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Pollination biology of two phenological forms of *Neotinea ustulata* (Orchidaceae) in Austria, with field experiments to clarify the biological significance of the plants' "burnt tips"

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The pollination biology of the burnt-tip orchid *Neotinea ustulata* with its two phenological forms was investigated in different protected areas around Vienna and in the Hohe Tauern National Park. The main pollinators of the spring form are *Tachina fera* and *T. magnicornis*, while some bumblebees, other wild bees and Cerambycidae are rarer pollinators. The main pollinator of the summer form is the tachinid fly *Nowickia ferox*. Choice experiments with intact versus manipulated *Neotinea* plants implied that the burnt tip of the inflorescence is an important visual and olfactory signal for the tachinid flies. The two phenological forms fulfil all criteria of biospecies (different morphology, different genetics, different smell, different ecological demands, as earlier investigations of other authors have found) with the consequence of complete reproductive isolation. It is justified to give *Neotinea aestivalis* the species rank instead of the typological systematization as a subspecies or just a variety.

Paulus H.F., 2022: Bestäubungsbiologie der beiden phänologischen Formen von *Neotinea ustulata* in Österreich, mit einigen Feldexperimenten zur Klärung der biologischen Bedeutung der „verbrannten Blütenspitze" der Pflanzen.
In der Umgebung von Wien und im Nationalpark Hohe Tauern wurden die Bestäubungsbiologie der beiden phänologischen Formen des Brandknabenkrautes *Neotinea ustulata* untersucht. Hauptbestäuber der Frühlingsform *N. ustulata* sind die beiden Raupenfliegen *Tachina fera* und *T. magnicornis*. Seltenere Bestäuber sind Hummeln, andere Wildbienen und Bockkäfer. Der Hauptbestäuber der Sommerform ist dagegen die Tachinidae *Nowickia ferox* neben gelegentlichen Besuchen durch Hummeln. Wahlexperimente im Gelände mit unveränderten versus manipulierter Pflanzen machten wahrscheinlich, dass die „verbrannte" Spitze der Infloreszenz ein wichtiges optisches und olfaktorisches Signal für die Raupenfliegen ist. Die beiden phänologischen Formen sind voneinander reproduktiv völlig isoliert, da sie morphologisch, ökologisch, und wie bereits andere Studien gezeigt haben, genetisch und über den Duft so verschieden sind, dass alle Kriterien für biologische Arten erfüllt sind. Es ist daher gerechtfertigt, beide Formen als getrennte Arten zu betrachten und *Neotinea aestivalis* als Art zu führen anstelle der völlig typologischen Systematisierung als Subspezies oder gar Varietät.

Keywords: *Neotinea ustulata*, species rank of *Neotinea aestivalis*, pollination biology in Austria, Diptera, Tachinidae, *Tachina fera*, *Nowickia ferox*, *Bombus*.

Introduction

The orchid family, the second largest plant family in the world, accounts for around 25,000–28,000 described species, depending on whether a more typological or biological definition of species is used (Willis 2017, Fay 2018). In Austria we have about 80–85 different species depending on the acceptance of species status of some taxa (especially in the genera *Dactylorhiza, Nigritella* and *Epipactis*) (e.g. Redl 2003 or Griebl 2013). A special characteristic in the morphology of orchid flowers is the form of pollen presentation, packed in so-called pollinia or pollinaria. The complete number of pollen grains, mostly packed in tetrades, can be transferred to the stigma by a pollinator with only a single or at least very few visits. This is an adaptation for getting a maximum of reproductive success for the many thousand to even a million seeds within one single flower. This kind of transport might be extremely successful in the case of an allogamic transfer to the next flower, but the risk of losing the entire male reproductive success after a "wrong" transfer (geitonogamy or autogamy) is also large. This is possibly one of the main selective pressures to evolve mechanisms to avoid self-pollination. It seems that the most common way to avoid or at least reduce self-pollination was the evolution of various kinds of deception by means of sometimes extraordinary methods of attraction without giving any reward. The "trick" is that a pollinator should become frustrated and stop further visitations. I called this hypothesis the "self-pollination-avoidance hypothesis" for the evolution of deceptive pollination modes within the whole family of orchids (Paulus 1988, Paulus & Gack 1990, Nilsson 1992). Experimental work with a fly-pollinated South African orchid *Disa pulchra* demonstrates that addition of nectar in the normally empty flowers increases the visitation rates and, as a consequence, the rate of self-pollination (Jersáková & Johnson 2006). Deceived pollinators learn very quickly to avoid deceptive flowers, thus provoking further visits to slightly different flowers of the same species. Exploiting the capacities of insects to learn to recognize flowers just visited, high individual variability was an evolving consequence in some orchid species to gain more visits (e.g. Paulus 2007, 2018 or Stejskal et al. 2015).

In fact, estimations on how many orchid species are deceptive reach from about one third (Renner 2005) to about half of the worldwide species (Dressler 1990). An exact number is only known for the European or Western Palaearctic species with more than 80 % of all species (Paulus 2005). It is to be expected that the number of deceptive species worldwide is also much higher. Deceptive orchids employ various strategies to lure in their pollinators, which range from the advertisement of false nectar or pollen sources (most of the European genera) to the imitation of brood sites or even mating partners (*Ophrys* and *Serapias lingua* in Europe) (van der Pijl & Dodson 1966, Gumprecht 1976, 1977; Paulus 2005, 2014; Jersáková et al. 2006; Johnson & Schiestl 2016). Each type of deception is associated with a particular combination of floral traits, in most cases influenced by their reliance on particular groups of pollinators (Paulus 2005, 2019; Johnson & Schiestl 2016; Valenta et al. 2017). Conspicuous visual displays coupled with reduced floral scent emission and the attraction of male and female pollinators are traditionally considered an indication of food-deceptive pollination (Galizia et al. 2004; Jersáková et al. 2006, 2012, 2016). The often less conspicuous visual displays in European orchids, the exclusive attraction of either male or female insects and the emission of particular scent bouquets are, in contrast, associated with pollination by sexual deception or brood-site mimicry (Ayasse et al. 2011; Vereecken & Schiestl, 2009; Bohman et al. 2016; but see Streinzer et al. 2010). For many deceptive orchids, the investigation of the floral traits employed can provide an accurate estimate of their pollination strategy, which often follows the classification of pollination syndromes. A summary of these syndromes for European orchids is given by Paulus (2005). Most of the members of the genera *Orchis, Anacamptis* or *Neotinea* are nutritive deception flowers, which means that the flowers appear like many other bee flowers but the nectar spurs are empty. However, some orchid species have intermediate characteristics and therefore are much more difficult to place (Jersáková et al. 2016). One of these is the case of the endangered European orchid species, *Neotinea ustulata* (L.) R.M. Bateman, Pridgeon & M.W. Chase with its two phenologically separated flower forms. I investigated the pollination biology of these two forms in greater detail during the last years, and want to provide additional data to an earlier publication of which I was a part (Martel et al. 2021), with a focus on the Austrian situation, together with further experiments to learn more about the attracting signals.

Despite being widely distributed, *Neotinea ustulata* is a strange European orchid because of its remarkable flowers with a colour combination of a reddish-brown inflorescence tip and the labellum with similarly coloured dots. Observations across different locations in Europe support the early records that *N. ustulata* is mainly pollinated by tachinid flies (Godfery 1933, Vöth 1984), which seem to be attracted at least visually by the dark tip. Some first experimental choice tests in the field with *Tachina fera* flies (Tachinidae) – comparing visitation rates in inflorescences with intact and removed dark tips – support these observations (Paulus in Martel et al. 2021).

The second remarkable fact is that *N. ustulata* has two phenologically extremely divergent varieties systematized either as varieties (var. *ustulata* and var. *aestivalis*) or as subspecies. However, the relative importance of tachinids as pollinators seems to differ, playing a predominant role for the late flowering var. *aestivalis*. One of the unsolved questions in this respect is why the two forms exist and how they evolved. As the main flower traits are very similar between both forms, the question is whether two different pollinator spectra or even two specialized pollinators acted as selective agents. This suggests that pollinator-mediated selection may have driven the phenological divergence, and consequently variation in floral traits within *N. ustulata*. Morphological (e.g., slightly larger flowers in var. *ustulata:* see morphometrical studies in Martel et al. 2021), visual (i.e., spectral reflectance pattern) and chemical (e.g., pattern of semi- and low-volatile compounds) floral traits distinguish *N. ustulata* var. *ustulata* from var. *aestivalis* (Martel et al. 2021). Another explanation might be that differences in phenology, pollinator spectra and floral traits thereby seem associated with adaptation to insects that are available within their respective habitat and blooming period, as is usual in most more generalized pollination systems of members of the related genera *Orchis, Anacamptis* or other *Neotinea* (Vöth 1999, Paulus 2005, Claessens & Kleynen 2011, 2016).

