Source: https://www.collieuganei.it/dove-si-trovano/)
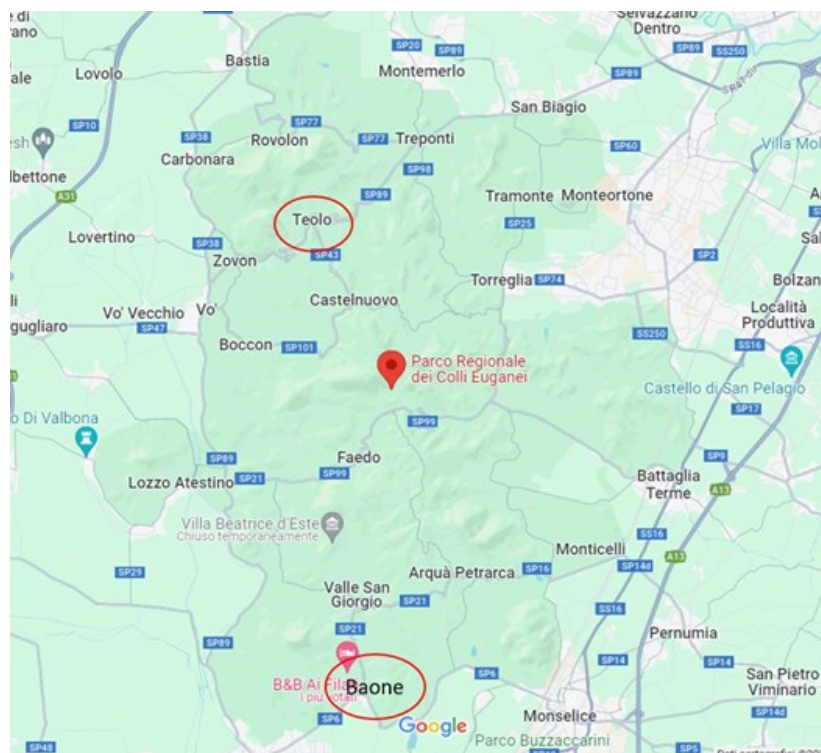

Figure 12 Detailed map of Regional Park of Euganean Hills, where it's possible to locate the municipalities of Teolo and Baone (Source: Google Maps)

The third study area were the study has been carried out was Bosco Nordio, a full reserve located in the municipality of Chioggia, in Venice province. Bosco Nordio extends for 113 hectares on the most ancient sand dunes of the whole north Adriatic Sea. These dunes were formed about 2,000 years ago thanks to sediments transported by Po and Adige rivers; the forest, on the contrary, was born more or less 1,000 years ago. Because of its development over the years and its peculiar position, Bosco Nordio hosts a series of important and relevant habitat types and species of flora and fauna: for this reason, it's become a site of Natura 2000 Network. The particular morphology of soil, made of a series of dune cords and a shallow aquifer, is able to affect deeply the forest composition: in the higher and drier parts of the dunes, it's possible the growth of holm oak (*Quercus ilex*), while in the humid infra-dune depressions we assist to the development of English oak (*Quercus robur*). Together with these species, it's also possible to find ash (*Fraxinus ornus*) and poplar (*Populus nigra* and *Populus alba*). With regards to pinewood, it was once quite diffused, but nowadays only a few groups composed of big trees have remained (*Pinus pinaster* and *Pinus domestica*). A relevant feature for the richness of biodiversity is the presence of open areas without any forest: these zones are called "grey dunes". Here, it's possible to find shrub species typical of the coast and adapted to sandy, hot and dry soils: some lichens of the genus *Cladonia*, a few mosses and herbs; in the transition zone between grey dunes and forest, a peculiar environment called "coastal dunes with juniper" develops, characterised by species typical of Mediterranean scrub, for example juniper (*Juniperus communis*) and wild asparagus (*Asparagus acutifolius*). However, this unique environment risks to disappear, as forest advances and tend to occupy open areas, despite their presence is fundamental to the existence of certain animal species, like nightjar (*Caprimulgus europaeus*). In Bosco Nordio, thanks to many interventions of environmental re-compositions, more or less 15 ponds have been created with the aim of maximising biodiversity and differentiating habitats. Along the banks of these ponds, species like willow (*Salix cinerea*, *Salix alba*, *Salix rosmarinifolia*), poplar (*Populus nigra* and *Populus alba*) grow, together with a series of plants listed as rare at national and regional levels: Hibiscus (*Hibiscus pentacarpos*), wild celery (*Sium latifolium*) and marsh plantain (*Plantago altissima*) (Veneto Agricoltura, 2024).

