Pollinator diversity and reproductive success of *Epipactis helleborine* (L.) Crantz (Orchidaceae) in anthropogenic and natural habitats

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Background. *Epipactis helleborine* is an Eurasian orchid species which prefers woodland environments but it may also spontaneously and successfully colonise human-made artificial and disturbed habitats such as roadsides, town parks and gardens. It is suggested that orchids colonising anthropogenic habitats are characterised by a specific set of features (e.g.: large plant size, fast flower production). However, as it is not well known how pollinator diversity and reproductive success of *E. helleborine* differs in populations in anthropogenic habitats compared to populations from natural habitats, we wanted to compare pollinator diversity and reproductive success of this orchid species between natural and anthropogenic habitat types.

Methods. Pollination biology, reproductive success and autogamy in populations of *E. helleborine* from anthropogenic (roadside) and natural (forest) habitats were compared. Eight populations (four natural and four human-disturbed ones) in two seasons were studied according to height of plants, length of inflorescences, as well as numbers of juvenile shoots, flowering shoots, flowers, and fruits. The number and diversity of insect pollinators were studied in one natural and two human-disturbed populations.

Results. Reproductive success (the ratio of the number of flowers to the number of fruits) in the populations from anthropogenic habitats was significantly higher than in the natural habitats. Moreover, plants from anthropogenic habitats were larger than those from natural ones. In both types of populations, the main insect pollinators were Syrphidae, Culicidae, Vespidae, Apidae and Formicidae. With respect to the type of pollinators' mouth-parts, chewing (39%), sponging (34%) and chewing-sucking (20%) pollinators prevailed in anthropogenic habitats. In natural habitats, pollinators with sponging (55%) and chewing mouth-parts (32%) dominated, while chewing-sucking and piercing-sucking insects accounted for 9% and 4% respectively.

Discussion. We suggest that higher reproductive success of *E. helleborine* in the populations from anthropogenic habitats than in the populations from natural habitats may result from a higher number of visits by pollinators and their greater species diversity, but also from the larger size of plants growing in such habitats. Moreover, our data clearly show that *E. helleborine* is an opportunistic species with respect to pollinators, with a wide spectrum of pollinating insects. Summarising, *E. helleborine* is a rare example of orchid species whose current range is not declining. Its ability to make use of anthropogenically altered habitats has allowed its significant spatial range expansion, and even successful colonisation of North America.

1 **Pollinator diversity and reproductive success of *Epipactis helleborine* (L.) Crantz**

2 **(Orchidaceae) in anthropogenic and natural habitats**

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16   **Abstract**

17   **Background.** *Epipactis helleborine* is an Eurasian orchid species which prefers

18   woodland environments but it may also spontaneously and successfully colonise human-made

19   artificial and disturbed habitats such as roadsides, town parks and gardens.

20   It is suggested that orchids colonising anthropogenic habitats are characterised by a specific set

21   of features (e.g.: large plant size, fast flower production). However, as it is not well known how

22   pollinator diversity and reproductive success of *E. helleborine* differs in populations in

23   anthropogenic habitats compared to populations from natural habitats, we wanted to compare

24   pollinator diversity and reproductive success of this orchid species between natural and

25   anthropogenic habitat types.

26   **Methods.** Pollination biology, reproductive success and autogamy in populations of *E.*

27   *helleborine* from anthropogenic (roadside) and natural (forest) habitats were compared.

28   Eight populations (four natural and four human-disturbed ones) in two seasons were studied

29   according to height of plants, length of inflorescences, as well as numbers of juvenile shoots,

30   flowering shoots, flowers, and fruits. The number and diversity of insect pollinators were studied

31   in one natural and two human-disturbed populations.

32   **Results.** Reproductive success (the ratio of the number of flowers to the number of fruits) in the

33   populations from anthropogenic habitats was significantly higher than in the natural habitats.

34   Moreover, plants from anthropogenic habitats were larger than those from natural ones. In both

35   types of populations, the main insect pollinators were Syrphidae, Culicidae, Vespidae, Apidae

36   and Formicidae. With respect to the type of pollinators' mouth-parts, chewing (39%), sponging

37   (34%) and chewing-sucking (20%) pollinators prevailed in anthropogenic habitats. In natural

38   habitats, pollinators with sponging (55%) and chewing mouth-parts (32%) dominated, while

39   chewing-sucking and piercing-sucking insects accounted for 9% and 4% respectively.

40   **Discussion.** We suggest that higher reproductive success of *E. helleborine* in the populations

41   from anthropogenic habitats than in the populations from natural habitats may result from a

42   higher number of visits by pollinators and their greater species diversity, but also from the larger

43   size of plants growing in such habitats. Moreover, our data clearly show that *E. helleborine* is an

44   opportunistic species with respect to pollinators, with a wide spectrum of pollinating insects.

45   Summarising, *E. helleborine* is a rare example of orchid species whose current range is not

46   declining. Its ability to make use of anthropogenically altered habitats has allowed its significant

47   spatial range expansion, and even successful colonisation of North America.

48

49   **Introduction**

50   Orchidaceae is one of the most diverse and species-rich (20 000 - 30 000 species) plant

51   families (Bauman, Kunkele & Lorenz, 2010; Djordjević et al., 2016a), with many species that

52   are seriously endangered and require conservation efforts to maintain their populations.

53   Destruction of natural habitats is causing extinction of many orchid species (Swart &

54   Dixon, 2009). However, some orchid species, especially in temperate regions of Europe and

55   North America, found anthropogenic habitats as suitable as natural ones (Pedersen, Watthana &

56   Srimuang, 2013). A recent study of orchids in Turkey has indicated that graveyards are places

57   where orchid species occur frequently (Löki et al., 2015). In addition, Djordjević et al. (2016b)

58   have noted that *Himantoglossum calcaratum*, *Anacamptis pyramidalis* and *Ophrys* species often

59   grow in habitats along the roads in western Serbia. Moreover, the same authors have shown that

60   *Orchis purpurea* can be an indicator of ruderal habitat type. The most common colonisers of

61 secondary habitats in Central Europe are *Epipactis* and *Dactylorhiza* species (Adamowski, 2004;

62 Adamowski, 2006; Esfeld et al., 2008; Rewicz, Kołodziejek & Jakubska-Busse, 2016).

63 Moreover, Jurkiewicz et al. (2001) noted that populations of *Epipactis atrorubens*, *E. helleborine*

64 and *Dactylorhiza majalis* were observed on several mine tailings in southern Poland.

65 Colonisation of such habitats is fuelled by disturbances of surface soil layers and exposure of

66 deeper layers including bedrock (i.e. quarries where limestone or chalk are exposed).

67 Additionally, the surface layer of soil and vegetation could be destroyed, which weakens the

68 competitive potential of original vegetation (Jakubska-Busse, 2008).Orchids that colonize

69 anthropogenic habitats are characterized by a specific set of features: fast growth resulting in

70 large plant size and fast flower production (Forman et al., 2009).

71   An important aspect of orchid population biology is its unique reproductive system

72 (Machaka-Houri, et al. 2012), which in this case means mass production of very small and light

73 seeds (from 0.31 µg to 24 µg, depending on the species) (Arditti, 1967). However, the high

74 number of seeds does not lead to high recruitment of seedlings. Low reproductive success, which

75 is defined as the ratio of the number of flowers to the number of fruits (Doust & Doust, 1988),

76 may be caused by high level of morphological adaptation of flowers to particular pollinators.

77 More than 60% of all orchid species are pollinated by a single pollinator (Tremblay, 1992;

78 Tremblay et al., 2005).

79   Human disturbance, especially habitat transformation (including its impact on soil,

80 moisture conditions, and changes of the floristic composition of plant communities), is regarded

81 as a principal cause of pollinator decline on a global scale (Goulson, Lye & Darvill, 2008), as

82 well as an important factor directly and indirectly changing the species structure of pollinating

83 insects (Clemente, 2009). Deficiency of suitable pollinators may also be a reason for low

84   reproductive success. Thus, autogamy (self-autonomy) is an alternative way of seed production.