As the fruit set was high even in localities where *Tachina* species seemed to be rare (Vöth 1999), I supposed that other pollinators must also be acting. To find out more about such possibly accidental pollinators, I frequently visited several growing sites around Vienna in the last years. Localities with many individuals of *Neotinea ustulata* are the "Perchtoldsdorfer Heide" near Vienna, the Lobau part of the Donau-Auen National Park along the river Danube, or in parts of the "Wiener Wald" at Gießhübl near Vienna. To find out if pollinator communities in the mountains are similar or even identical, we made some observations at the "Mitteralm" at 1500–1600 m above sea level in the Hohe Tauern National Park near Fusch (Salzburg). Because of the relatively late flowering time (beginning of July, instead of May or June) these plants could belong to the summer form, but from the plant characteristics they belong to the spring form of *N. ustulata*.

A further aim was to investigate the pollination biology of the summer form of *N. ustulata* to find out which insects are mainly attracted and really pollinate this form. The summer variety is only very locally distributed in the wider surroundings of Vienna. There are only very few observations on pollinators. Known are the records of Mrkvicka (1991) who observed the small longhorned beetle *Pseudovadonia* (=*Leptura* auct.) *livida* (F.) (Cerambycidae) and the picture of an Oedemerid beetle *Chrysanthia* spec. (Danesch & Danesch

1962). I was able to observe more visitors at two localities near Vienna during the last years. One growing area with 40–80 individuals is in the so-called "Aumüllerwiese-Schattenwiese" in the "Lainzer Tiergarten" of Vienna. The other locality is the "Weiderwiese" near Purkersdorf, west of Vienna. Further distribution points are the "Dianawiese" within the "Sparbacher Tierpark" near Vienna, and I was able to newly discover an occurrence in the "Frauenwiese" near Loretto (northern Burgenland). To my surprise, I found about 50 plants at the end of June 2020. Why these plants had been overlooked all these years is puzzling as this wet meadow is a prominent protected area with a well-known flora (e.g. Kuyper et al. 1978).

Material and methods

All observations were done in the field directly in the habitats over the last 20 years. Observations of pollination were done especially in the Lobau (Donau-Auen National Park) in and near Vienna, the Neusiedler See – Seewinkel National Park near Illmitz (Burgenland), and on two protected smaller meadows – the "Frauenwiese" in northern Burgenland and the "Aumüllerwiesen" in the Lainzer Tiergarten in Vienna.

Only those insects which did remove or are clearly able to remove pollinaria were considered as pollinators. Others are just visitors and play no role for pollination.

As orchid flower visiting insects are normally rare to see, experimental choice tests in the field can only be done under very special conditions. As pollinator bees learn very quickly to avoid deceptive orchid flowers just visited, such tests are only possible if many still inexperienced pollinator individuals are flying in the study area. During all these years, only twice did I have the opportunity to conduct such choice experiments. Once at the Gießhübl meadows near Mödling (Wienerwald) (48⁰05′54.25″N / 16⁰13′12.59″E, 433 m) with the spring form and *Tachina fera* in May 2015. The other field experiments were done near Schlagl-Gloggnitz (47⁰38′20.50″N / 15⁰54′02.46″E, 926 m) with the summer form. These plants had been removed from the Schattenwiese/Aumüllerwiese (48⁰10′02.88N / 16⁰12′09.22″E, 372 m) just before the cut of this meadow, and transferred to the flying side of *Nowickia ferox* near Schlagl. On the meadows near Gießhübl in normal years there are about 25–50 plants of *N. ustulata*. For experimental work, a few plants were manipulated in the ways described in more detail in the respective chapters (Figs. 10 and 11). As in all cases the total numbers of visits by the flies are low, and a repetition of more observations was not possible because of the learning capacities of all flies, which avoid further interactions after unsuccessful visits. Unfortunately, the following year (2021), the number of plants was catastrophically low due to extreme dryness, so that no further experiments could be done. I present just the original numbers of the results. A statistical analysis would not reveal more than is already offered by the basic numbers. All photos in the text are by the author.

Results and reviews

Further observations on the spring form of burnt orchid or burnt-tip orchid *Neotinea ustulata* subsp. *ustulata* (L.) R.M. Bateman, Pridgeon & M.W. Chase

The burnt-tip orchid is an inhabitant of nutrient poor grasslands and warm mountain meadows until 1900–2000 m above sea level. Start of flowering is end of April or beginning of May, in mountain areas later. The small flowers have a very short (about 2 mm) downwards-directed nectar spur without any reward. The smell is slightly of sweet honey. Systematic investigation of the pollination biology did not exist until the intensive observations of Walter Vöth near Vienna. He confirmed the incidental observation of the tachinid fly *Tachina (Echinomyia) magnicornis* by Godfery (1933). Vöth (1984) made more intensive observations of this parasitic fly on caterpillars. The flies landed on the top of the orchid and lowered the proboscis into a flower, seeking nectar (Fig. 1). In many cases the pollinaria were attached at the underside of the proboscis tip (Fig. 2). As I could see myself, the flies sitting on the top of the inflorescences dabbed many of the top flowers – even those which were still closed. Continuing down the inflorescence they found the opened flowers and inserted their proboscis. As some of these visitors had up to 5 pollinaria attached to the tip of their proboscis, they should be very good pollinators. Vöth (1984) noticed some other visitors (small butterflies and diurnal moths) but these were never able to extract pollen. A summary of our recent observations together with data on genetics, morphometrics, olfactory compounds and some data on pollination is given by Martel et al. (2021)

Observations near Vienna

From my own observations, *Neotinea ustulata* subsp. *ustulata* is also visited by bumblebees and some other solitary bees. These bees land at the bottom of the inflorescences and move towards the top as bumblebees usually do on vertical inflorescences (Pyke 1978). Despite their long proboscides, they were able to remove pollinaria that were attached to the upper side of the tip.

Near Vienna (Gießhübl and Perchtoldsdorfer Heide) I found the following pollinating bumblebee species:
Bombus ruderarius queens (many observations)
Bombus terrestris queens (many observations)
Bombus pascuorum worker (many observations)
Bombus pratorum males (many observations)
Psithyrus vestalis queens (a few observations)
Psithyrus rupestris queens (a few observations)
Osmia rufohirta male (one single observation) (Fig. 4C)
Anthophora plumipes (a few observations)
Anthophora aestivalis (one single observation) (Fig. 4D)

Whereas the bumblebees were regularly able to remove pollinaria, the two species of *Anthophora* always had difficulties due to their extremely long proboscides, which almost prevented these bees from finding the entrance to the flower's spur. In some cases they were successful but then instantly lost the just attached pollinaria.

Some own investigations in the higher mountains: population in the Hohe Tauern National Park, Großglockner Hochalpenstrasse

During several "pollination biology" field courses with my students and with my co-workers (Johannes Spaethe and Johann Neumeyer), we mainly studied bumblebee ecology to demonstrate the potential of bumblebee communities to contribute to understanding of ecological evolutionary strategies (see Neumeyer & Paulus 1999). But only two of the bumblebee species acted as pollinators in *N. ustulata* (see Martel et al. (2021): *Bombus mucidus* worker (Fig. 3A) and

Bombus pyrenaeus worker (Fig. 3B).

In two cases I even observed a cerambycid beetle (*Leptura annularis* = *Strangalia arcuata*) visiting flowers (Fig. 4E).

While *Tachina fera* and *T. magnicornis* were only rarely seen around Vienna, *Tachina fera* was common on the Mitteralm in the Hohe Tauern National Park. Many observations of pollination by this fly could be observed at the beginning of July during several years.

Males of *Tachina* often patrol at grassland sites looking for females. They mostly use exposed small plants as resting places to keep watch for flying females. All similar flying objects were inspected by a quick approach. As individual males often use the same resting places, this looks similar to territorial behaviour. Other conspecific males are attacked, other flying objects (other flies, honeybees or small flying beetles) are briefly inspected. If there are flowering orchids like *Dactylorhiza fuchsii* or, even better, *Neotinea ustulata* in the area, these are used preferably.

But these males sitting on top of *Neotinea ustulata* show the same behaviour already described by VÖTH (1984). After landing on the "burnt" tip, some males turn downwards to inspect the nearest flowers of the inflorescence for nectar with erected proboscides. After visiting two or three flowers the flies return to the tip. During this inspection pollinaria could be removed and are possibly attached on the underside of the proboscis tip. Thereafter, most flies move on to the next observation point. If this is another *Neotinea ustulata*

Fig. 1:Males of *Tachina fera* mainly land on the top of the burnt-tip orchid *Neotinea ustulata*, then sometimes turn downwards to inspect the nearest flowers of the inflorescence for nectar. – Abb. 1: Männchen von *Tachina fera* landen überwiegend auf der "verbrannten" Infloreszenzspitze von *Neotinea ustulata*, um sich dann nach unten zu den nächst gelegenen Blüten zu wenden, um nach Nektar zu suchen. – Hohe Tauern National Park, Mitteralm near Fusch 4 July 2006.