3.2   Experimental design

In all the three sites chosen as study areas, an experimental design with random blocks has been used. The five tested treatments corresponding to five different visual stimuli were positioned randomly in six blocks. Thus, a total of 30 traps have been used for each study area. A different longhorn beetle species was targeted in the three different study area, namely *Xylotrechus stebbingi* for Teolo, *Xylotrechus antilope* for Baone, and *Neoclytus acuminatus* for Bosco Nordio. The position of the six blocks in each study site is shown in Figure 13.
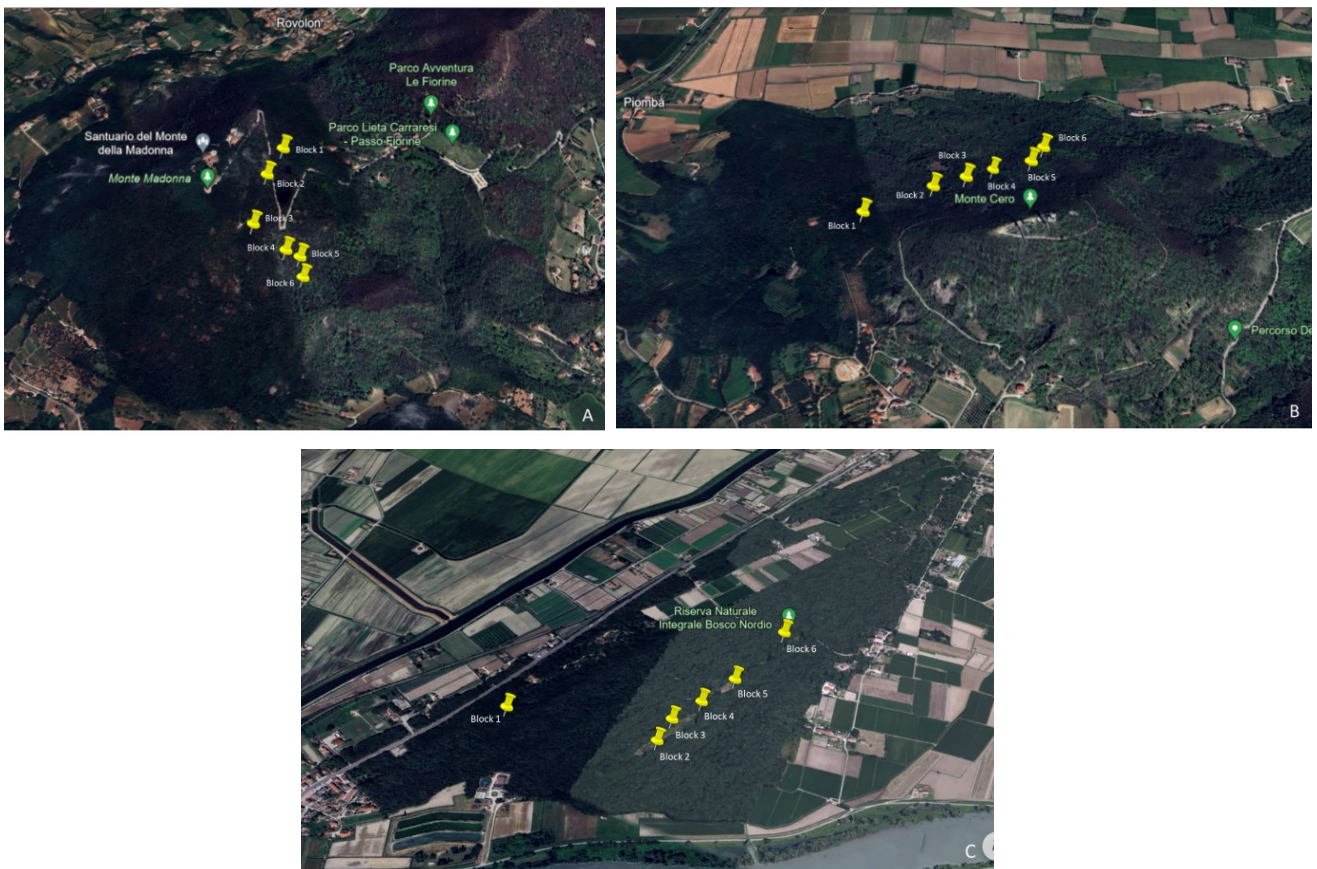

Figure 13 Maps with representation of blocks' organization within each study area: A) Teolo; B) Baone; C) Bosco Nordio

Within each block, traps were set at a height between 8 and 15 m above the ground on the most suitable trees irrespective of the tree species (Fig. 14).

Figure 14 Traps installed on trees at the canopy level

3.3   Trap type

Traps used to carry out this study were panel-traps (also known as crossvane traps) (Wita Prall Crossvane panel), produced by the Austrian company Witasek PflanzenSchutz GmbH. These traps are made of a plastic lid, a bottom with square opening, a main part with a length of about 80 centimetres to which the box containing the attractants is fixed, a cylindrical container to collect the insects, and a rope for suspending the whole apparatus (Fig. 15).

Figure 15 Traps used for the study. In this picture the five treatments used to target *Xylotrechus stebbingi* are shown.