85   A question arises which reproductive system is preferred by orchids rapidly and successfully

86   colonising anthropogenic habitats (Light & MacConaill, 2006). *Epipactis helleborine* can be a

87   suitable model species, as it occurs in both natural and disturbed habitats, is able to undergo both

88   auto- and allogamy, and furthermore, has been successfully established in North America.

89   Ehlers, Olesen & Gren (2002) have investigated the reproductive success of *E. helleborine* in

90   natural habitats, however, there is a lack of knowledge concerning the reproductive success and

91   pollinator diversity of *E. helleborine* in relation to certain types of natural and anthropogenic

92   habitats. Moreover, in the literature there are no detailed data about the diversity of insect

93   pollinators of *E. helleborine* in relation to the type of their mouth-parts. Such information is

94   important as some types of mouth-parts allow insects to collect food from morphologically

95   different types of flowers, as a result such insects can be pollinators of many taxonomically

96   different plant groups. In addition, insects with specialised mouth-parts can collect food only

97   from one or two morphologically different types of flowers, which means that they can only be

98   pollinators of a narrow number of plants (Gilliot 2005). Some orchid species have flowers which

99   are morphologically adapted for nectar and pollen collecting by insects with different types of

100 mouth-parts, while other can be pollinated only by insects with one, sometimes strongly

101 specialised, type of mouth-parts.

102   In this study, we compared the reproductive success and pollinator diversity of *E.*

103 *helleborine* from natural and anthropogenic habitats. Specifically, we addressed the following

104 questions: a) what is the composition of and the differences in the pollinator fauna of *E.*

105 *helleborine* from anthropogenic and natural habitats in terms of insect diversity and diversity of

106 insect mouth-parts? b) does the number of capsules produced through autogamy and natural

107 pollination differ between populations from anthropogenic and natural habitats? c) is the

108 reproductive success of *E. helleborine* different in populations from anthropogenic and natural

109 habitats?

Materials and methods

Studied species and study area

112 *Epipactis helleborine* (L.) Crantz (broad-leaved helleborine) is a Eurasian species

113 (Kolanowska, 2013) introduced in the 19th century (Owen, 1879) to several regions of North

114 America (Procházka & Velísek, 1983). It usually grows in deciduous and coniferous forest

115 communities, on edges and in clearings in woodland, up to 2 000 m a.s.l. (Delforge, 2006).

116 Furthermore, this species inhabits different types of anthropogenic habitats such as: roadsides,

117 cemeteries, poplar plantations, gravel pits, quarries, railway embankments, and mine tailings

118 (Świercz, 2004; Świercz, 2006; Kiedrzyński & Stefaniak, 2011), and may also appear

119 spontaneously in urban areas such as town parks and gardens (Kolanowska, 2013). Some studies

120 have shown that *E. helleborine* is a species with a broad ecological tolerance, which is not highly

121 specialised, and often acts as a pioneer in human-disturbed areas (Piękoś-Mirkowa & Mirek,

122 2006; Tsiftsis et al., 2008). *Epipactis helleborine* is thus a fine example of apophytism – i.e. a

123 native species growing in disturbed or human-made habitats (Sukopp, 2006; Rewicz et al.,

124 2015). Orchids that colonise anthropogenic habitats are characterised by a specific set of

125 features: fast growth resulting in large plant size and fast flower production (Forman et al.,

126 2009).

127 We accepted the definition of reproductive success after Doust & Doust (1988) as the

128 ratio of the number of flowers to the number of fruits. All shoots found in each population were

129 measured. The following parameters were recorded for each plant in a population: number of

130   juvenile shoots (JS), number of flowering shoots (NFS), number of flowers (NF), number of

131   fruits (capsules) (NFR), and height of plants (HP). Population size includes the total number of

132   shoots (juvenile and flowering). The area occupied by a given population was determined by the

133   most remote ramets of *E. helleborine*. Border posts indicating the surface area of the population

134   were placed at intervals of one meter from each other, and then the edges of the study area were

135   delineated using a piece of string. The population area measured using a square net was

136   approximately 1 m^2 (Tab. 1). Density of each population was also measured as shoots/m^2. The

137   consent to conduct the research was issued by the Regional Directorate of Environmental

138   Protection in Białystok, permit no. WPN6400.74.2013.MW and the Ministry of the Environment

139   – permit no. 35/17258/12/RS.

140   Eight populations of *E. helleborine* occurring in Poland were studied in two seasons,

141   2011 and 2012 (July – flowering and pollinators, September – seed capsules collection). The

142   identified habitat types were divided in to two categories. One included human-disturbed

143   habitats, such as roadsides (population A1 – between a road and a wooden fence in the village of

144   Guszczewina; A2 – close to a car park in Hajnówka; A3 – in a thicket by a roadside in Sulejów;

145   A4 – on a roadside bordering *Pinus sylvestris* forest in Sulejów) (Fig. 1). The other one grouped

146   natural habitats (population N1 – in a forest of *Galio sylvatici-Carpinetum betuli* Oberd. 1957 in

147   Kotowice (Jakubska & Orłowski, 2003); N2 – in a forest of *Galio sylvatici-Carpinetum betuli*

148   Oberd. 1957 in Kaczawskie Mts. (Kwiatkowski, 2006); N3, N4 – *Galio sylvatici-Carpinetum*

149   *betuli* Oberd. 1957 in the Strict Reserve of the Białowieża Primeval Forest (Faliński, 2001) (Fig.

150   1, Tab.1).

151   The detailed review of the vascular flora of the studied habitats (the sample area was 30

152   m^2) is presented in Table S1. For each species recorded in this study, its relative frequency of

153   occurrence was calculated (Brower & Zar, 1984). Frequency (Fi) was calculated according to the

154   formula: $Fi = (j_i / k) \times 100\ \%$, where: j – the number of populations in which species 'i' was

155   recorded, and k – the total number of populations (Table S1).

156   In total, 68 species of vascular plant species were recorded; 38 (ten species of trees) in

157   natural and 38 (five species of trees) in anthropogenic habitats. The most frequently occurring

158   plants in natural habitats were*: Carpinus betulus*, *Veronica chamaedrys* and *Rubus* sp., registered

159   in all the study plots (*Fi*=100%). The most frequently occurring plants in populations in

160   anthropogenic habitats were: *Achillea millefolium*, *Conyza canadensis, Dactylis glomerata*,

161   *Galium aparine*, *Medicago lupulina*, *Poa annua, P. nemoralis* and *Vicia cracca.*

162

163   ***Pollinators of E. helleborine***

164   Pollinators were both caught and observed in two populations of *E. helleborine* in two

165   anthropogenic habitats: a roadside in the village of Guszczewina (A1), and close to a car parking

166   in the city of Hajnówka (A2), and in one population in a natural habitat - in the Białowieża

167   Primeval Forest (N3) (Fig.1, Tab.1).

168   Insects from fresh orchid flowers were caught using entomological hand nets in two

169   periods: 15.-23.07.2011 and 13.-22.07.2012. The fieldwork was carried out during days with

170   sunny weather. In the anthropogenic (A1, A2) *E. helleborine* populations, the insects were

171   caught by two two-people teams between 9 a.m. and 7 p.m. In the population (N3) from natural

172   habitat, insects were caught by four people to get comparable hours effort between populations

173   from anthropogenic and natural habitat. Insects were collected from 10 shoots (20 in the natural

174   habitat) growing close to each other. Specimens were collected until the transfer of pollinia by

175   pollinators was observed. Insects were killed using ethyl acetate and preserved in 75% ethanol

176   (except bumble-bees *Bombus* spp. which are protected by Polish law – these specimens were

177   only photographed). Insects which arrived with attached pollinia or departed the flower with

178   pollinia were recognised as pollinators. The number of captured insects corresponded to the

179   number of visits, with the exception of the *Bombus* species, for which only the number of visits

180   was counted. Identification of the insects to order/family levels was done based on Gilliot

181   (2005), Hůrka (2005) and Oosterbroek (2006), while entomological nomenclature of insect

182   mouth-parts was provided according to Gilliot (2005).

