Ecological interactions of the sexually deceptive orchid *Orchis galilaea*

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**Abstract**

Plant species dependent on highly specific interactions with pollinators are vulnerable to environmental change. Conservation strategies therefore require a detailed understanding of pollination ecology. This two-year study examined the interactions between the sexually deceptive orchid, *Orchis galilaea*, and its pollinator *LasioGLOSSum marginatum*. Relationships were investigated across three different habitats known to support *O. galilaea* (garrigue, oak woodland, and mixed oak/pine woodland) in Lebanon. Visitation rates to flowers were extremely low and restricted to male bees. The reproductive success of *O. galilaea* under ambient conditions was 29.3% (±2.4), compared to 89.0% (±2.1) in plants receiving cross-pollination by hand. No difference in reproductive success was found between habitat types, but values of reproductive success were positively correlated to the abundance of male bees. Pollination limitation can have negative impacts on the population growth of orchids, and this study provides clear evidence for more holistic approaches to habitat conservation to support specific interactions.

**Introduction**

Orchidaceae is one of the largest and most diverse families of the flowering plants in the plant kingdom, containing an estimated 25,000–30,000 species (Kull and Hutchings 2006). The majority of species within the family have highly specialized adaptations to enhance pollination by insect pollinators (Vereecken et al. 2010; Kindlmann and Roberts 2012).

Orchids adopt different strategies to attract pollinators, but approximately a third do not offer nectar, and their pollen is rarely considered a food source (Dressler 1993). In fact, many orchid species from the Mediterranean region do not possess nectar, attracting pollinators through food deception (*Dactylorhiza* genus), shelter deception (*Serapias* genus), or sexual deception (*Ophrys* genus) (Dressler 1993; Vereecken et al. 2010; Kindlmann and Roberts 2012).

Globally, there are 400 species of orchids in 18 genera that are sexually deceptive, attracting bees, wasps or other insects (Cozzolino and Widmer 2005; Jersáková et al. 2006). Sexually deceptive orchids mimic mating signals of female insects (e.g. sex pheromones, shape of the female bee), which induces pre-copulatory behavior in males (Vereecken 2009; Xu et al. 2012). Throughout the Mediterranean region, orchids in the genus *Ophrys* mainly exhibit this behavior, and in Lebanon, *Orchis galilaea* is the only known sexually deceptive orchid belonging to the *Orchis* genus. This is also Lebanon’s only narrow endemic orchid species (Cozzolino and Widmer 2005). Self-fertilization (autogamy) in *O. galilaea* does not occur (Bino et al. 1982), and flowers produce a musk-like scent that mimics the female sex pheromones to attract male bees of *LasioGLOSSum marginatum* (Bino et al. 1982). *LasioGLOSSum marginatum* is distributed throughout Europe, particularly in the Mediterranean region. It is a social, polylectic species that forms the largest colonies of any bee in the halictine tribe (McGinley 1986).

*Orchis galilaea* is considered an endangered and threatened species (Kretzschmar et al. 2007), and whilst Lebanon provides a stronghold for the species, populations have been recorded in neighboring Palestine and Jordan (Bino et al. 1982). It grows in a variety of different habitat types at altitudes ranging from 100 to 1130 m above sea level (Delforge 2006). It is found on alkaline soils (~pH 8), especially on moderately dry and stony subsoil, and is associated with bleached rendzina and calcareous red soils ‘Terra Rossa’ (Kretzschmar et al. 2007). *Orchis galilaea* affords protection from herbivory by growing amongst thorny shrubs in phrygana communities, especially those consisting of *Sarcopoterium spinosum* (thorny burnet). It is also found in garrigue habitats of *Quercus calliprinos* (Palestine oak) and *Calycotome villosa* (spiny broom), as well as in open grassland surrounded by bushes of *Q. calliprinos*. However, it is also present in open pinewoods, with sparse shrub cover.

Sexually deceptive orchids usually have narrow ecological niches and, as with *O. galilaea*, are typically pollinated by specialized bees. Consequently, they are highly vulnerable to local extinction (Kindlmann and Roberts 2012). Such intricate plant-pollinator interactions are prevalent in the Mediterranean region, which highlights the importance of targeted approaches for the conservation of endangered species (Vereecken et al. 2010). Studies investigating the population ecology of threatened and vulnerable species have the potential to enhance our understanding of how species should be managed. The reproductive success of plants (defined as the percentage of flowers that develop into fruits) is an important aspect of population ecology due to its direct influence on...
recruitment and therefore the potential for a species to become extinct (Jacquemyn et al. 2002a).