The capture container was of "wet" type, meaning that it was filled with propylene glycol which kill and preserve trapped specimens without having any attractive or repellent effect towards the target insects.

An identification code is assigned to each trap with information about: study area, sampling period, target species, block and treatment.

3.4   Attractants and visual stimuli used

Traps were baited with a mix of two substances: the first one was represented by one or more than one component of sexual pheromones released by each target species, while the second was ethanol, a highly volatile alcohol, usually produced by stressed plants and used by longhorn beetles to identify hosts. These substances were bought at Synergy Semiochemicals (Canada) and they were released through a heat-sealed polyethylene box fixed to the trap (Fig. 16).

Figure 16 Heat-sealed polyethylene box, fixed to the trap, releasing attractants.

Considering a temperature of 20°C, the release rate of the substance is estimated by the producer as 1-2 mg/day. The study lasted 11 weeks and only a replacement of attractants was performed at the fifth week.

Considering the visual stimuli used, five treatments were chosen and they're shown in Figure 17: i) black background, ii) yellow background, iii) black background with yellow dots, iv) black background with the shape and colour of stripes present on the elytra, v) black background with realistic representation of the target species (Fig. 17A: *Neoclytus acuminatus*; Fig. 17B: *Xylotrechus antilope*; Fig. 17C: *Xylotrechus stebbingi*).
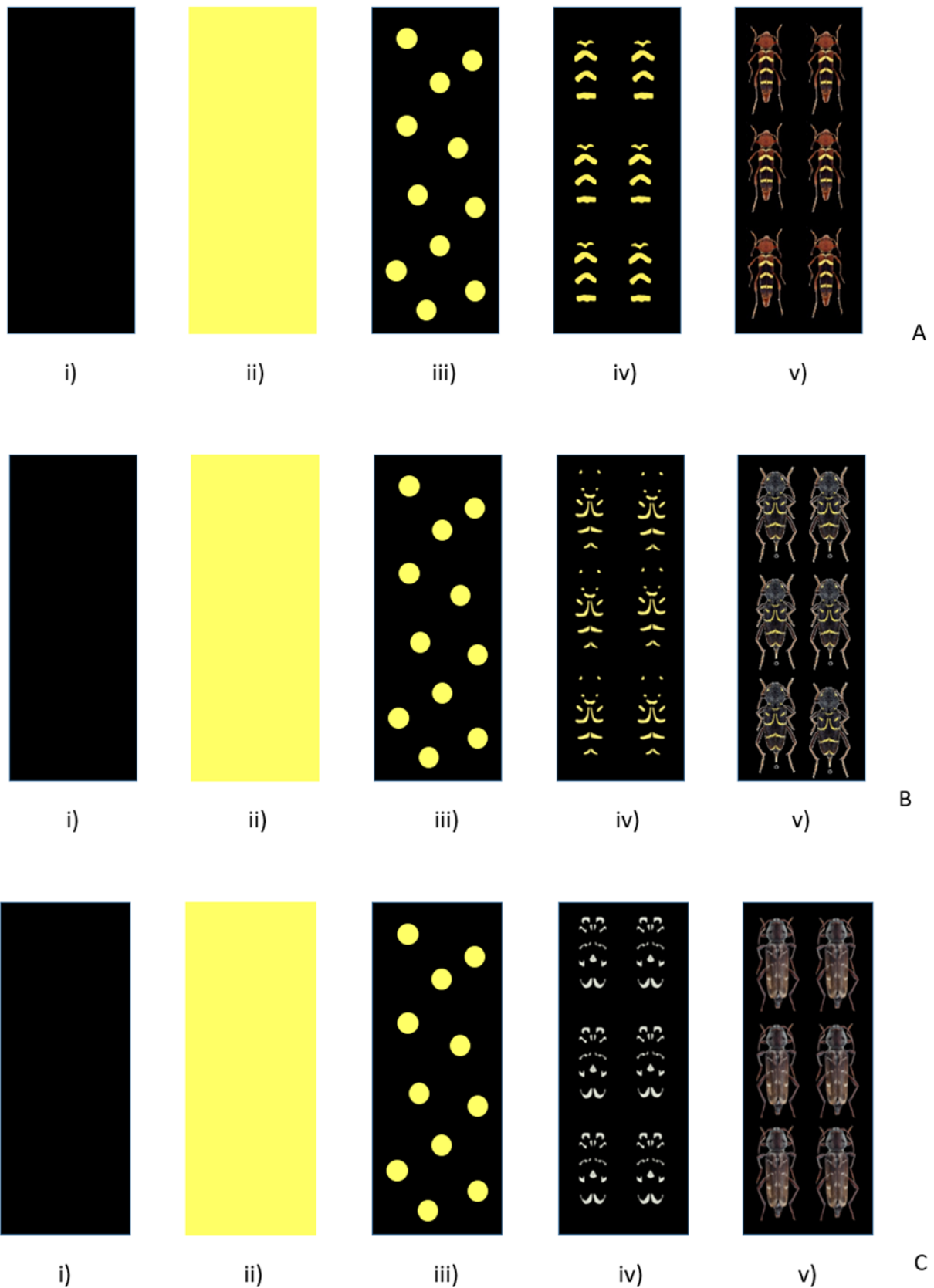
Figure 17 Visual stimuli used on traps respectively for A) *Neoclytus acuminatus*, B) *Xylotrechus antilope*, C) *Xylotrechus stebbingi*: i) black background; ii) yellow background; iii) black background with yellow dots; iv) black background with silhouette of target species elytra coloured in yellow; v) black background with realistic image of target species