183   ***The ability of E. helleborine to undergo autogamy***

184   The autogamy experiment was carried out from July to September 2012. Ten shoots in the

185   early stage of flowering (closed buds) were selected in each population for the autogamy

186   experiment. Flowers on each shoot were counted and inflorescences were covered by bags made

187   from a mosquito net. We also used ten control plants (not covered by mosquito-net bags). After

188   three months, the isolators were removed and the number of fruit sets was counted. Viability of

189   seed was examined by the tetrazolium test (live seed with stained embryos and dead seed with

190   unstained embryos) (Van Waes & Debergh, 1986).

191   ***Data analysis***

192   The software package STATISTICA PL. ver. 10 (Stat-Soft Inc. 2011) was used for all the

193   statistical analyses (van Emden, 2008). Diversity of pollinator fauna in natural and anthropogenic

194   habitats was evaluated using the chi-squared test. To compare reproductive success between

195   habitats, we used the Student's *t*-test (we used values of individuals). Correlation between the

196   number of flowers and the number of fruits (we used average) in inflorescence in different

197   habitats was evaluated using the Spearman's correlation The relationship between reproductive

198   success and the height of plant and number of flowers was investigated by linear regression

199   model (Meissner, 2010). To compare the number of fruits (capsules) produced by autogamy in

200   different habitats (we used average) we used the Mann-Whitney *U* test.

201

202   **Results**

203   ***Pollinators of E. helleborine***

204   Pollinators of *E. helleborine* collected during this study belonged to six orders and 24

205   families of insects (Tab. 2, Fig. 2). In the case of the populations from the anthropogenic

206   habitats, taxonomic diversity of pollinators was higher, with 19 families grouped in five orders,

207   while in the natural habitats we noted only 14 families from four orders (statistically significant

208   values, chi-squared test, χ^2= 0.001161, df = 6, p =0.05). In the population from the natural

209   habitat, the most frequent families were Syrphidae (111 visits) and Vespidae (44). In the

210   populations from the anthropogenic habitats, the most frequent families were Syrphidae (57),

211   Vespidae (48) and Apidae (43). In both types of habitats, Diptera and Hymenoptera clearly

212   dominated, with 41% and 52% of all the pollinators observed in the populations from the

213   anthropogenic habitats, and with 59% and 37% observed in the population from the natural

214   habitat (Fig. 3). Coleoptera were the third main group of pollinators making up 6% of the

215   populations from the anthropogenic habitats and 4% of the population from the natural habitat.

216   Occasionally, single individuals of grasshoppers (Orthoptera) and earwigs (Dermaptera) were

217   also noted as pollinators of *E. helleborine* in the populations from the anthropogenic habitats and

218   scorpion flies (Mecoptera) in the population from the natural habitat.

219   In the populations from the anthropogenic habitats, the main dipteran pollinators were

220   hoverflies (Syrphidae), making up 63% of dipteran pollinators and 26% of all the observed

221   pollinators, with the most frequent species *Meliscaeva cinctella* and *Episyrphus balteatus,*

222 followed by mosquitoes (Culicidae) (18% of dipteran and 7% of all the pollinators). The main

223 hymenopteran pollinators were wasps (Vespidae – 42% of hymenopteran and 22% of all the

224 pollinators, with the most frequent species *Dolichovespula saxonica*), bees (Apidae – 38% and

225 20%, respectively, with the main pollinator *Apis mellifera*), and ants (Formicidae– 16% and 8%

226 respectively) (Fig. 3). In the population from the natural habitat, true flies (Syrphidae) accounted

227 for 90% (53% of all the pollinators; with *Meliscaeva cinctella* as the most frequent species), and

228 mosquitoes (Culicidae) made up 7% of pollinators (4% of all the pollinators), while the main

229 hymenopteran pollinators were wasps (Vespidae– 58% of hymenopterans and 21% of all the

230 pollinators; with *Dolichovespula saxonica* as the most frequent pollinator), bees (Apidae– 24%

231 and 9%, respectively), and ants (Formicidae– 17% and 6%, respectively) (Fig. 3).

232   According to the type of mouth-parts, the pollinators of *E. helleborine* can be ascribed to

233 four groups: 1/ sponging insects (Diptera excluding Culicidae– 44% of all the noted pollinators),

234 2/ chewing (= mandibulate) insects (Hymenoptera excluding Apidae, Coleoptera, Dermaptera,

235 Orthoptera and Mecoptera– 36%), 3/ chewing-sucking insects (Apidae– 14%), and 4/ piercing

236 and sucking insects (Culicidae– 6%) (Fig. 4).

237   In the population from the natural habitat, the main groups of pollinators of *E.*

238 *helleborine* were sponging (55%) and chewing insects (32%), while the chewing-sucking and

239 piercing and sucking insects made up respectively 9% and 4% of all the pollinators. In the

240 populations from the anthropogenic habitats, the most frequent pollinators belonged to the

241 groups of chewing (39%), sponging (34%), and chewing-sucking (20%) insects, and only 7% of

242 the noted insects were characterised by piercing and sucking mouth-parts.

243 ***Reproductive success***

244   Reproductive success in the populations from anthropogenic habitats (average from 2011-

245   2012 – 87.1%) was significantly higher than in the populations from the natural habitats (average

246   from 2011-2012 – 72.3%) (Student's *t*-test, p=0.02, df=14). In the populations from the

247   anthropogenic habitats, reproductive success ranged from 77.8% (A4 – in 2011) to 100% (A2 –

248   2012), while in the populations from the natural habitats, it ranged from 44.4% (N1 – 2011) to

249   85.0.3% (N1 – 2012) (Tab. 3). The number of flowers in the populations from the natural

250   habitats ranged from 20 (in 2011) to 22 (in 2012), while in the populations from the

251   anthropogenic habitats from 15 (2011) to 14 (2012). Differences between habitats of the number

252   of flowers were statistically significant (Student's *t*-test, p=0.03).

253   The strongest correlation was found between the reproductive success and height of

254   plants (r = 0.82, p < 0.05) in the populations from the anthropogenic habitats. No significant

255   correlation was found between reproductive success and population density. In the populations

256   from natural habitats, a weak correlation was found between reproductive success and density of

257   populations (r = 0.40, p < 0.05). The regression analysis was significant only in the case of

258   populations from the anthropogenic habitats between reproductive success and height of plants,

259   as well as between reproductive success and the number of flowers (Fig. 5).

260   ***Autogamy***

261   The mean number of capsules (20) produced in the autogamy treatment in the populations

262   from the anthropogenic habitats was significantly higher than the number of capsules produced

263   in the population from the natural habitat (12 capsules) (Mann-Whitney *U* test, Z=3.30, p =

264   0.0008). In the case of autogamy in the populations from the natural and anthropogenic habitats,

265   the number of capsules was strongly positively correlated with the number of flowers per

266   inflorescence (Spearman's correlation, r = 88, p<0.05) (Fig. 6). In the case of natural pollination,

267   in both the populations from anthropogenic and natural habitats, the number of fruits was the

268   same (19) and the number of capsules was also strongly positively correlated (Spearman's

269   correlation, respectively: r = 96 and r = 98, p<0.05) with the number of flowers in the

270   inflorescence.