Fig. 2: *Tachina fera* is the main pollinator of the spring form of the burnt-tip orchid *Neotinea ustulata*. – Abb. 2: *Tachina fera* ist der Hauptbestäuber der Frühjahrsform von *Neotinea ustulata*. – Hohe Tauern National Park, Mitteralm near Fusch, 21 July 2010.

plant, pollination can occur. It is important to note that nectar seeking only occurred in *Neotinea* and never in *Dactylorhiza*, though it sometimes also occurred on *Thymus* or *Origanum* flowers, which interestingly have a very similar "burnt" colour to the top of *Neotinea*. This colour type seems to be an attractive signal for these *Tachina* flies.

During the several observation years, some of these males had up to 10 pairs of pollinaria attached to the proboscis. As this number of pollinaria obviously disturbed most of the flies, they began intensive cleaning behaviour using their front legs. After a short time most of the pollinaria were removed. Only a few pollinaria remained. These observations demonstrate that there must be a strong selection pressure on a good adhesion property of the pollinaria's viscidia. It also shows that there is a heavy loss of pollen in this orchid.

The question is if only males act as pollinators. Only males have this type of semi-territorial behaviour. Do females also visit the orchid flowers? To find this out, we caught all individuals of *Tachina* to check whether they had pollinaria on their proboscides, and how many. As I had also observed visits by bumblebees several times, we also caught all bumblebee and larger hoverfly individuals to study their proboscides for pollinaria. After inspection, all individuals were released again. As we were well experienced in determination of free-flying bumblebees in this area (Neumeyer & Paulus 1999), the results of these counts are summarized in Table 1

The checked individuals had removed a total of 179 pollinaria (=100 %). 86.5 % were found on the proboscides of *Tachina fera*, most of them in males (= 78.7 %). This means that the main pollinators of *Neotinea ustulata* subsp. *ustulata* are males of *Tachina fera*, and only some of their females (7.8 % of the pollinaria). Of the eight occurring bumblebee species

Tab. 1: List of all individuals of *Tachina fera* and *Bombus* species checked for pollinariae of *Neotinea ustulata* subsp. *ustulata* at their proboscides. The data are the sum of the years 2010–2013 from the "Mitteralm" (ca. 1500 m) (Hohe Tauern National Park, near Fusch), each beginning of July. – Tab. 1: Liste aller auf Pollinarien von *Neotinea ustulata* am Rüssel untersuchten *Tachina* und Hummelarten auf der Mitteralm (Nationalpark Hohe Tauern nahe Fusch) der Jahre 2010–2013, jeweils Anfang Juli.

species	**sum of checked individuals**	**individuals with pollinaria**	**number of pollinaria attached on the proboscis/indiv.**	**sum of pollinaria**
Tachina fera-♂♂	78	37	2♂♂: 10 3♂♂: 7 18♂♂: 4 14♂♂: 2	141 = 78,7 %
Tachina fera-♀♀	29	6	1♀: 4 5♀♀: 2	14 = 7,8 %
Bombus mucidus w (=worker)	21	4	1w: 6 3w: 2	12 = 6,7 %
Bombus pascuorum w	44	3	3w: 2	6 = 3,3 %
Bombus hortorum w	21	0		
Bombus ruderarius w	12	0		
Bombus lucorum w	55	0		
Bombus soroeensis w	17	0		
Bombus wurflenii w	13	0		
Bombus sichelii w	9	0		
Pipiza sp. (Syrphidae)	1	1	1: 2	2 = 1,1 %
Leptura annularis (Cerambycidae)	2	1	1: 0 1: 4	4 = 2,2 %
Sum	301	52		179 = 100 %

Fig. 3: Bumblebees as pollinators of the spring form of the burnt-tip orchid *Neotinea ustulata* subsp. *ustulata*: left *Bombus pyrenaeus*, right *Bombus mucidus*. – Abb. 3: Hummeln als Bestäuber der Frühlingsform von *Neotinea ustulata:* links *Bombus pyrenaeus*, rechts *Bombus mucidus*. – Hohe Tauern National Park, Kasereckwiesen, 1700 m, 12 July 2012.

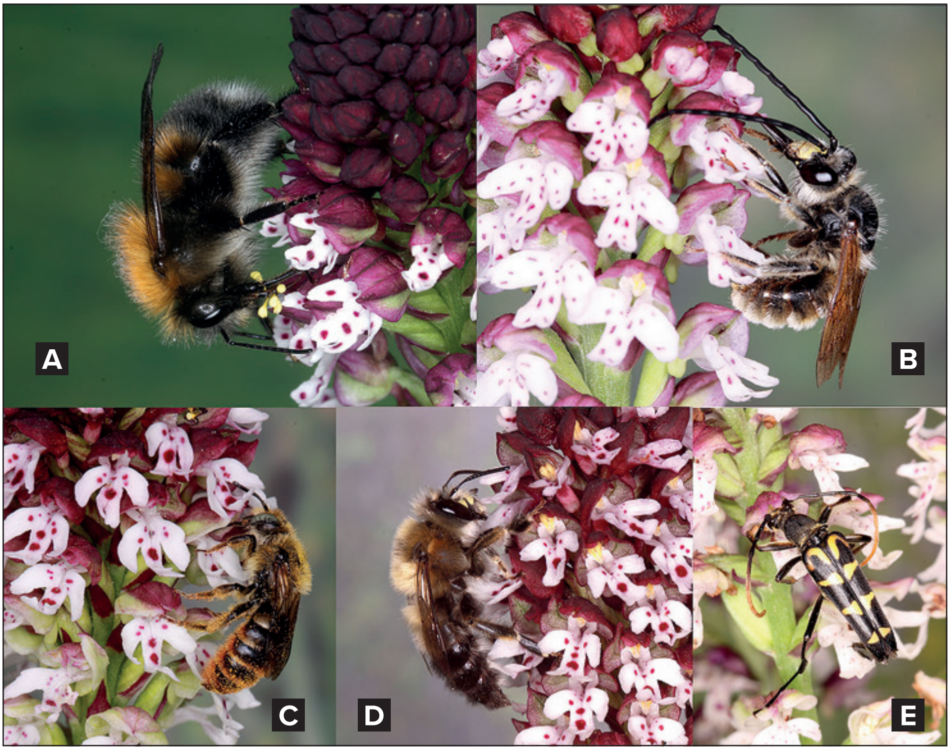

Fig. 4: *Neotinea ustulata* is rarely visited by other wild bees and sometimes even beetles. – Abb. 4: *Neotinea ustulata* wird nur selten von anderen Wildbienen und gelegentlich auch von Käfern besucht. – A. *Bombus hypnorum* male (Apidae) on *N. ustulata* subsp. *aestivalis*: Wien, Lainzer Tiergarten, Aumüllerwiese 27 June 2009, B. *Eucera pollinosa* male (Anthophoridae) on *N. ustulata* subsp. *aestivalis*: northern Burgenland, Frauenwiesen near Loretto 23 June 2020, C. *Osmia rufohirta* male (Megachilidae) on *Neotinea ustulata* subsp. *ustulata*: Wienerwald, Groisbach-Alland 25 April 2020, D. *Anthophora aestivalis* male (Anthophoridae) on N. *ustulata* subsp. *ustulata*: near Vienna, Gießhübl 30 April 2020, E. *Leptura annularis (=Strangalia arcuata* (Panzer, 1793) (Coleoptera, Cerambycidae) on *N. ustulata* subsp. *ustulata*: Hohe Tauern National Park, Mitteralm near Fusch 20 July 2010.

in this area, only two (*Bombus mucidus* and *B. pascuorum*) also visited *Neotinea ustulata*. These two species had 10.0 % of all pollinaria.

Why tachinid flies are attracted by this orchid species, and with which signals, is only partly investigated (Martel et al. 2021). To understand what kind of signals are responsible for this strong attraction, we needed to conduct further experiments.

The stimulus effectiveness of the burnt colour of the top of the inflorescence was tested by Paulus (in Martel et al. 2021) by choice tests between total plants and plants with removed tips. To ensure that the two plants to be tested had the same attractivity, they were tested before as intact plants. They were chosen by *Tachina fera* equally. After manipulation, the flies preferred plants with intact tops over plants with removed tips. After reattachment of the removed top, both plants again had the same attractivity. This demonstrates that the top is an important signal for choosing the orchid inflorescence. But this test did not differentiate between colour alone and a possible (additional?) olfactory signal of this top. To find this out, I repeated these kinds of choice experiments in the summer of 2020, but with the summer form (subsp. *aestivalis*) and with a different, but very similar, tachinid fly, *Nowickia ferox*.