3.5 Trap checks and collection of samples

Traps were exposed from the beginning of May to the beginning of August and four trap checks were performed in this period. Check and collection of samples were repeated every three weeks at the end of each sampling period. In Table 1 a summary of the entire sampling time, for all the target species considered, is reported: for each species, the beginning and the ending of single sampling periods are indicated. In the first column, each period is indicated with letter "T" (which indicates the "time") and the progressive number of sampling.

Table 1 Summary of the entire sampling time for all the target species, with indications about starting and ending of each single period.

	Xylotrechus stebbingi		Xylotrechus antilope		Neoclytus acuminatus	
	start	end	start	end	start	end
Period 1 (T1)	17/05/2023	06/06/2023	19/05/2023	06/06/2023	15/05/2023	07/06/2023
Period 2 (T2)	07/06/2023	21/06/2023	07/06/2023	22/06/2023	08/06/2023	23/06/2023
Period 3 (T3)	22/06/2023	11/07/2023	23/06/2023	12/07/2023	24/06/2023	13/07/2023
Period 4 (T4)	12/07/2023	01/08/2023	13/07/2023	02/08/2023	14/07/2023	03/08/2023

During each collection, containers were emptied and insects were put inside plastic vials filled with alcohol 70%, which were singly labelled with information about day of collection and the unique code of each trap. After that, the trap collector cup was refilled with propylene glycol.

3.6 Species identification in lab

Collected material was brought to the lab, in the DAFNAE Department in Padova (Agripolis) for sorting. Cerambycids were divided from all the other types of insects. Then individuals belonging to both target and non-target species were (Fig. 18) identified to species and counted. Finally, information collected was put in an Excel sheet to perform the statistical analysis.

Figure 18 Sorting of collected insects

3.7 Statistical analysis

The effect of visual stimuli was tested using generalized linear mixed models with a negative binomial distribution (log link function). For each of the three sites, a model was built for the target species and the other three most abundant species. The abundance of each species was considered as response variable. The treatment (i.e., visual stimuli) was considered as categorical explanatory variable (five levels). The block identity was included in the models as a random factor. For each model, the black trap ("BL") was used as a baseline for the other four treatments. Data collected from each trap and pooled over the sampling rounds were treated as a distinct statistical unit. All the analyses were carried out in R software (R Core Team, 2021).

4.   RESULTS

4.1   General results

During the entire sampling period and considering the three different sites, a total of 46 long-horn beetle species were captured. A total of 38 species were collected in Teolo. Among them, the four most abundant species were *X. stebbingi*, the target species, with 1,434 individuals, *X. antilope* with 1,213 individuals, *T. pallidus* with 547 individuals and *P. testaceus* with 407 individuals. In Baone, 39 species were collected. *X. antilope* was the target species, with 491 insects. The other three most abundant species were *X. stebbingi* with 1,729 individuals, *C. sartor* with 432 individuals, and *P. testaceus* with 277 individuals. In Chioggia, a total of 22 species were collected. The target species was *N. acuminatus*, with 569 insects. The other three most abundant species were *T. pallidus* with 113 individuals, *P. testaceus* with 84 and *X. antilope* with 53 individuals.