271   In the population from the natural habitat, the number of fruits produced by open-

272   pollination was slightly higher than the number of fruits produced by autogamy (Mann-Whitney

273   *U* test, Z= 1.30, p = 0.48). The proportion of dead seeds (with unstained embryo) resulting from

274   autogamy varied from 70.5% to 75.4%, and was higher compared to natural pollination (which

275   varied from 48.8% to 50.3%) (Tab. 4).

276   **Discussion**

277   ***Pollinators of E. helleborine***

278   Pollinator availability is one of the key aspects of reproductive success of angiosperm

279   species including orchids. According to Kearns, Inouye & Waser (1998) and Potts et al. (2003),

280   in approximately 90% of all angiosperm species, insect pollinators are involved in sexual

281   reproduction of these plants. In orchids, approximately 70% of the species are closely related to

282   specific insect pollinators (Neiland & Wilcock, 1998). Although our knowledge of how human-

283   induced habitat disturbance affects diversity and composition of pollinator fauna is still

284   fragmentary (Aizen & Vázquez,2006); it is suggested that pollinator diversity decline is mainly

285   due to the transformation of the environment associated with urbanisation, agricultural

286   development and transformation of the area. Numerous studies have confirmed declining

287   diversity and changes in species composition in cities and other anthropogenic habitats,

288   especially in the case of bees (Apidae) and hoverflies (Syrphidae) (e.g.: Schweiger et al., 2007;

289   Banaszak-Cibicka & Żmihorski, 2012). Regarding these two insect groups, we have found that

290   only hoverflies occurred in much smaller numbers in anthropogenic habitats compared to natural

291   ones, while in the case of bees, these values were very similar (Tab. 2). In contrast, the effect of

292   small-scale disturbance can be positive for pollinator fauna, especially in forest habitats.

293   Quintero, Morales & Aizen (2010) have noted that local species richness, abundance, and

294   diversity of insect pollinators were higher in the case of disturbed areas than in natural ones. Our

295   results clearly correspond with those latter observations, as in our studies in natural habitats

296   members of only 14 insect families were noted, while in anthropogenic areas 19 families of

297   insect pollinators occurred. Many authors suggest that higher insolation can provide an

298   explanation of such a phenomenon (e.g.: Herrera, 1995; Thompson, 2000; Hegland & Boeke,

299   2006). Herrera (1995) and Quintero, Morales & Aizen (2010) have noted that small patches and

300   other forest gaps with high insolation can be characterised by higher pollinator activity,

301   particularly of small-sized insects, which may increase local richness and abundance of day-

302   active pollinators. Moreover, forest areas exposed to higher insolation are often characterised by

303   higher flower diversity and, as a result, by higher abundance of insect pollinators (Thompson,

304   2000; Hegland & Boeke, 2006). Sometimes it is even suggested that small-scale disturbed forest

305   areas might act as "diversity oases" for flowering plants (Romey et al., 2007). The higher

306   pollinator diversity in anthropogenic habitats of the studied sites may be explained by the higher

307   insolation of anthropogenic habitats as the most common species were small and medium-size

308   flowering plants, whereas the tree species dominated in natural habitats (Table S1). The

309   anthropogenic habitats probably were located near the natural habitats, so the edge effects maybe

310   also had an important role influencing the high pollinator diversity.

311   Traditionally, the orchid *E. helleborine* demonstrates different morphological and

312   physiological adaptations to attract social wasps as pollinators (e.g.: Judd, 1971; Müller, 1988;

313   Claessens & Kleynen, 2011). According to the literature, its main pollinators are wasps

314   belonging to the following genera: *Vespula, Vespa*, and *Dolichovespula* (Claessens & Kleynen,

315   2011). However, at least in some regions of the orchid distributional range, additional insect

316   groups, such as flies and beetles, may play an important role in pollination (Jakubska et al., 2005;

317   Claessens & Kleynen, 2014). As shown above, the pollinators of *E. helleborine* noted during our

318   studies belonged to six orders and 24 insect families (Fig. 3). All these insects could be

319   characterised by four different types of mouth-parts adapted to collect food in different ways

320   (Fig. 4). Similar results were noted by Jakubska et al. (2005) who observed five coleopteran, four

321   hymenopteran, two dipteran, and one lepidopteran family acting as pollinators of this orchid

322   species and also belonging to four groups according to the type of mouth-parts. In comparison

323   with our results, only piercing and sucking insects were not recorded as pollinators of *E.*

324   *helleborine* by the above-mentioned authors, while, on the other hand, they noted sucking insects

325   (Lepidoptera), which we did not observe. Such high taxonomical diversity of insects, as well as

326   their diverse morphological adaptations of mouth-parts (all five main types of insect mouth-

327   parts) used for collecting nectar and pollen, clearly suggest that *E. helleborine* is an opportunistic

328   species according to pollinators. As it was shown by Jacquemyn, Brys & Hutchings (2014), such

329   a strategy can be also used in some other species belonging to this orchid genus. In their

330   summary of knowledge concerning pollinators of *E. palustris*, the authors have noted members

331   of six families of Coleoptera, 22 of Diptera, 12 of Hymenoptera, and one of Heteroptera

332   (wrongly placed among Hymenoptera). However, it seems that a few insect groups play a much

333   more important role in pollination biology of *E. helleborine* than the others. In our studies, both

334   in natural and anthropogenic sites, sponging (flies, mainly Syrphidae) and chewing insects

335   (mainly Vespidae and Formicidae but also Coleoptera) dominated, with the chewing-sucking

336   insects (Apidae) as the third group according to the frequency of occurrence (Tab. 2, Figs. 3-4).

337   Such differences may result from: geographical location, diversity of pollinator fauna, weather

338   conditions, and especially air temperature, which may change emission of attractants contained

339   in the nectar of *Epipactis* (Ehlers & Olesen, 1997). The important role of Vespidae, as well as of

340   Syrphidae and Apidae, was observed not only in other Polish populations of *E. helleborine*

341   (Jakubska et al., 2005), but also in other regions of Europe (e.g.: Claessens & Kleynen, 2014).

342   Similar patterns were also observed in other species of *Epipactis* such as *E. palustris* (Vöth,

343   1988; Jacquemyn et al., 2014), *E. atrorubens* (Jakubska-Busse & Kadej, 2011), *E. consimilis*

344   (Ivri & Dafni, 1977), *E. turcica* (Fateryga, 2012) and *E. veratrifolia* (Jin et al., 2014). As a result,

345   at least some insect species visit *Epipactis* not only for its highly energetic pollen and/or nectar,

346   but also to look for prey, i.e. other insects attracted by the flowers (Rico-Gray & Oliveira, 2007).

347   For example, Vespidae, Crabronidae or Ichneumonidae, but also some Syrphidae flies, can be

348   classified as such predators. As was shown by Turlings, Tumlinson & Lewis (1990) and

349   Brodmann et al. (2008), at least some *Epipactis* species (including *E. helleborine* and *E.*

350   *purpurata*), produce green-leaf volatiles (GLVs) whose chemical composition is very similar to

351   those emitted by damaged plant tissues when the plant is attacked by caterpillars. The latter are

352   known as one of the most important prey for wasps. Surprisingly, Jin et al. (2014) have noted

353   that females of some Syrphidae can lay their eggs on orchids attacked by aphids (Aphidoidea),

354   which are the main food for their hatched larvae. The explanation of this phenomenon was

355   provided by Stökl et al. (2010), who noted that flowers of *E. veratrifolia* are visited by some

356   aphidophagous Syrhipidae as the orchid produces α- and β-pinene, β-myrcene and β-

357   phellandrene. These substances are very similar to aphid-derived kairomones, which normally

358   are emitted as alarm pheromones by several aphid species. Hoverflies were also noted as

359   important pollinators of *E. helleborine*, both during our studies (Tab. 2, Fig. 3) and by Jakubska

360   et al. (2005). Moreover, aphids are regularly noted as feeding on this orchid species (pers.