Neotinea ustulata subsp. *aestivalis*

It has long been known that, after the spring form, in some regions there also exists a summer form. First citations are from Vogge & Angelbrodt (1886 cited in Bergfeld 2018), later from Neumann (1905) or Zimmermann (1910) (under "*Orchis ustulatus* L. *biflorens*") and Gumprecht (1981) in SW-Germany (Kaiserstuhl). Early reports for Switzerland are from Danesch & Danesch (1962). First reports for Austria are from Timpe (1987,1989) (southern Burgenland, Steiermark), for Bavaria from Wucherpfennig (1992). A recent review was given by Bergfeld (2018). Meanwhile we know that the summer form is widely distributed in Europe. In Southern Europe the form seems to occur in mountain areas only (unpubl. observations by M. Hirth in Northern Greece). The summer form of the burnt tip orchid starts flowering at the end of June, long after the spring form, and grows in totally different habitats. Plants of the summer form are also much higher than the spring form (up to 80 cm!), presumably because grassland plants are already quite high at this time of year. Besides the clearly separated flowering time and the much taller habitus, flower morphology is only statistically different with an overlap (Martel et al. 2021). But the smell is different: a little like lemon in the summer form, while the spring form smells more like honey. First gaschromatical investigations were done by Martel et al. (2021). According to Kümpel & Mrkvicka (1990) flowers of aestivalis used to be more open in comparison to the spring form. But the differences are small. In fact, some of the older intensive morphometric studies find only weak differences (Tali et al. 2004, 2006; Haraštová-Sobotková et al. 2005). Martel et al. (2021) find a better separation in their multivariate statistical analyses. Kümpel (1988) described the summer form as *Orchis ustulata* var. *aestivalis*, later together with Mrkvicka they gave the rank of a subspecies (Kümpel & Mrkvicka 1990).

As mentioned before, the results on morphology are ambiguous. As summarized in Haraštová-Sobotková et al. (2005), indirect gradient analysis has not shown a distinct separation of early- and late-flowering individuals in the ordination space. The new statistical analyses of Martel et al. (2021), however, found a small but clear difference in all inflorescence measurements. The differences in flower morphology given by Kümpel & Mrkvicka (1991) are not consistent because the two flower forms exist in both types of *ustulata*. As cited by Martel et al. (2021), however, according to MANOVA, populations of early- and late-flowering plants can be distinguished by plant height, leaf length, numbers of basal (rosette) and stem leaves, and even better by certain ratios of these numbers (Fig. 13).

All genetic analyses done by Haraštová-Sobotková et al. (2005), on the other hand, are definite, and consistently distinguish two groups. Random amplified polymorphic DNA (RAPD) markers have shown that the early- and late-flowering populations differ significantly from one another. "Principal coordinate analysis (PCoA) based on a presence/absence matrix of RAPD bands separated the two groups, implying that the difference in flowering phenology could form an effective barrier to gene exchange. Partitioning of genetic diversity in analysis of molecular variance (AMOVA) has shown that the genetic divergence between the two groups, early- and late-flowering populations, is somewhat greater (33 %) than the genetic variability among populations within a particular group (23 %). After elimination of the effect of sample origin from the model, direct gradient analysis (RDA) has shown that the early- and late-flowering groups differ significantly in their RAPD spectra. To conclude, these results indicate the presence of two genetically, phenotypically and phenologically distinct taxa". The astonishing conclusion from a phylogenetic point of view is that, because of the weak morphological differentiation1), they could not decide on another classification, as the taxonomic rank of variety, than subspecies. This is the result of complete typological rather than biological thinking because one of the most important criteria for a biospecies is the evidence of reproductive isolation. The result of a distinct genetic separation between both forms is best interpreted as the consequence of an isolation in reproduction, and of different ecological demands. Beside the strong phenological separation of at least 4 – 6 weeks, both forms grow in completely different habitats: *N. ustulata ustulata* on dry meadows from the end of April to the beginning of June, in higher elevations later, *N. ustulata aestivalis* on wetter meadows (often

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1) Weak morphological difference is of course no argument against systematization of two forms as two different species. In insects or spiders there exist numerous examples of extremely similar species, but with good arguments that these are indeed different biospecies. Another real problem in botanical systematics is that there is no distinct definition of subspecies. Some colleagues use the term for geographically separated populations of the same species (like in zoology), but some use it in the sense of micro-species, or even just as a form of variety. In any case, subspecies is a category **within** the species, with the logical consequence that subspecies are not reproductively isolated from the other individuals of this species.

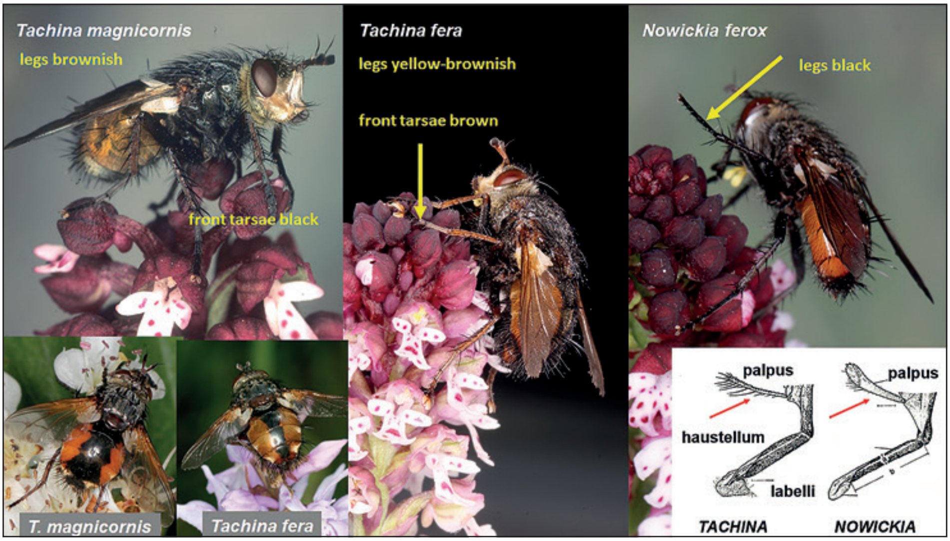

Fig. 5: The three main pollinators of *Neotinea ustulata* are *Tachina magnicornis* and *T. fera* of the spring form, and *Nowickia ferox* of the summer form. These species are not easy to separate. The main characteristics for separation are indicated here. A further differentiation of *Tachina* and *Nowickia* is the shape of the mouthpart palpus (characters from Tschorsnig & Hering 1994). – Abb. 5: Die drei Hauptbestäuber von *Neotinea ustulata* sind *Tachina magnicornis* und *T. fera* auf der Frühlingsform und *Nowickia ferox* auf der Sommerform. Diese drei Arten sind nicht immer leicht zu unterscheiden. In der Abb. sind die wichtigsten Unterscheidungsmerkmale dargestellt. *Tachina* und *Nowickia* unterscheiden sich außerdem in der Gestalt des Palpus. Merkmale nach Tschorsnig & Hering (1994).

together with *Dactylorhiza incarnata*, *Epipactis palustris* or even *Gentiana pneumonanthe*) from the end of June until the middle of August and later.

But the situation regarding the late flowering types is possibly not so quite so clear. Reinicke & Rietdorf (1991) demonstrated that there are possibly three types of *N. ustulata*. Besides the common early variant and the very local late form, there possibly exists a more widely distributed third type which sometimes blooms in the same habitats as the early form. This is supported by morphometric studies by Tali et al. (2006). In their Fig. 2 (p. 18), a cluster analysis from different populations throughout Europe (mainly northern parts) resulted in three types: the early form (*ustulata*), the very late form (*aestivalis*) and a third form which might be provisionally named "*pseudoaestivalis*".

How to recognize *Tachina* and *Nowickia* species

Tachina and *Nowickia* species mainly visit flowers with easily reached nectar, like *Eupatorium*, *Origanum* or *Thymus,* which interestingly have similar colours to *Neotinea ustulata*. This orchid's buds and tips, in particular, correspond with the brownish-purple colour of these nectar offering flowers. Separation of the three species is not easy in the field. Therefore, I provide a picture with all three forms indicating the main differences (Fig. 5).

Situation in eastern Austria (own investigations)

Lainzer Tiergarten (Vienna): Aumüllerwiese

A small population of *N. ustulata* subsp. *aestivalis* is long known from the "Aumüller Wiese" (Lainzer Tiergarten in Vienna), on the clearly wet part of this meadow near open small ponds. I have controlled these plants for some years, normally starting around the end of June or beginning of July with blooming. In some years there are about 25–30 plants. The plants grow together with *Epipactis palustris*, *Gymnadenia cf. densiflora* and many later flowering *Gentiana pneumonanthe*. This meadow is well known and is mowed very late (October). On the "Schattenwiese", nearby, I had only ever found just single plants in some years. In 2020, in the southern part of this meadow, I discovered a further population of about 50–60 plants in late June. Here, the cut time in 2020 was before 20 September. This is too early for successful fruiting of these rare plants.

Weiderwiese near Purkersdorf

This four-part grassland belongs to the old Weidlingauer Bürgerspitalswald 2 forest, now known as the Purkersdorfer Bürgerspitalswald[2)] (Fig. 6). The grassland parts belong to a periodically wet Molinieta with a central part that is only rarely cut. This part harbours the last remaining *Iris sibirica*, which were more frequent in earlier times, and sometimes many *Epipactis palustris*. The three other parts are regularly cut. In 2020 all parts were already cut on 30 July, with the result that all *Neotinea aestivalis* plants had not been able to fruit.