4.2 Effect of visual stimuli on target and non-target species at each site

4.2.1 *Xylotrechus stebbingi*

At Teolo site, no significant effect of the tested visual stimuli was observed for the target species *X. stebbingi*. In particular, the number of individuals collected in black traps (52,83 ± 11,35) did not differ form the number of individuals collected by the other tested treatments (Fig. 19A). A similar non-significant trend was observed for the non-target species *X. antilope* (Fig. 19B), *T. pallidus* (Fig. 19C), and *P. testaceus* (Fig. 19D).
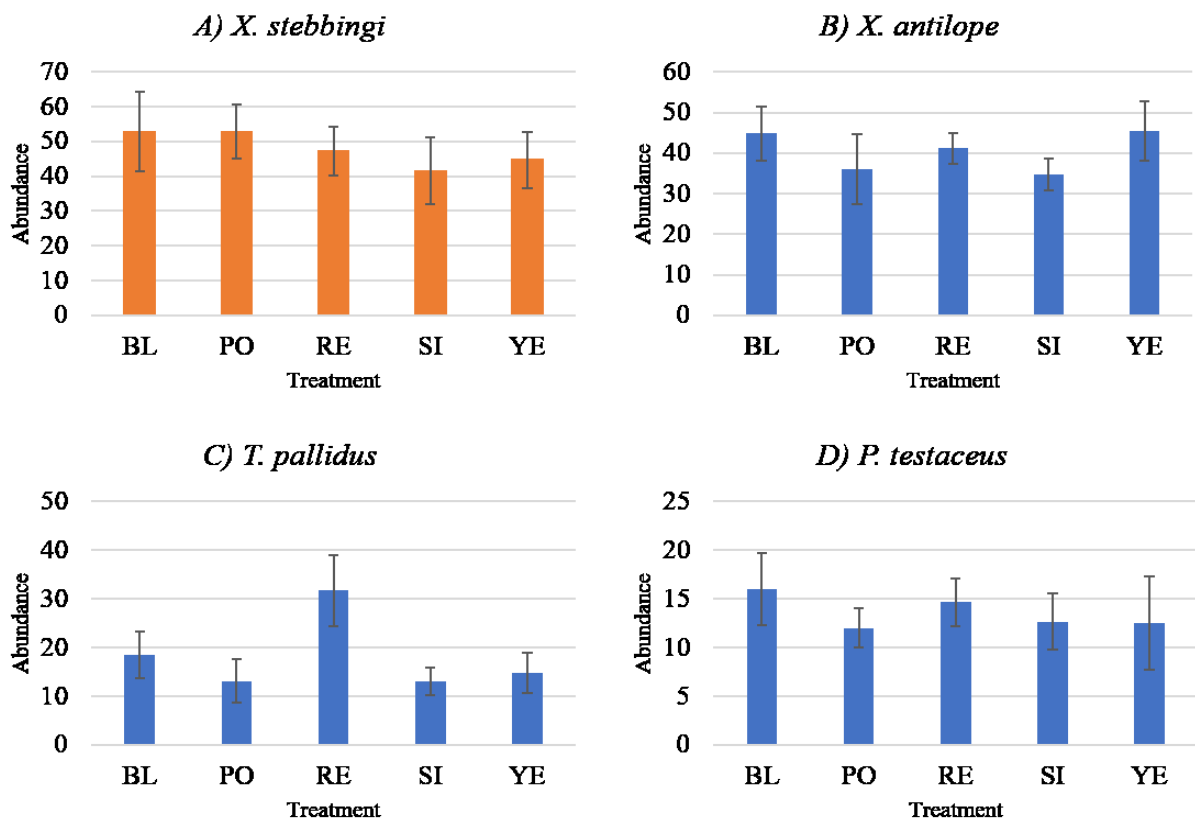

Figure 19 Mean ± Standard error of catches for A) *X. stebbingi* (target species); B) *X. antilope*; C) *T. pallidus*, D) *P. testaceus*. (BL = black; PO = pois, with yellow dots; RE = real; SI = silhouette; YE = yellow). P-values: * = 0.01 - 0.05; ** = 0.001 - 0.01; *** = < 0.001 (statistical differences referred to BL)