361   observations). Thus, we can suppose that such chemical mimicry is more common among

362   *Epipactis* species than was shown until now. Overall, these results indicate that *E. helleborine*

363   has a diverse group of pollinators, which may promote this species in very rapidly changing

364   areas transformed by man and which is one of the key features of apophytes.

365   ***Reproductive success and effect of autogamy of E. helleborine seeds***

366   Autogamy in *E. helleborine* was observed by many authors, some of them (Richards &

367   Porter, 1982; Robatsch, 1983) have claimed that this species shows optional autogamy (mixed-

368   mating) (Ehlers & Pedersen, 2000; Claessens & Kleynen, 2011). However, Ehlers, Olesen &

369   Gren (2002) have suggested that autogamy in *E. helleborine* is rare and that this phenomenon

370   occurs only in specific conditions, i.e. when suitable pollinators are lacking. Our results provide

371   evidence that autogamy occurs in populations from both anthropogenic and natural habitats of *E.*

372   *helleborine* (Tab. 4). Despite some differences in the number of fruits between the populations of

373   the two habitats, we have found no significant differences in the number of fruits formed

374   between autogamy and natural pollination, which is congruent with work of Weijer (1952). In

375   our opinion, autogamy is a common phenomenon in the life cycle of *E. helleborine*.

376   According to Grime's (1979) theory, some species can tolerate environmental

377   disturbances. Hágsater and Dumont (1996) have suggested that orchids belong to the group

378   between ruderal and stress-tolerant plants. Recent studies of Rewicz et al. (2016) have

379   highlighted a positive impact of disturbed anthropogenic habitats on occurrence of some orchids

380   species, even against the general thesis that orchids are competitively weaker than other plant

381   species. In this particular case, it may be caused by reduction in the vigour of other plants by

382   some management practices. Djordjević et al. (2016a) suggested that disturbed habitats can be

383   preferred by orchids. It is possible that *E. helleborine* from the anthropogenic habitats has more

384   space with a favourable light regime. Furthermore, some ecological conditions, such as soil

385   moisture, soil pH, and organic matter could also be conducive to the growth of *E. helleborine*,

386   which results in its larger size in anthropogenic habitats.

387   The relationship between plant height and reproductive success was confirmed by

388   Machaka-Houri et al. (2012) in their studies on *Orchis galilea*, as well as on *Ferocactus*

389   *cylindraceus*, *F. wislizeni*, and *Lotus corniculatus* (Ollerton & Lack, 1998; McIntosh, 2002). Our

390   results also suggest that there is no association between reproductive success and the number of

391   flowers on a sprout in populations from both anthropogenic and natural habitats. It appears that

392   the height of the plant and the number of flowers in orchids enhances attractiveness for insect

393   pollinators (Kindlmann & Jersakova, 2005). Specimens from the populations from the

394   anthropogenic habitats we studied were higher and had more diverse pollinating fauna. Plants

395   with bigger shoots and more flowers are more tempting for pollinators, which results in more

396   efficient transport of pollinia (van der Piper & Waite, 1988). Moreover, no significant correlation

397   between density of plants and reproductive success in populations from the anthropogenic

398   habitats was noted and in populations from the natural habitats such correlation was weak.

399   Similar results were obtained in other studies (Sih & Baltus, 1987; Ågren, 1989; Alexanderson &

400   Agren, 1996; Ehlers, Olesen & Gren, 2002).

401   In conclusion, our research demonstrates that the spectrum of insects pollinating *E.*

402   *helleborine* is much wider than it has been suggested in the literature. Increased variety of

403   possible pollinators allows faster and better adaptation to the human-changed environment. The

404 reproductive success of *E. helleborine* was higher in anthropogenic habitats, which might have

405 been a higher number of visits and greater species diversity of pollinators, as well as by a larger

406 size of the plants. Moreover, autogamy was not uncommon as the reproductive strategy, and we

407 found no significant differences between the number of fruits formed by autogamy and by

408 natural pollination. In addition, this study contributes to a better understanding of why *E.*

409 *helleborine* is one of the few Eurasian orchid species that has been naturalised in North America.

410 The study confirms the general thesis that orchid species which are not highly specialised in

411 relation to the type of pollinator have wider distribution ranges and are less rare than orchid

412 species that have a high level of pollinator specialisation (Swarts & Dixon, 2009).

413   Summarising, our study helps to explain why *E. helleborine* is one of the (few) orchid

414 species that manages to successfully use anthropogenic habitats in a manner comparable to that

415 of natural ones. The question how wide the tolerance range of this species is still remains open.

416 To answer the question, we plan further research on pollinator diversity and reproductive success

417 of *E. helleborine* in other types of anthropogenic habitats (the surroundings of industrial

418 facilities, fly ash, mine tailing, highway, railway, urban parks, etc.).

419

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426

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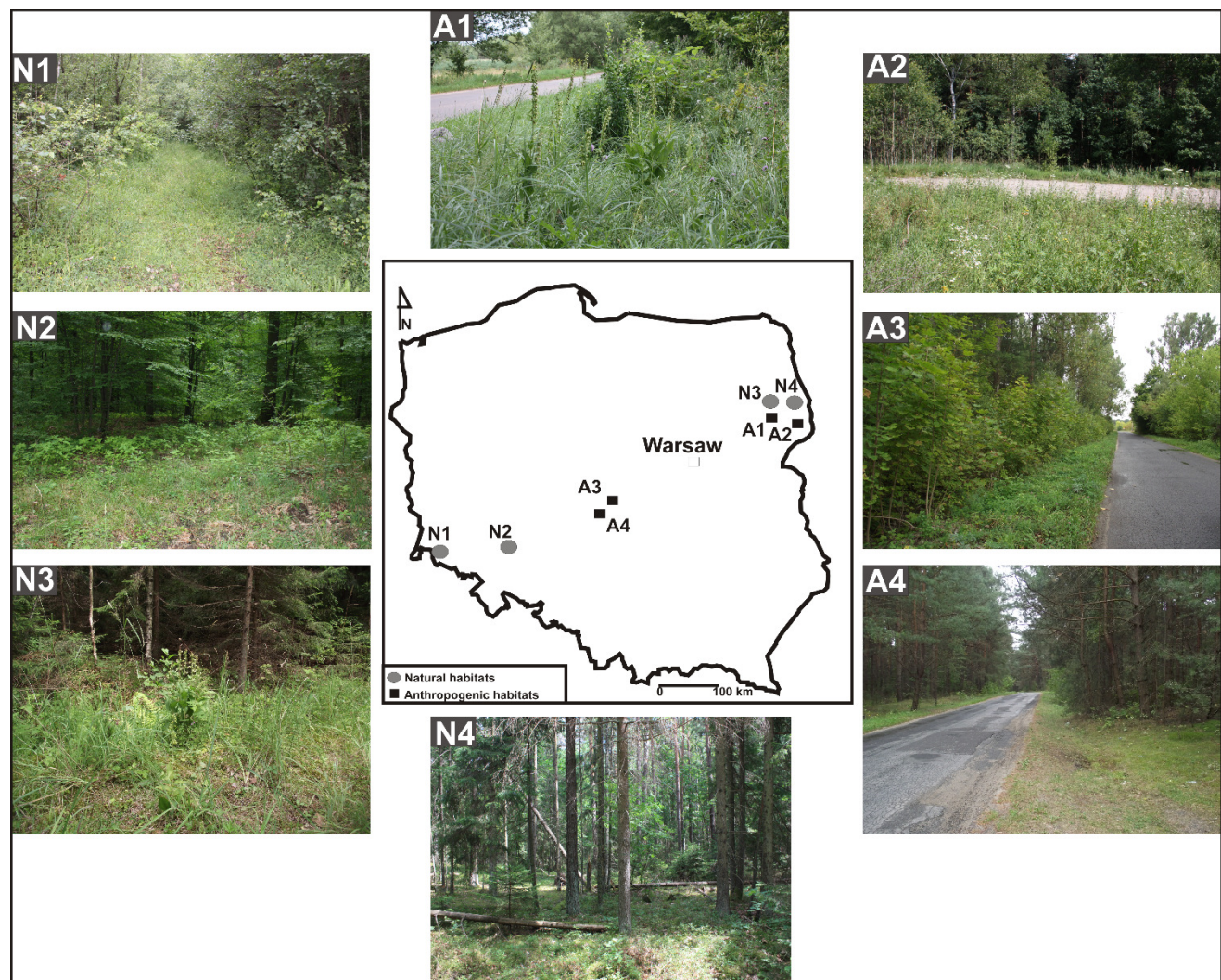
Figure 1. Habitats of *E. helleborine*: A – anthropogenic habitat, N – natural habitat, A1 –

between a roadanda wooden fence in the village of Guszczewina, A2 – close to a car park in