I visited this grassland directly adjacent to the A1 motorway west of Vienna on 24 June 2020 and was able to find about 75 specimens of the summer form full in bloom in the wetter parts of the grassland. Interestingly, in July 2021 not a single plant was flowering.

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2) https://www.geschichtewiki.wien.gv.at/Weidlingauer_B%C3%BCrgerspitalwald_2; http://www.botanische-spaziergaenge.at/viewtopic.php?f=30&t=2086; https://www.bpww.at/de/artikel/biosphere-volunteer-das-war-die-pflegesaison-2020#&gid=1&pid=19

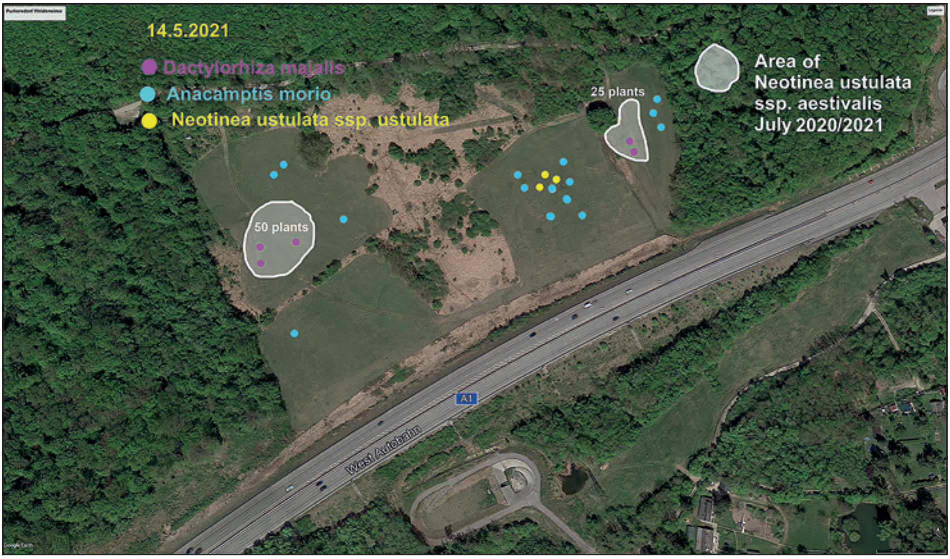

Fig. 6: Small-scale mapping of individual orchid plants on the Grassland „Weiderwiese" near Purkersdorf during spring and summer 2020 and 2021. Local distributions of the orchids *Neotinea ustulata* subsp. *ustulata* (yellow dots), *Anacamptis morio* (blue) and *Dactylorhiza majalis* (purple) in spring time (14 May 2021) and summer time (end of June/beginning of July) (white small areas: *N. ustulata* subsp. *aestivalis* only). The spring form was found only in the dry parts together with *Anacamptis morio,* whereas the summer form occurred in the wet parts of the meadow where the march orchid *Dactylorhiza majalis* was found in spring. This orchid belongs to the typical indicators of wet conditions. Both forms of the burnt-tip orchid can be found in the same area, but are ecologically well separated as the consequence of their different ecological demands. – Abb. 6: Kleinräumliche Kartierung der Individuen verschiedener Orchideen auf der Weiderwiese nahe Purkersdorf im Frühling und Sommer der Jahre 2020 und 2021. Die lokale Verteilung von *Neotinea ustulata* subsp. *ustulata* (= Frühlingsform) (gelbe Punkte), *Anacamptis morio* (blaue Punkte) und *Dactylorhiza majalis* (lila Punkte) im Frühling (14.5.2021). Im Sommer gab es ausschließlich *N. ustulata* subsp. *aestivalis* (weiß umrandete Kleinareale). Die Frühlingsform fand sich ausschließlich in dem zentralen, trockenen Teil zusammen mit *Anacamptis morio*, die Sommerform dagegen ausschließlich in den beiden feuchten Teilen der Wiese, auf denen im Frühling *Dactylorhiza majalis* steht. Letztere Orchidee ist ein typischer Feuchteanzeiger. Auf der Wiese kommen zwar beide Formen von *Neotinea ustulata* vor, sind aber wegen ihrer verschiedenen ökologischen Ansprüchen kleinräumlich klar getrennt.

In the uncut central part, about 10 *Epipactis palustris* were found in summer time. A remarkable aspect is the lack of *Gymnadenia conopsea* aggr., because these orchids were always found together with *Neotinea ustulata* subsp. *aestivalis* in other meadows near Vienna.

The Weiderwiese is the only locality known to me where both forms occur, but with a phenological distance of at least 5 – 6 weeks. On 15 Mai 2021, in the central part only, which is much dryer than the three other parts of the meadow, I found 8 plants together with *Anacamptis morio*. The summer form grew only in the wetter areas where *Dactylorhiza majalis* flowered in spring, as a good indicator for wetness. This means the two forms of *Neotinea ustulata* occur together sympatrically but not syntopically, because of the clear difference in ecological demands (Fig. 6).

Frauenwiese near Loretto (northern Burgenland)

I was recently able to newly discover one further occurrence of the summer form on the protected wet grassland called the “Frauenwiese”, near Loretto (Fig. 7). Here, I found more than 50 plants of the late summer form in full bloom in late June 2020.

Like on the “Weiderwiese”, *N. ustulata* subsp. *aestivalis* is associated here with typical wetland orchids like *Epipactis palustris*. Many *Dactylorhiza incarnata* and even *Dactylorhiza traunsteinera* flower in spring time (Fig. 7). Interestingly, two different *Gymnadenia* taxa of the *conopsea* group grow successively along these wet grasslands. The early form (starting end of May) only has a very weak smell during the day, but stronger at night, the later form (starting middle of June or later) has a strong smell both day and night.

The “Frauenwiese” is a Natura 2000 site[3] with a well-known flora (e.g. Kuyper et al. 1978). Whereas the two species of *Gymnadenia* are a case of an unsolved taxonomical problem (see Dworschak 2001, 2002, Trávníček et al. 2012), the occurrence of *Neotinea ustulata* subsp. *aestivalis* is surprising because these plants must have been overlooked all these years.

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[3] For a good description of this protected area, see page 4 of the enactment document: https://apps.bgld.gv.at/web/landesrecht.nsf/0/8E4051BC0D079496C1257B730025276F/$FILE/EUSchG%20Leithaprodersdorf%20Frauenwiesen%20und%20Johannesbach%20L103-10037-3%20-%20formatiert.pdf?Open;

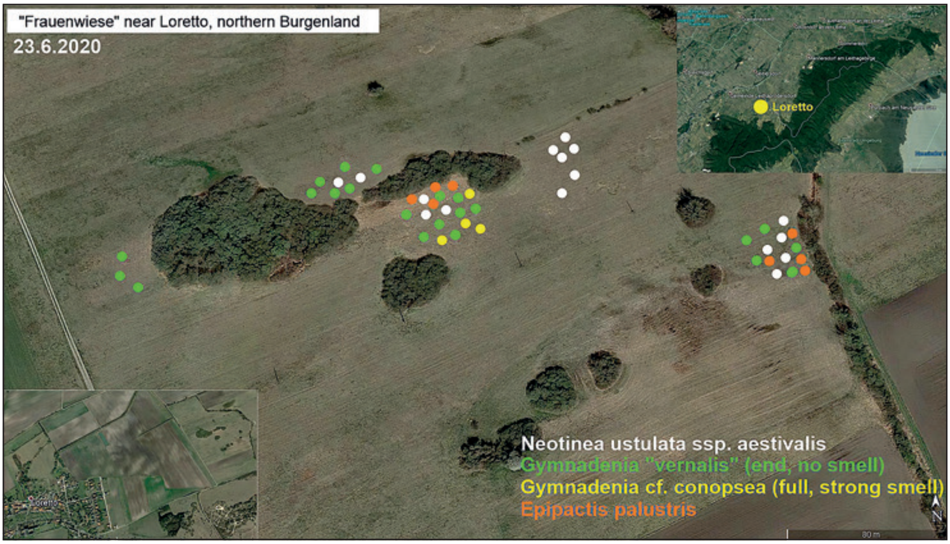

Fig. 7: Local distributions of the orchids *Neotinea ustulata* subsp. *aestivalis* (white dots), *Gymnadenia* cf. *vernalis* (green dots), *Gymnadenia* cf. *conopsea* (yellow dots) and *Epipactis palustris* (orange dots) on the “Frauenwiese” northeast of Loretto (northern Burgenland) on 23 June 2020. Each dot represents an individual or a group of plants of the respective species. *Dactylorhiza incarnata* and *D. traunsteineri* grow within the same habitats in springtime. – Abb. 7: Kleinräumliche Verteilung der Orchidee *Neotinea ustulata* subsp. *aestivalis* (weiße Punkte), *Gymnadenia* cf. *vernalis* (grüne Punkte), *Gymnadenia* cf. *conopsea* (gelbe Punkte) und *Epipactis palustris* (orange Punkte) auf der “Frauenwiese“ nordöstlich von Loretto (N-Burgenland) am 23.6.2020. Jeder Punkt stellt eine Einzel- oder kleine Gruppe von mehreren Pflanzen der betreffenden Arten dar. An denselben Kleinhabitaten stehen im Frühling außerdem *Dactylorhiza incarnata* und *D. traunsteineri*.