Studies on the ecology of *O. galilaea* are very limited, particularly with regards to pollination. However, Bino et al. (1982) investigated the pollination ecology of *O. galilaea* in Palestine, and whilst it was confirmed that *O. galilaea* adopts a sexually deceptive pollination strategy, many aspects of this interaction and the implications for reproductive success and population dynamics are not known. For example, although *O. galilaea* is able to grow in a range of habitat types, its reproductive success might depend on the abundance of its pollinator, *L. marginatum*, which in turn is likely to be influenced by habitat type and the availability of forage plant species (Potts et al. 2006). A key concern for Mediterranean orchids is the lack of sufficient pollen transfer due to inadequate pollination (Pellegrino et al. 2015), which could be linked to habitat quality (Chi and Molano-Flores 2015). Consequently, a more holistic strategy for the conservation of rare endemic species might be required, rather than species-specific measures.

The aim of this study was to investigate the interactions between *O. galilaea* and its pollinator *L. marginatum*, and to investigate whether the abundance of *L. marginatum* was related to the reproductive success of *O. galilaea*. Due to the occurrence of *O. galilaea* in a number of habitat types, a further aim was to investigate whether reproductive success differed between habitats. Specifically, the study tested the following hypotheses: (1) male *L. marginatum* are the sole pollinators of *O. galilaea* in Lebanon; (2) the reproductive success of *O. galilaea* is not pollinator limited; (3) the reproductive success of *O. galilaea* is related to *L. marginatum* abundance; (4) the abundance of *L. marginatum* is related to habitat type and the availability of forage plant species; and (5) the reproductive success of *O. galilaea* is related to habitat type.

Materials and methods

Study sites

Throughout Lebanon, sites were selected according to the three different habitat types in which *O. galilaea* typically grows: garrique, oak woodland, and mixed (oak/pine) woodland. Populations of *O. galilaea* were identified from field explorations during the flowering period from February to May in 2010 and 2011, allowing individuals to be readily identified. This included visiting areas previously cited in the literature and from personal communications with naturalists, and colleagues (García and Guzmán 2002). Some sites were not accessible due to the presence of landmines and issues with personal safety and security. In total, thirteen sites were selected; most were along the western slopes of Mount-Lebanon, ranging in altitude from 291 to 1256 m above sea-level (Appendix Table 1). Irrespective of habitat type, all sites were either on Terra Rossa (five sites), or mixed soils (eight sites). The minimum distance between any two sites was 720 m, whilst the average distance between sites was 5.75 km. Twelve of the sites were studied for two full years (2011 and 2012), whilst in 2012, due to human interference, one site (Zedbine 1) was replaced (Kafount).

Within populations, the distribution of *O. galilaea* is mostly patchy rather than consisting of randomly scattered individuals (N. Machaka-Houri, personal observation). For the purpose of this study, a population of *O. galilaea* was defined as all flowering individuals located within a 20–50 m radius from the first patch found in a particular location. A patch was defined as a continuous aggregation of at least two individuals separated by at least two meters from neighboring patches (Dauber et al. 2010; Tscheulin and Petanidou 2010). Based on the 200 m dispersal range of *L. marginatum* (S. Roberts, personal communication, 2010), selected populations of *O. galilaea* were at least 500 m apart (Coates and Duncan 2009; Dauber et al. 2010). Within each population, a maximum of four distinct patches of *O. galilaea* plants were selected for measuring reproductive success (Tscheulin and Petanidou 2010).

Habitat site descriptions

Trees and shrubs common to all three habitat types were *Quercus calliprinos*, *Sarcopoterium spinosum*, *Calycotome villosa*, *Cistus creticus* and *C. salviifolius*. However, oak woodlands were dominated by *Quercus infectoria*, *Pistacia palaestina* and *C. salviifolius*. The mixed woodlands were dominated by *Pinus pinea* or *P. brutia*, *Quercus infectoria* and *Cistus creticus*, whilst the garriques were dominated by *Sarcopoterium spinosum*, *Cistus creticus* and *C. salviifolius*.

Pollinator surveys

Pollinator surveys were carried out in 2011 and 2012 during March and April, when *O. galilaea* was flowering. In order to assess the abundance and visitation rates of pollinators, two methods were used: (i) direct (video) observation, and (ii) pan trapping. These methods were carried out only in favorable weather conditions: at temperatures between 20°C and 25°C and wind speed 0–5.4 m s⁻¹ (Moron et al. 2009), avoiding cloudy or rainy days (Dauber et al. 2010; Tscheulin and Petanidou 2010).