Hajnówka, A3 – in a thicket by a roadside in Sulejów, A4 – on a roadside bordering a*Pinus*

sylvestris forest in Sulejów, N1 – a forest of*Galio sylvatici-Carpinetum betuli* Oberd. 1957 in

Kotowice, N2 –a forest of*Galio sylvatici-Carpinetum betuli* Oberd. 1957in Kaczawskie Mts., N3

and N4 – *Galio sylvatici-Carpinetum betuli* Oberd. 1957in thenatural habitat (Strict Reserve of

the Białowieża Primeval Forest).

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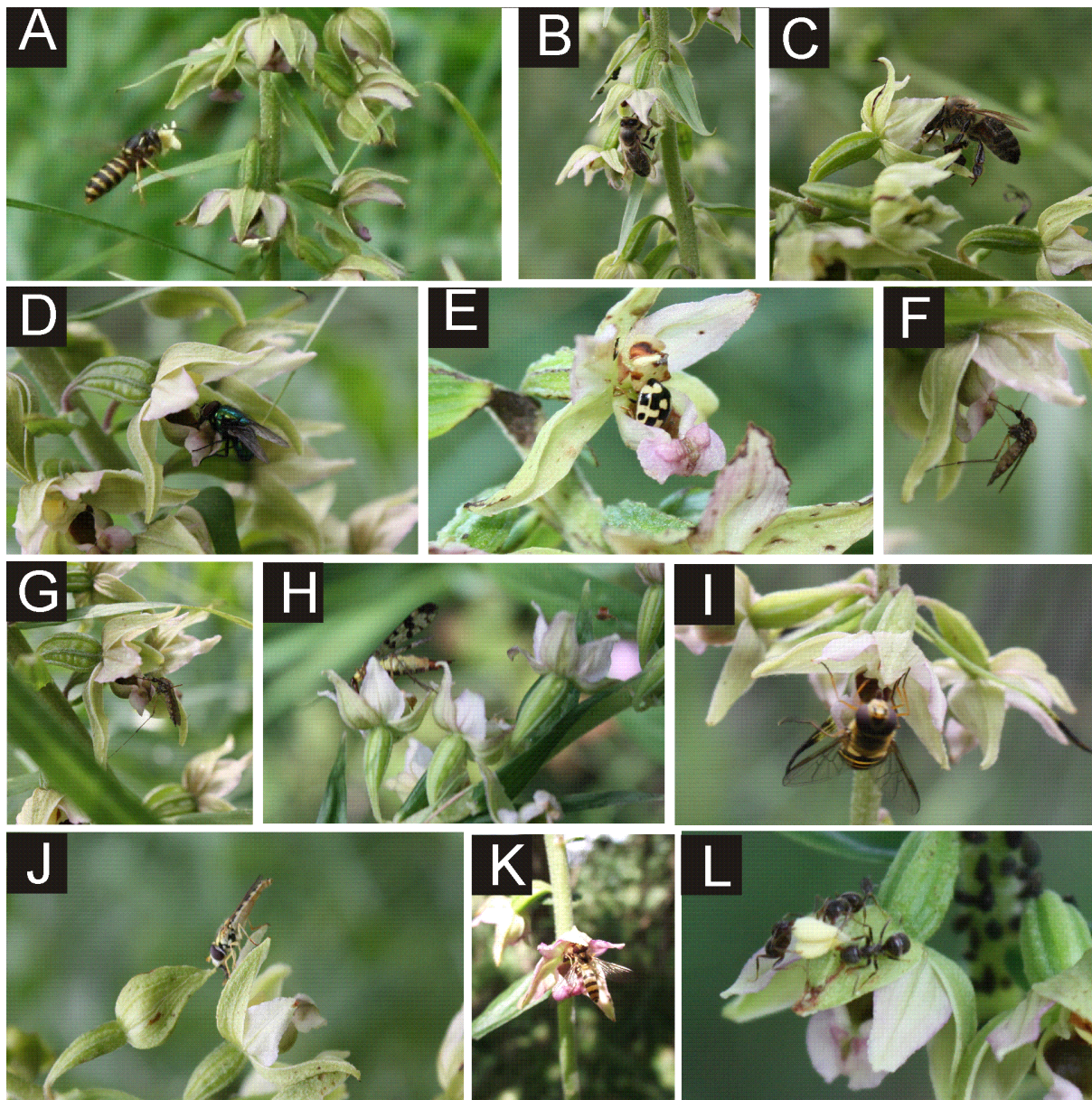
Figure 2.Pollinators of *E. helleborine*: A – wasp (Vespidae) with pollinia attached to its head,

B-C – honeybees (Apidae), D – carrion fly (Calliphoridae), E – ladybird (Coccinellidae), F-G –

mosquito (Culicidae), H – scorpionfly (Panorpidae), I-K – hoverflies (Syrphidae), L – ants

(Formicidae) (photo: A. Rewicz 2012/2013).