It is worth noting that another protected area, the "Stotzinger Heide" is quite close. But this area is a classical Pannonian dry or arid grassland of the so-called Cirsio-Brachypodion type, with the occurrence of orchids typical for this kind of habitat, all flowering in May/ beginning of June: *Ophrys sphegodes, Ophrys holosericea* (in most of the last years nearly one thousand (!) plants[4)]), *Ophrys insectifera* (quite common), *Orchis purpurea, Orchis militaris* and many *Neotinea ustulata* subsp. *ustulata*. The original reason for the official protection of this area was an occurrence of the European ground squirrel (*Spermophilus citellus*), which meanwhile seems to have disappeared. But the protection status is much more justified by the extraordinarily large number of these rare orchids[5)] which are not mentioned in the original floristic description of this area (Krauscher 1983).

New observations of the pollination biology of the summer burnt-tip orchid

Nearly nothing was known about the pollination of the summer form of *N. ustulata*.

Danesch & Danesch (1962: p. 111) found an oedemerid beetle *Chrysanthia* and Mrkvicka (1991) observed the small longhorned beetle *Pseudovadonia livida* (sub *Leptura livida*). Ever since, the summer form has been thought to be pollinated by beetles. But the photo with the oedemerid beetle was taken in Graubünden (Switzerland) at 1900 m above sea level on 2 July 1990, and from the picture alone it is not certain that this was indeed the summer form. Since this small longhorn beetle is rare, and since I never observed

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[4)] I visited this area in 2017 for the first time and counted about 600–700 *Ophrys holosericea*.

[5)] http://forum.flora-austria.at/viewtopic.php?t=882

Fig. 8: *Nowickia ferox* (Tachinidae) was first discovered as a regular pollinator together with many of the summer form of *Neotinea ustulata* subsp. *aestivalis* on the Frauenwiese (northern Burgenland near Loretto): 20 June 2020. – Abb. 8: *Nowickia ferox* (Tachinidae) als Bestäuber entdeckte ich zusammen mit vielen der Sommerform *Neotinea ustulata* subsp. *aestivalis* auf der Frauenwiese (Loretto, N-Burgenland, 20.6.2020).

any flower visits by this beetle, I believe that it is not a regular pollinator. Several times I was, however, able to observe bumblebees pollinating the late form: *Bombus hypnorum* (27 June 2009) and *B. terrestris* (4 July 2009).

Frauenwiese

On 20 June 2020, I observed an old male of *Eucera pollinosa* trying to find the entrance of the flower's spur with its very long proboscis. But even though it worked for nearly one minute, it was unable to remove pollinaria (Fig. 4B). I also observed several individuals of the fly *Nowickia ferox,* which look very similar to *Tachina*. After a closer look at home I realized that these belong to another genus. These flies behave like *Tachina*, landing primarily on the top of the inflorescences and thereafter turning down to the nearest open flowers to put their proboscis into the spur. In nearly all cases pollinaria were removed (Fig. 8).

Schattenwiese (Lainzer Tiergarten)

On 20 July 2020, several males of *Bombus soroeensis, B. terrestris* and *B. lucorum* visited flowers, all removing pollinaria. Interestingly, I again observed 5 individuals of *Nowickia ferox* visiting different plants of *N. ustulata* subsp. *aestivalis*. As described before, they also landed on the top of the inflorescences, but one male landed on the side and moved to the top, visiting several flowers. They all removed pollinaria and should be very successful pollinators.

Fig. 9: *Bombus terrestris* male (left) and *B. lucorum* male (right) are also rarely pollinators of the summer burnt-tip orchid *Neotinea ustulata* subsp. *aestivalis*. – Abb. 9: *Bombus terrestris*-Männchen (links) und *B. lucorum*-Männchen (rechts) sind ebenfalls selten Bestäuber der Sommerform des Brandknabenkrautes *Neotinea ustulata* subsp. *aestivalis*. – Weiderwiese near Purkersdorf 26 June 2020.

Weiderwiese (Purkersdorf)

On 24 June 2020 I observed several males of *Bombus terrestris* and one male of *Bombus lucorum* (Fig. 9) visiting flowers of *N. ustulata* subsp. *aestivalis*. On 2 July 2020 I observed some individuals of the small longhorned beetle *Pachytodes erraticus*. But none of these specimens removed pollinaria. The beetles normally sat on the flowers of *Inula salicina* to feed on pollen. I caught one male of *Nowickia ferox* with about 6–7 pollinaria on its proboscis, which surely originated from *N. ustulata* subsp. *aestivalis* as there were no other orchids in the vicinity. On 20 July 2020 I still saw many *Neotinea* plants in peak flowering period. Unfortunately, the meadow was cut on 30 July 2020. No plants could be found any more to count fruiting success.

What is the significance of the burnt-tip: Experimental field work with *N. ustulata*

The difficulty with this experimental work is that it can only be done when there are enough flies active in the test area lacking previous experiences with *Neotinea ustulata*. This is rarely the case. The other method is to sum up results of many years of observations.

N. ustulata subsp. *ustulata*

In the middle of May 2017 I had the opportunity for some experimental observations with the spring form and flying *Tachina fera* near Gießhübl (near Vienna). Most of the *Tachina* were visiting *Thymus* (possibly *praecox*) or *Knautia* for nectar. Two picked plants of *N. us-*

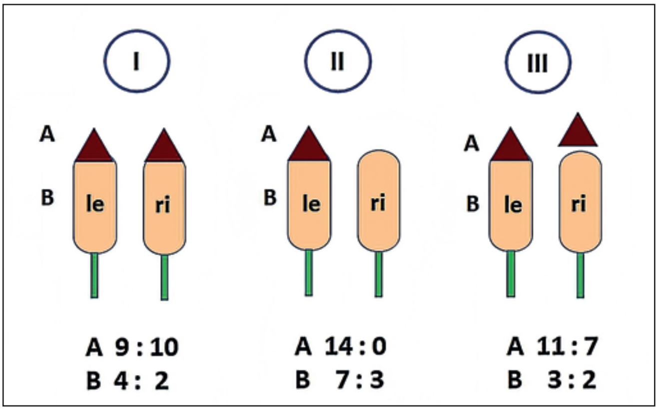

Fig. 10: Three types of experiments as choice tests with flies of *Tachina fera*. I. two intact plants, II. right plant with the burnt tip removed, III. right plant with the removed tip re-attached. Two types of responses were counted: A: landings on the top, B: landings on the side of the inflorescence. Numbers are the results each of a 60 min test. Total number of visits in I: 25, in II: 24 and in III: 23. – Abb. 10: Drei Typen von Wahlexperimenten mit der Fliege *Tachina fera* wurden durchgeführt: I. zwei intakte Infloreszenzen, II. rechte Kappe der Pflanze wurde entfernt, III. die entfernte Kappe der rechten Pflanze wurde wieder befestigt. Gezählt wurden zwei Typen von Landungen: A. Landungen einer Fliege auf der Kappe, B. Landungen an der Seite der Infloreszenz. Jeder Experiment-Typ wurde 60 min. beobachtet und gezählt. Gesamtzahlen der Landungen für Typ I: 25, Typ II: 24 und Typ III: 23.