4.2.2 *Xylotrechus antilope*

In the experiment targeting *X. antilope* carried out in Baone we found a significant effect of the tested visual stimuli. In particular, traps that were completely yellow (24,50 ± 5,57) caught significantly more individuals than the commonly used black traps (12,67 ± 2,04), whereas there was no difference between the latter black traps and the other tested treatments (Fig. 20A). A significant effect was observed also for the non-target species *C. sartor* (Fig. 20B) and *P. testaceus* (Fig. 20C), but not for the non-target species *X. stebbingi* (Fig. 20D), for which no significant difference was found for any of the tested treatment. In particular, for *C. sartor* completely yellow traps caught much more individuals (67,83 ± 24,90) than black traps (1,50 ± 0,92). In the case of *P. testaceus* (Fig. 20C), the silhouette treatment (11,67 ± 1,48) and the real treatment (12,50 ± 3,68) caught significantly more species than black one (5,67 ± 0,88).
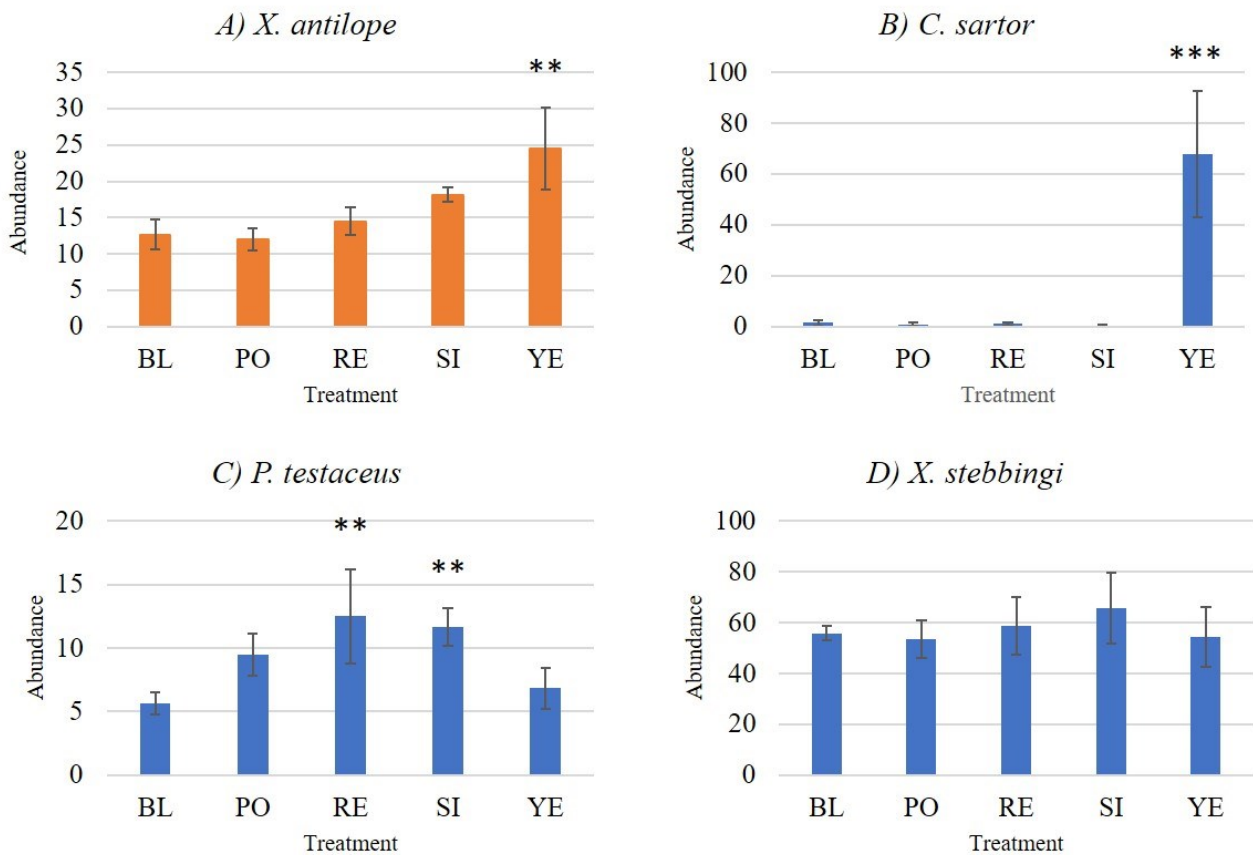

Figure 20 Mean ± Standard error of catches for A) *X. antilope* (target species); B) *C. sartor*; C) *P. testaceus*, D) *X. stebbingi*. (BL = black; PO = pois, with yellow dots; RE = real; SI = silhouette; YE = yellow)
P-values: * = 0.01 - 0.05; ** = 0.001 - 0.01; *** = < 0.001 (statistical differences referred to BL)