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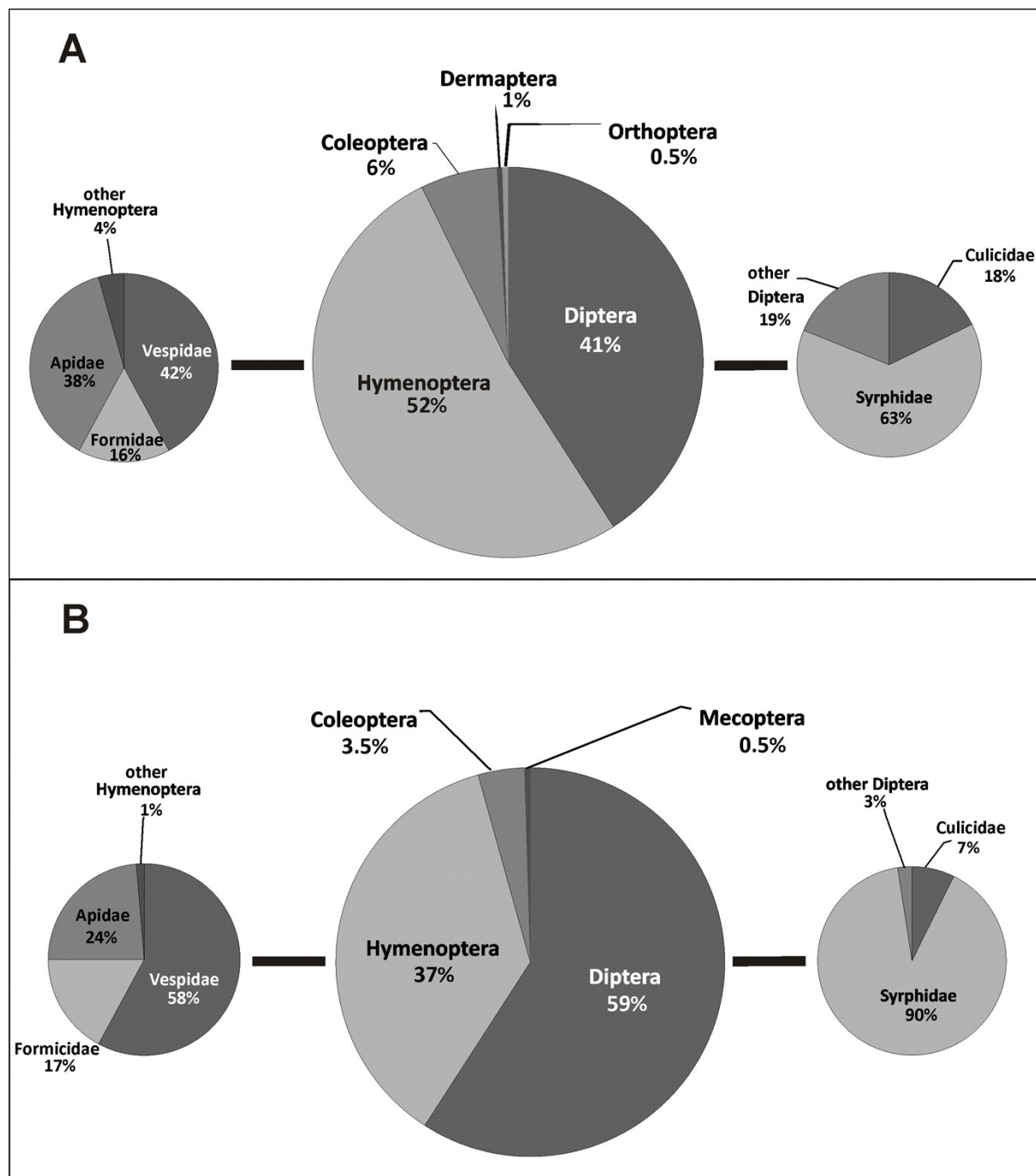
Figure 3. Taxonomic diversity of *E. helleborine* pollinators in anthropogenic (A) and

natural (B) habitats.

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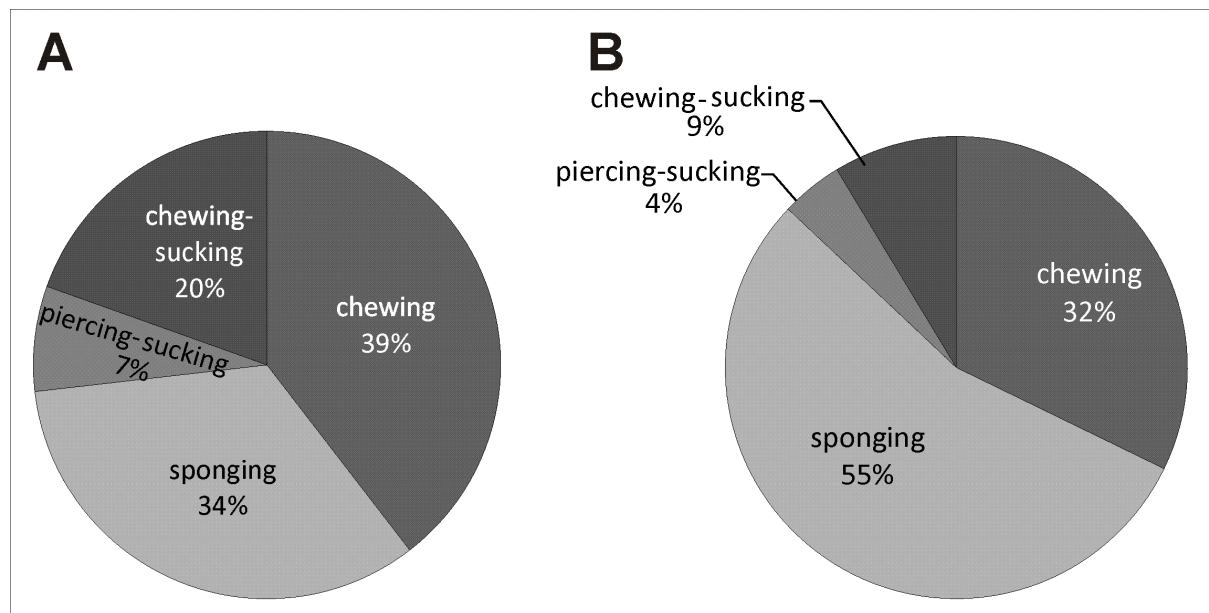
649 **Figure 4. Diversity of *E. helleborine* pollinators in anthropogenic (A) and natural (B)**

650 **habitats based on insect mouthparts**.

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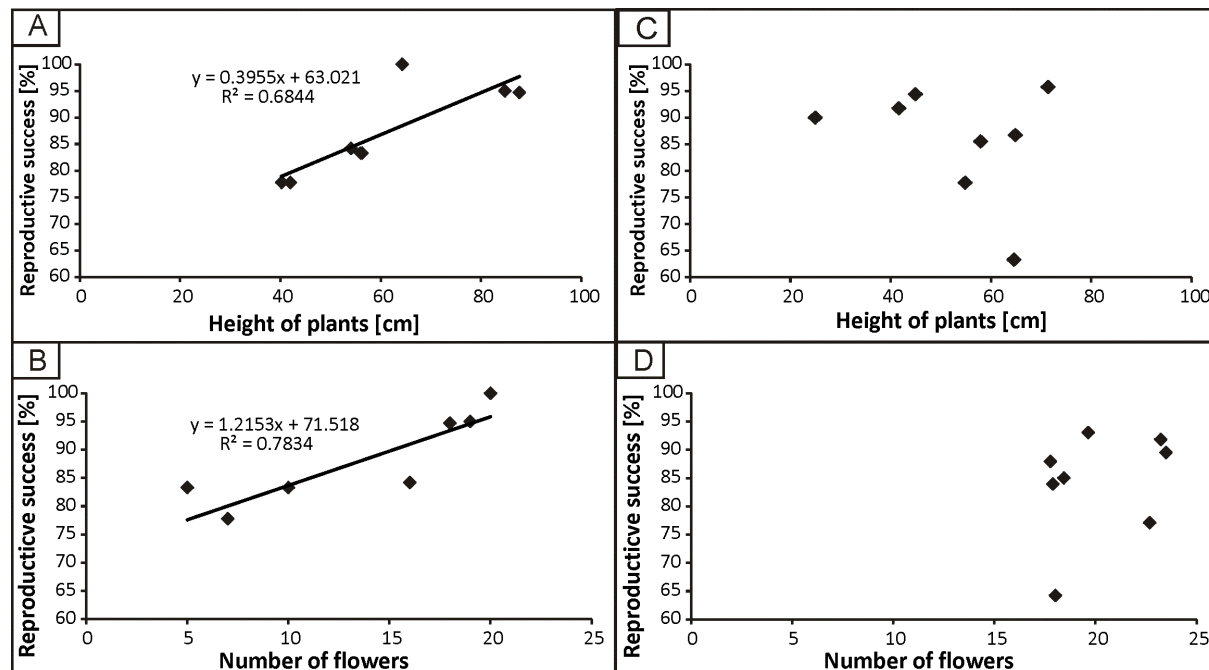
655   **Figure 5. The dependence of reproductive success from height of plants and number of**

656   **flowers in populations from anthropogenic (A, B) and natural (C, D) habitats.**