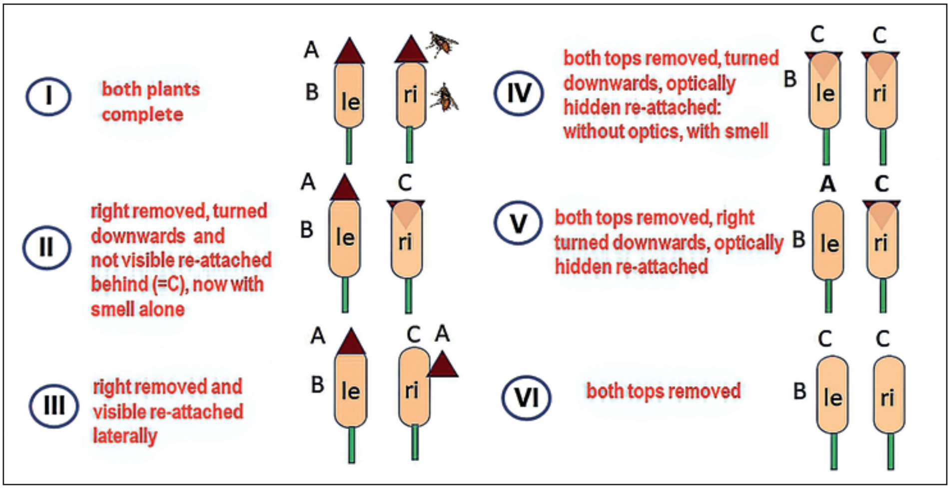

Fig. 11: Six types of choice experiments with free flying summer tachinid fly *Nowickia ferox* and *Neotinea ustulata* subsp. *aestivalis*. Similar as in experiments with the spring form (Fig. 10) I used intact plants and different types of removed and re-attached tips in choice tests between two plants. I. both plants complete, II. right tip removed and re-attached but more or less concealed from the flies, III. right tip visibly re-attached, IV. tips of both plants removed and re-attached but not visible, V. left tip completely removed, right cap re-attached but not visible, and VI. both tips completely removed. A= landing on the tip, B= landing laterally, C= landing on the top of the inflorescence with the concealed or removed tip. Each test situation was presented for 30 minutes. – Abb. 11: Dargestellt sind die 6 Typen von Wahl-Experimenten mit frei fliegenden *Nowickia ferox* und *Neotinea ustulata* subsp. *aestivalis*. Ähnlich wie bei den Experimenten mit der Frühlingsform (Abb. 10) wurden zunächst intakte Pflanzen und dann verschiedene Kombinationen mit entfernten Spitzen und wieder befestigte Spitzen der Infloreszenzen in Wahltests angeboten. Test I: zwei intakte Pflanzen, Test II: rechte Kappe entfernt, aber mehr oder weniger für die Fliegen unsichtbar wieder befestigt, Test III: rechte Kappe wieder sichtbar befestigt, Test IV: beide Kappen entfernt und wieder für die Fliegen nicht sichtbar befestigt, Test V: linke Kappe völlig entfernt, rechte Kappe wieder sichtbar befestigt, Test VI: beide Kappen völlig entfernt. A= Landungen auf der Kappe, B= Landungen seitlich an der Infloreszenz, C= Landungen auf der Spitze, Kappe aber entfernt oder unsichtbar seitlich wieder befestigt. Jede Testsituation wurde 30 Minuten geboten.

tulata were put in two small, water filled vases. They were presented to flying *Tachina* flies side by side, so that they could choose the left or the right plant to land on.

Three types of experiments were conducted (Fig. 10):
two unmanipulated plants,
one plant with removed top, the other plant intact
one plant with re-attached top, the other plant intact.

At first the two plants were presented unmanipulated (exp. Type I) to see if both inflorescences had the same attractivity (frequencies of choice nearly 1: 1, see Fig. 10). Then I started the main choice tests by cutting the dark tip of one of the plants (exp. Type II) to see if the burnt tip is an important signal for the *Tachina* flies. The result clearly indicated that the tip is important, as the plant with the removed tip now received no visits in comparison to the intact plant. After re-attaching the tip, attractivity increased again (Type III) (choice frequency now 11: 7).

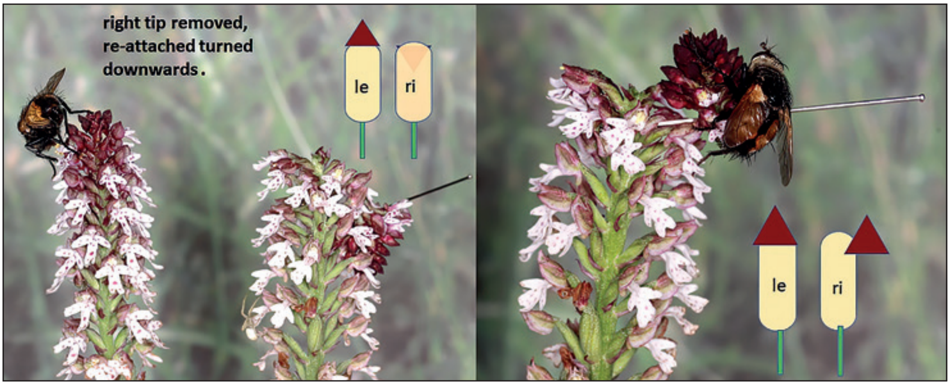

Fig. 12: Two examples of the test situation of *Neotinea* subsp. *aestivalis* and the fly *Nowickia ferox*. Left picture shows the experiment type II: right tip removed and re-attached but concealed to present smell only. A fly has landed on the tip of the intact plant. The right picture shows experiment type III (only the right plant is shown): the previously cut tip was re-attached on the top, so both signals (visual and smell) could attract together. – Abb. 12: Fotos von zwei Beispielen der Testsituationen nach einer Landung von *Nowickia ferox*. Linkes Bild zeigt die Situation von Testtyp II (linke Pflanze intakt, rechte Pflanze mit der Spitze entfernt und unsichtbar wieder seitlich befestigt): eine Fliege ist auf der intakten Infloreszenzspitze gelandet, rechtes Bild zeigt die Testsituation III, hier nur rechte Blüte dargestellt: eine Fliege ist auf der wieder befestigten Spitze gelandet.

Despite the low numbers of reactions overall, these tests indicate that the burnt tip might be an important releasing signal. The landing responses on the lateral parts of the inflorescences were indiscriminate ("B" in Fig 10). This means the tip of the inflorescence seems to be an important attractor for the main pollinator, the *Tachina* flies, and therefore for pollination success. Bumblebees or solitary bees never landed on the top of the plant but always on the sides.

Some of these experimental field work data were published by Paulus in Martel et al. (2021). But these results did not differentiate between the visual cue of the tip and a possible special smell of this tip. We suppose an olfactorial sexual component in the attraction of male tachinid flies, as it was supposed for *Anacamptis papilionacea* (Scopece et al. 2009). It is possible that both types of signal are responsible for the attraction, or even the smell alone! Therefore, both signal types have to be tested separately. This is precisely what I tried with *N. ustulata* subsp. *aestivalis* and the newly discovered main pollinator, the tachinid fly *Nowickia ferox*.

N. ustulata subsp. *aestivalis*

In two different areas with about 25–30 free flying *Nowickia ferox* visiting mainly *Origanum,* I conducted six types of choice experiments with the summer form *Neotinea ustulata* subsp. *aestivalis* to investigate the colour and smell significance of the burnt tip of this orchid. Similar to the experiments with the spring form (Fig. 10), I first used intact plants and then different types of removed and re-attached tips in choice tests between two plants (Fig. 11). The results of all observations and counts are summarized in Tab. 2.

Some of these results are important for an understanding of the tip. Both colour and a special smell seem to be important for the attraction of the fly *Nowickia*. Plants without this coloured tip became nearly entirely unattractive (exp. Type VI). The colour of the tip seems to be more important than the smell (exp. Type II and III), but the smell is also important (Type V). Similar to the experiments with the spring form and another tachinid fly (*Tachina*), it became evident that the tip of the burnt-tip orchid seems to be an important releasing signal for the attraction of the main pollinator of the summer form, *Nowickia ferox*.

Tab. 2: Summary of the choice tests with two intact plants and different types of removed and reattached tips in the choice test Types I – VI, as seen in Fig. 11. Sum of all landings: n = 72. – Tab. 2: Zusammenfassung aller sechs Typen (I – VI) Wahltests zwischen zwei kompletten (I) und in verschiedener Weise manipulierten Infloreszenzen wie sie in Fig. 11 dargestellt sind. Summe aller Landungen N = 72.

experiment type	left plant	right plant	proportions left : right	preliminary explanations
I	A=5 B=3	A=5 B=2	$A_{(le)} : A_{(ri)}$=5:5= 1.0 : 1.0 $B_{(le)} : B_{(ri)}$=3:2= 1.5 : 1	top was preferred
II	A=8 B=3	A=2 B=3	$A_{(le)} : A_{(ri)}$=8:2= 4.0 : 1 $B_{(le)} : B_{(ri)}$=3:3= 1.0 . 1.0	plant with visible top was preferred over plant with invisible top but with smell
III	A=7 B=3	A=5 B=2 C=0	$A_{(le)} : A_{(ri)}$=7:5= 1-.4 : 1 $B_{(le)} : B_{(ri)}$=3:2= 1.5 : 1	intact plant (le) was slightly preferred over manipulated plant
IV	C=3 B=1	C=3 B=2	$A_{(le)} : A_{(ri)}$=3:3= 1 : 1 $B_{(le)} : B_{(ri)}$=1:2= 1 : 2	no preferences
V	A=2 B=2	C=5 B=2	$A_{(le)} : C_{(ri)}$=2:5= 1.0 : 2.5 $B_{(li)} : B_{(re)}$=2:2= 1.0 : 1.0	plant without visible top but with smell slightly preferred over plant without top or smell
VI	C=1 B=1	C=0 B=2	$C_{(le)} : C_{(ri)}$= 1:0 $B_{(le)} : B_{(ri)}$= 2:3	weak attraction, no preferences

Discussion

Pollination system

First observations of pollination of *Neotinea ustulata* involved the tachinid flies *Tachina magnicornis* (Vöth 1984). Individual observations of more accidental visits are given in Paulus (2005) and in the book by Claessens & Kleynen (2016). Further data are summarized in Martel et al. (2021). A more quantitative investigation is presented in this publication. 86.5 % of pollinaria carried by pollinator individuals in a special growing site of the spring form of *Neotinea ustulata* were found on the proboscides of *Tachina fera*, most of them in males (78.7 %). This means that the main pollinators are *Tachina fera* males, and only to a lesser extent females (7.8 % of the pollinaria). Sometimes I could also observe bumblebees and other wild bees visiting the flowers. But in all cases, these were only single individuals. Even if they may contribute to pollination, from an evolutionary point of view they will not (or only slightly) contribute to flower selection. Interestingly, the summer form is more frequently pollinated by *Nowickia fera*, a near relative of *Tachina* species. *Nowickia fera* is active in summer, whereas *Tachina* flies in two generations, one in spring, the other in late summer. The late phenology of the summer form of *Neotinea ustulata* might be selected by this tachinid fly. But this has yet to be proven in other parts of the distribution area of *N. ustulata* subsp. *aestivalis*. Therefore, it is possible that the special colour in both forms of *Neotinea ustulata*, the so-called "burnt tip", together with the supposed smell of *Neotinea ustulata* are signals mainly for *Tachina* and *Nowickia*. To find out if this "burnt tip" is a signal for *Tachina* and *Nowickia* I started with some preliminary field experiments. Results were that the dark-coloured tip seems important for attraction and that the smell of this tip is possibly also important. But this did not explain why Tachinid flies were attracted exactly by these compounds. Therefore, further experiments with more data for better statistical analyses and a more detailed investigation of the chemical compounds are needed.