4.2.3 *Neoclytus acuminatus*

In the experiment targeting *N. acuminatus* carried out in Chioggia we found a significant effect of the tested visual stimuli. In particular, the silhouette treatment caught significantly more individuals (25,67 ± 4,26) than black traps (16,17 ± 4,39), whereas there was no difference between the other tested treatments and the latter black traps (Fig. 20A). Considering the non-target species, a significant effect of the tested visual stimuli was found for *X. antilope* (Fig. 21B) but not for *T. pallidus* (Fig. 21C) and *P.testaceus* (Fig. 21D). In particular, for *X. antilope,* completely yellow traps (2,83 ± 0,87) and black traps with yellow dots (2,17 ± 0,48) caught significantly more individuals than black traps (0,67 ± 0,33).
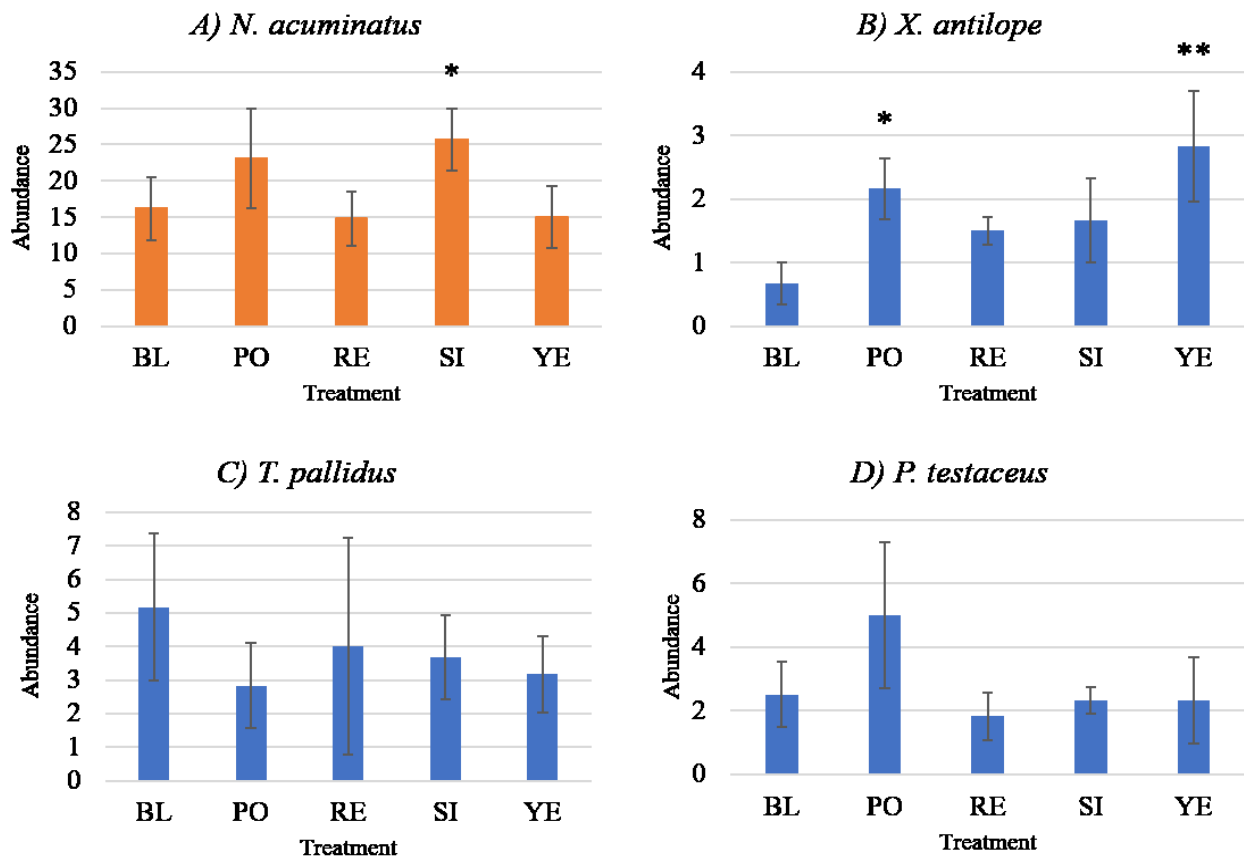

Figure 21 Mean ± Standard error of catches for A) *N. acuminatus* (target species); B) *X. antilope*; C) *T. pallidus*, D) *P. testaceus*. (BL = black; PO = pois, with yellow dots; RE = real; SI = silhouette; YE = yellow)

P-values: * = 0.01 - 0.05; ** = 0.001 - 0.01; *** = < 0.001 (statistical differences referred to BL)

5. DISCUSSION AND CONCLUSIONS

Surveillance programs used for longhorn beetles commonly rely on the use of baited traps located at or nearby entry points, as this approach resulted to be one of the most efficient in intercepting specimens of exotic species introduced via international trade (Poland and Rassati, 2019). Most of the surveillance programs carried out so far have relied on black traps resembling the bark of standing trees, as this colour was assessed to be the most efficient when compared with white or light-coloured traps in previous studies. Recent publications, however, clearly highlighted that other trap colours can be more attractive than black, especially for flower-visiting longhorn beetle species or for species displaying particular colours or particular configurations on their elytra (Cavaletto et al., 2021). In fact, a longhorn beetles can rely on the exploitation of some visual stimuli to detect not only feeding substrates, but also their mates. However, no study investigated so far whether the application of particular visual stimuli to baited traps can improve their efficiency in capturing longhorn beetles. Results obtained in our study showed that it is indeed possible, as depending on the species certain visual stimuli allowed to catch more individuals compared to the commonly used black traps. The observed patterns appears to be linked to the ecology of the species, as well as feeding and mating habits, despite it was not possible a clear explanation to be found for all the species analyzed.