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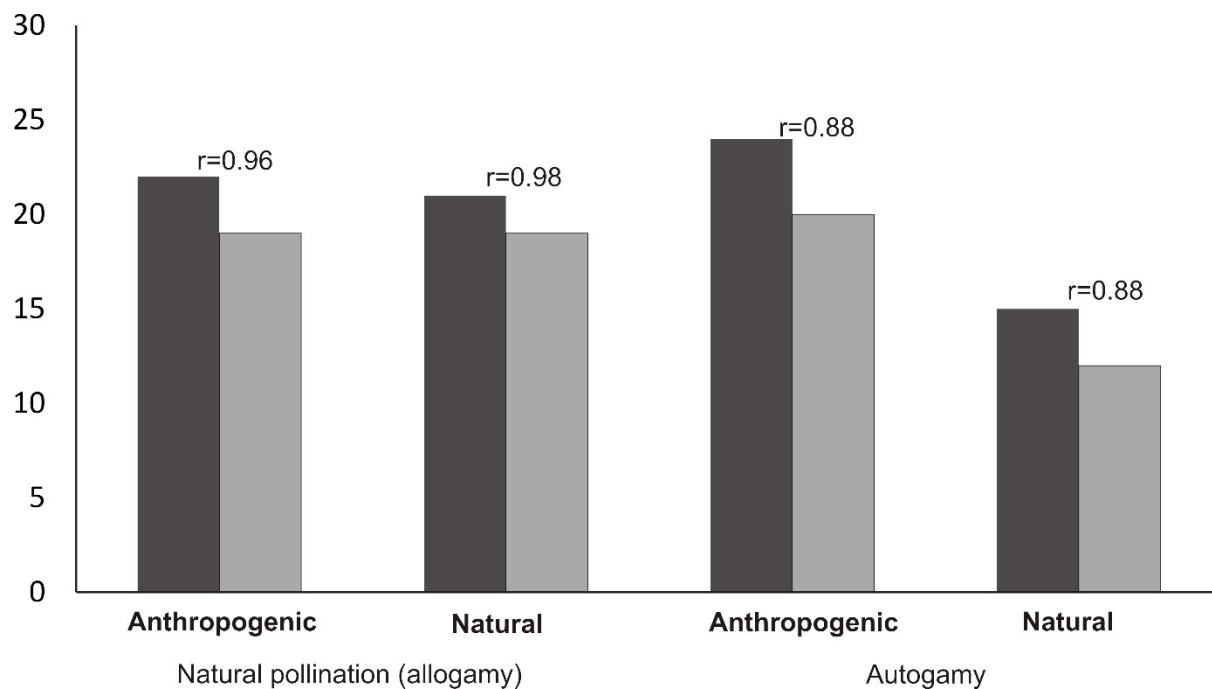
Figure 6. Mean number of flowers and fruits in analyzed populations and Spearman
correlation between number of flowers and fruits

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Table 1. Studied populations of *Epipactis helleborine*.

Population code	Locality	Coordinates	Altitude (m asl)	Area (m²)	Population size (number of shoots)	Density (shoots/m²)
anthropogenic habitats						
A1*	roadside (Guszczewina)	N 52.831600 E 23.794836	148	36	127	3.53
A2*	roadside (Hajnówka)	N 52.734217 E 23.603314	181	108	102	0.94
A3	roadside (Sulejów)	N 51.353793 E 19.883155	166	460	80	0.17
A4	roadside (Sulejów)	N 51.349757 E 19.882484	167	46	152	3.30
natural habitats						
N1	mixed forest (Kotowice)	N 51.041241 E 17.176701	128	100	300	3.00
N2	mixed forest (Kaczawskie Mts)	N50.963255 E 15.963255	480	40	150	3.75
N3*	mixed forest(Białowieża Primeval Forest)	N 52.800743 E 23.914125	51	120	34	0.28
N4	mixed forest (Białowieża Primeval Forest)	N 52.7650022 E 23.884316	44	400	41	0.10

666 *asterix indicates populations where pollinator fauna were analyzed
667

669 **Table 2. Pollinators of *Epipactis helleborine* in natural and anthropogenic habitats:**

670 **C - chewing, S - sponging, PS - piercing and sucking, CS - chewing and sucking.**

Taxon of pollinator		Type of mouthparts	Habitat type	
Order	Family		Natural	Anthropogenic
Orthoptera	Acrididae	C	0	1
Dermaptera	Forficulidae	C	0	1
Diptera	Calliphoridae	S	1	7
	Culicidae	PS	9	16
	Lauxanidae	S	0	1
	Muscidae	S	1	3
	Scathopagidae	S	0	1
	Sepsidae	S	0	1
	Syrphidae	S	111	57
	Tachinidae	S	1	2
	Tephritidae	S	0	1
	Tipulidae	S	0	1
Mecoptera	Planorpidae	C	4	0
Hymenoptera	Apidae	CS	18	43
	Formicidae	C	13	18
	Ichneumonidae	C	1	4
	Pamphiliidae	C	0	1
	Vespidae	C	44	48
Coleoptera	Cantharidae	C	0	10
	Cerambycidae	C	1	0

	Coccinellidae	C	0	4
	Elateridae	C	2	0
	Melyridae	C	4	0
	Nitidulidae	C	1	0
Total			208	220

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672

673 **Table 3. Reproductive success of *Epipactis helleborine* in natural and anthropogenic habitats:**

674 D (n/m^2) - density of population, HP - height of plants, NFS - number of flowering shoots, NF -

675 number of flowers, NFR - number of fruits, RS - reproductive success, ± standard deviation.

Anthropogenic habitats							Natural habitats						
2011							**2011**						
site	D (n/m^2)	HP	NFS	NF	NFR	RS	site	D (n/m^2)	HP	NFS	NF	NFR	RS
A1	0.28	84.79±24.04	39	20±9.20	19±9.48	95.00±7.20	N1	0.83	61.96±17.91	25	18±11.8	8±7.80	44.40±5.13
A2	0.93	54.08±15.84	26	19±8.63	16±7.83	84.20±8.30	N2	0.77	57.36±15.99	30	18±10.8	13±8.36	72.20±8.78
A3	0.9	56.29±11.80	22	12±6.20	10±5.92	83.30±10.4	N3	0.21	34.53±15.27	28	18±12.5	14±11.99	77.80±9.54
A4	0.4	41.98±12.64	38	9±9.25	7±9.21	77.80±12.34	N4	0.88	48.36±23.60	33	24±14.8	20±13.15	83.30±11.40
Average	0.63	59.28	31.25	15	13	85.08	Average	0.67	50.55	29	20	14	69.42
2012							**2012**						
A1	0.38	87.65±22.48	43	19±8.37	18±8.28	94.70±6.54	N1	1.24	66.67±13.60	15	20±6.34	17±5.19	85.00±7.45
A2	0.77	64.3±19.51	45	20±14.1	20±13.87	100.00±10.30	N2	0.97	62.15±16.58	24	19±11.4	14±10.00	73.70±6.78

A3	2.39	56.0±19.07	13	6±6.20	5±5.81	83.30±8.56	N3	0.22	55.22±20.32	25	24±13.4	15±10.45	62.50±5.45
A4	0.33	40.24±15.97	46	9±8.73	7±8.66	77.80±8.34	N4	0.81	46.07±23.91	32	25±14.3	20±14.00	80.00±9.45
Average	0.97	62.05	36.75	14	12	88.95	Average	0.81	57.53	24	22	17	75.30

676

678 **Table 4. Ratio of dead and alive seeds developed in autogamy and natural pollination in**
679 **analyzed populations** (ns – Non-significant results).

Trait	Populations		Mann-Whitney U test, $p<0.05$
	Anthropogenic	Natural	
	Natural pollination (allogamy)		
live seed (%)	49.7±0.5	51.2±2.1	ns
dead seeds (%)	50.3±0.7	48.8±2.6	ns
	Autogamy		
live seed (%)	24.6±2.9	29.5±3.7	p<0.05
dead seeds (%)	75.4±10.2	70.5±8.6	p<0.05

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