Possible evolution of the pollination system in *N. ustulata*

To understand in which way *Neotinea ustulata* could have modified its attraction mode, we have to consider how the next relatives attract pollinators. *Neotinea* species, like the other members of the related genera *Orchis* and *Anacamptis* s.lat., deceive floral visitors for pollination by not offering any reward for their services (Sprengel 1793, Nilsson 1981, Dafni 1984, Paulus 2005). This is also the case in the two forms of *N. ustulata*. Indeed, both varieties are visited by some generalist insects like bumblebees or solitary bees seeking nectar, and the flower lip has UV light-absorbing coloured spots, like other typical bee flowers, as first discovered by Osche (1983). But the main pollinators are members of the fly family Tachinidae, especially *Tachina magnicornis* and *fera* in the spring form and *Nowickia ferox* in the summer form. However, other floral traits such as a strong and complex scent, the evolutionary innovation of a dark tip on the inflorescence and the common visits by mainly male flies suggest that a pollinator system other than just food deception might have evolved. The production of special alkenes is a derived trait within *Neotinea ustulata* and the most abundant alkenes have been also detected in the cuticular extracts of *Tachina* flies (Martel et al. 2021), which supports the idea of some kind of chemical mimicry. Sexual chemical mimicry in tachinid-pollinated systems has been reported for the South American sexually deceptive *Telipogon* orchids (Martel et al. 2019) and something similar might occur in *N. ustulata*. *Neotinea ustulata* and *Telipogon peruvianus* share a similar pattern of alkanes and alkenes, and their bouquets are dominated by unsaturated hydrocarbons, (*Z*)-11-tricosene and (*Z*)-9-tricosene (Martel et al. 2016, 2021). The hypothesis is therefore that *N. ustulata* has evolved a mixed pollination system: food deception and a kind of not really understood sexual deception. Thus, the pollination system in *N. ustulata* might have evolved from a rather generalist food deceptive system to a specialist system which combines sexual deception and food deception. Indeed, specialization from food deception to sexual deception systems has occurred among European (Inda et al., 2012) and some non-European orchids (Johnson et al. 2013). Another European orchid, *Anacamptis papilionacea* (L.) R.M. Bateman, Pridgeon & M.W. Chase, was also proposed as having evolved a pollination system involving food deception and a kind of sexual deception (Scopece *et al*., 2009), but no experimental evidence supports this so far.

Taxonomical position of the two phenological forms

As Timpe (1987) and Kümpel & Mrkvicka (1990) have already pointed out, both forms never grow in the same habitats: the late form always grows in wet grassland habitats, whereas the spring-form can only be found in dry grassland or similar habitats. This strengthens a clear difference in ecological demands of both forms.

Both forms have different main pollinators: the spring form is mainly pollinated by *Tachina fera* and *T. magnicornis*, the summer form mainly by *Nowickia ferox*. But this might be just a consequence of late flowering because *Nowickia ferox* is a summer species whereas the first generation of *Tachina* species starts in spring, a second-generation late summer (Tschorsnig & Herting 1994). This means *Nowickia ferox* is active just in the gap time between the first and second generation of the two *Tachina*.

If one considers the criteria for a biospecies then the two forms belong to two different species! Biospecies are defined as: 1. morphological units (Fig. 13), 2. ecological units, and 3. genetic units. The classification as varieties in spite of clear ecological and genetic but weak morphological differences (as argued by Haraštová-Sobotková et al. 2005) contradicts the biological facts, especially the evidence of reproductive isolation of both “forms”. The classification as subspecies[6)] also denies the biological differences, because even as subspecies they would belong to the same species. This also ignores the genetic and ecological independencies as a consequence of reproductive isolation. There are further examples of clear differences in European orchids which are therefore justifiably classified as biospecies: *Gymnadenia conopsea* (s.l.)[7)] and *G. densiflora* (Stark et al. 2011) or *Dactylorhiza incarnata* and *D. ochroleuca* (Filippov et al. 2017).

The new combination at the species level:
Neotinea aestivalis (Kümpel 1988) Paulus H.F. comb.nov., basionym: *Orchis ustulata* var. *aestivalis* Kümpel 1988, Haussknechtia 4, 23–24.

Synonyma:
Orchis ustulata L. subsp. *aestivalis* (Kümpel 1988) Kümpel & Mrkvicka 1990
Neotinea ustulata (L.) subsp. *aestivalis* (Kümpel 1988) Jacquet & Scappaticci 2003
Neotinea ustulata (L.) var. *aestivalis* (Kümpel 1988) Tali, Fay & Bateman 2006

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6) In botany the term subspecies is used either like in zoology – for geographically separated, slightly different populations of a species – or in the sense of micro-species, which can even occur syntopically; nevertheless, formally this term is a subordination within a species with the biological consequence that these should not be reproductively isolated. This is the wrong way to express that even micro-species are in reality different biospecies.

7) *G. conopsea* s.l. seems to be an aggregate of further different biospecies (Dworschak 2001).

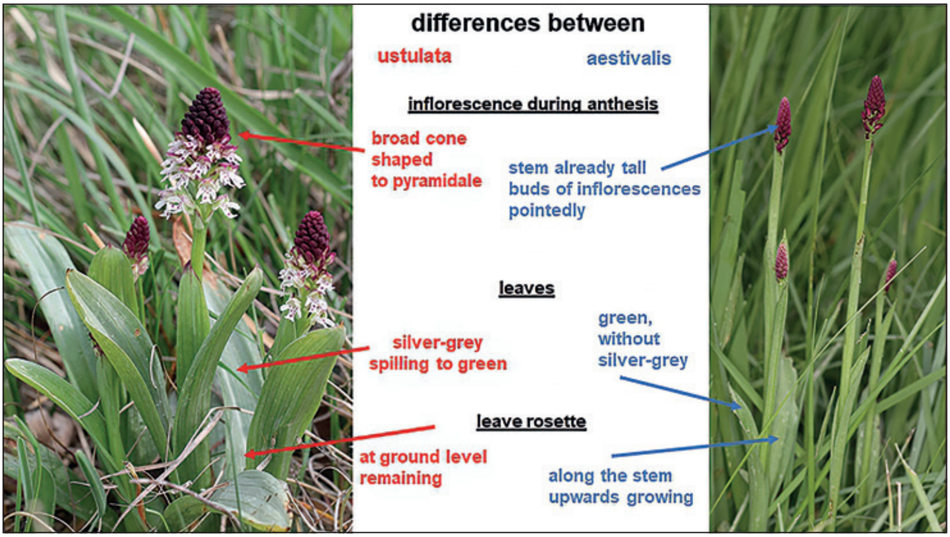

Fig. 13: Main morphological differences between the two phenological forms of the burnt-tip orchid *Neotinea ustulata;* left: spring form (Perchtolsdorfer Heide 2 May 2020), right: summer form (Wien, Lainzer Tiergarten, Aumüllerwiese 2 July 2016), both just starting flowering. The different characteristics of the two forms are written between the figures. These morphological differences together with ecology, phenology and genetics justify that the two forms are different biospecies. – Abb. 13: Wesentliche morphologische Unterschiede zwischen den beiden phänologischen Formen des Brandknabenkrautes *Neotinea ustulata*. Links: Frühlingsform *N. ustulata* subsp. *ustulata* (Wien, Perchtolsdorfer Heide 2.5.2020), rechts: Sommerform *N. ustulata* subsp. *aestivalis* (Wien, Lainzer Tiergarten, Aumüllerwiese 2.7.2016); beide abgebildeten Pflanzen gerade beim Aufblühen. Die entsprechenden Merkmale beider Formen sind zwischen den Fotos aufgeführt. Diese morphologischen zusammen mit ökologischen, phänologischen und genetischen Unterschieden begründen, dass beide Formen unterschiedliche Biospezies sind.

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