Regarding the species targeted in this study, *X. stebbingi* did not show any particular preference for the visual stimuli we offered on the traps. In other words, black traps caught a similar number of individuals compared to the other traps. This is however not surprising, given that *X. stebbingi* is a non-flower visiting species and it is characterized by a dark coloration (brownish-greyish) which likely does not represent a visual stimulus used during mate finding but only as a way to camouflage in the environment. The lack of any preference for any of the trap treatment observed in Baone (where the target species was *X. antilope*) reinforces the latter idea. On the contrary, *X. antilope* resulted to be significantly more attracted by yellow-coloured traps than black traps. This species is diurnal, with its maximum activity in the late morning to early afternoon (Molander et al., 2019), so it's reasonable to think that it exploits a chromatic visual channel to move in the environment. However, it is not a flower visiting species and the clear preference shown for yellow, which confirms results obtained also in previous studies (Cavaletto et al. 2021) may be linked to the yellow stripes present on the elytra, thus suggesting a mechanism involved in locating partners. In the latter case, the totally yellow traps might have acted as a super-stimulus, explaining why the species preferred totally-yellow vs. traps displaying the yellow stripes (i.e., silhouette) or the yellow-dots. Trends observed for this species at the sites where the latter was not the main target support this hypothesis. At the site where *X. stebbingi* was the target species (i.e., where there were not totally yellow traps but instead greyish

traps) the lack of any trend was observed. At the site where *N. acuminatus* was the target species, *X. antilope* resulted to be attracted more to totally yellow traps or black traps with yellow dots ("pois" treatment) than black traps. Finally, *N. acuminatus* resulted to prefer traps displaying the stripes present on the beetle elytra rather than black traps. Also *N. acuminatus* is not a flower visiting species active during the day, and the latter trend might be linked to the use of the visual pattern present on the elytra of both males and females as cue during mate searching, especially at short distance.

Regarding non-target species collected at the different sites, we observed various trends. *T. pallidus* was found as non-target species both in Teolo (where *X. stebbingi* was the target species) and Chioggia (where *N. acuminatus* was the target species), and in both cases the observed behaviour was exactly the same. Individuals of *T. pallidus* didn't show any preference. As for *X. stebbingi*, *T. pallidus* is a non-flower visitor and it appears to be mostly active during the night. In addition, it's body is brownish with some white stripes, and it is very likely that it does not use colour vision much during its life cycle. *P. testaceus* was found in all the three sites, but its behaviour resulted to be peculiar and not easy to explain. Despite in two of the three sites no difference was observed among the traps, at the site where *X. antilope* was the target species *P. testaceus* showed a preference for traps displaying the pattern and colour of the stripes present on *X. antilope* elytra and for traps displaying the picture of *X. antilope* adults rather than for black traps. *Phymatodes testaceus* is a crepuscular and nocturnal species, thus the preference for the latter visual stimuli is difficult to explain and requires further studies. Finally, *C. sartor* found at Baone site (where *X. antilope* was the target species) showed a clear preference for yellow traps over black traps. This is probably linked to the ecology of the species, which is a flower-visitor, and thus likely attracted to the colour indicating the feeding sites (i.e., flowers).

In conclusion, with this work it was possible to demonstrate that the inclusion of visual stimuli on baited traps represent a valid approach to significantly improve the efficacy of traps commonly used in surveillance programs. Different species were attracted to different visual stimuli or showed no preference pattern, often in line with their ecological habits: this is another fundamental element to be exploited to increase the capacity of traps towards particular species by using species-specific stimuli, especially in the context of surveillance for the early detection of non-native and exotic species, before they can establish and colonise a new environment, thus avoiding possible damages to local biodiversity. Future studied are needed to investigate more in depth the extent to which longhorn beetles exploit species-specific visual cues and possible differences existing between males and females.

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ACKNOWLEDGMENTS

At the end of this journey, thanking everyone is a duty but also difficult, because so many people accompanied me and deserve to be mentioned here.

First of all, a huge thank you goes to my supervisor, Prof. Rassati, for offering me this great opportunity and guiding me from the beginning, in a patient and attentive way which helped me writing this thesis.

Together with him, I also thank my supervisor, Dott. Santoiemma, for his crucial support when elaborating statistical results: without him, it would have been impossible to me.

Moving forward, a huge thank you to Dott. Cavaletto and Dott. Morgante for their help during field trips to collect samples and their subsequent sorting in the lab: thanks to you I learnt a lot and had so much fun, despite my phobia for insects!

A dear thought goes to my beloved friends, Maryna and Piper, with whom I spent two beautiful years full of memories that I will carry with me forever.

Thanks to Chloe, my beloved roommate and study partner, passionate about insects: I know you would be proud of me!

In general, thanks to all my classmates for the good moments spent together, both in the good and bad times. We did it, finally, and you all will always have a place in my heart and in my memories.

My gratitude goes to all my friends, who have been by my side throughout my journey: thank you for listening to me, supporting me and putting up with me.

The biggest thanks goes to my family, my mother, my father, my grandparents and all those who have been close to me, in one way or another: your immense support has been going on for years and has been fundamental for me to overcome obstacles that seemed insurmountable.

A special acknowledgment goes to my omnipresent and beloved Mia, the greatest and most beautiful gift I could ever receive. My little dog who never abandons me and never gives me a minute to breathe, who has always listened to me patiently while I repeated for my exams in all languages... this degree is for you too, little one.

And finally, I want to thank myself for being brave and choosing a path that was anything but easy; I thank myself for never giving up: you overcame all difficulties, you did it.