Direct observations

Video cameras were used to estimate insect visitation rates to *O. galilaea* rather than direct personal observation because pollination events were rare; the use of video cameras was more time effective. Video observations were recorded in selected patches within each population (Dauber et al. 2010; Tscheulin and Petanidou 2010). The video camera was set up to record insect activity simultaneously on between one and three orchid spikes (individual plants of *O. galilaea* only produce one flowering spike) from 09:00 to 16:30 h over a period of 21 days. The number of flowers visited was determined and visits to different flowers by the same individual insect was treated as a separate event (Nielsen et al. 2012). Each insect visitor was recorded and assigned to the best recognizable classification unit (Tscheulin and Petanidou 2010). The type of insect visit was also determined (Dauber et al. 2010); if the insect hovered around the flowers it was counted as an ‘approach’, whereas when the insect landed on a flower it was counted as a ‘visit’; if bees flew out with pollinia attached to their heads it was recorded as a ‘pollinia attachment’ (Bino et al. 1982). For each visit, the insect visitation time per flower was determined, which started when an insect first made contact with a flower and stopped when contact was broken (Kearns and Inouye 1993). The visitation rate was calculated by dividing the total number of approaches, visits, or pollinia attachments observed, by the total hours of observation.
Pan trapping
To determine the abundance of *L. marginatum*, five sets of three different colored pan traps (yellow, blue, and white), were set up at each site during the flowering season of the orchid (late March to April). Pan Traps were made from plastic bowls 13.5 cm in diameter and 6 cm deep. The bottom of the container was filled with water and a drop of detergent (Dafni et al. 2005). They were placed on the ground in the core area of each site from 09:00 h and removed at 17:00 h. Sets of pan traps were deployed at least 10 m apart and at least 1 m from the boundary of the study area. Traps were placed in areas exposed to the sun to maximize the visibility to insects. Pan traps that were deployed across all sites during each field season (2011 and 2012), for a total of 13 days each year during March/April.

All insects were identified to order and *Lasioglossum* to species level. Voucher specimens kept at the Natural History Museum of the American University of Beirut (AUB) were used to confirm records. The sex of the *L. marginatum* bees caught in the pan traps was also determined (Bino et al. 1982).

Reproductive success of *Orchis galilaea*
In each orchid population, 10 plants per patch were randomly selected and marked with wooden tags (Coates and Duncan 2009). When there were fewer than ten flowering individuals in a patch, all individuals were marked (Jacquemyn et al. 2002b). During the flowering season (March to April) the number of flowers on each tagged plant were counted (Hansen and Olesen 1999). To determine reproductive success, the number of fruit capsules per marked plant were counted.

Pollination limitation
The pollination experiment was undertaken to investigate pollinator limitation and reproductive success at three different sites according to habitat type (garrigue, oak woodland, and mixed (oak/pine) woodland). Only sites providing protection from human disturbance were used, these were Maasser (garrigue), Chhim (oak woodland) and Burjiein (mixed woodland) (Appendix Table 1). The experiment was conducted over two years (2011 and 2012). Three different treatments were investigated: (i) ambient pollination (not bagged), (ii) pollinator exclusion (bagged), and (iii) cross-pollinated and bagged.

Thirty flowering plants (when possible) were randomly selected at each of the sites. Twenty individual flowering spikes were covered with nylon mesh bags prior to flowering (Brzosko 2003). Ten of the bagged spikes were randomly selected for hand cross-pollination and ten were left bagged but untreated to test for spontaneous autogamy (seed production in the absence of pollinators). The bags were left in place until after fruit set (Brzosko 2003). The remaining ten plants acted as controls to investigate the potential for ambient (natural) pollination. Cross-pollination by hand was performed at the peak of the flowering season; five flowers per flowering spike were randomly selected for cross-pollination and marked. Cross-pollination involved removing orchid pollinia from plants at least two meters away with a toothpick and transferring it to the stigmatic surface of the recipient. This helps to ensure out-crossing (Elliott and Ladd 2002). Five flowers were also randomly selected and marked on the bagged untreated plants, and the un-bagged control plants (Huda and Wilcock 2008). The number of fruits produced according to treatment was determined at maturity (typically in June).

Vegetation surveys
To evaluate the plant community composition of the different habitats and therefore the potential availability of resources for *L. marginatum*, vegetation surveys were performed in 2011 and 2012. In each habitat a total of ten quadrats measuring 1 m × 1 m were used to assess vegetation composition. Five quadrats were positioned within patches of *O. galilaea* and five were randomly positioned outside of patches at least 10 m away. If there were fewer than five patches of *O. galilaea*, the number of quadrats assessed was equal to the number of patches available. Within each quadrat, all plant species were identified and assigned a percentage cover value. Plant species co-flowering with *O. galilaea* that are known to provide pollen for *L. marginatum* (S. Roberts, personal communication, 2010) were recorded. Plant nomenclature followed Tohmé and Tohmé (2007), except for orchids which were identified according to Delorge (2006).

Statistical analysis
Data obtained on the visitation rates of *L. marginatum* to flowers of *O. galilaea* according to habitat type (garrigue, oak woodland, and mixed (oak/pine) woodland) and year (year one and year two) were analyzed using SAS Studio (Version 3.5 2016). A mixed linear model was used to investigate responses with regards to the average number of approaches per hour, the average number of visits per hour, and the average number of pollinia attachments per hour. Habitat type and year, including the interaction between these factors were set as fixed effects. Year was specified as a repeated measure with an autoregressive covariance structure. Site was specified as a random effect. Degrees of freedom were calculated using the iterative Satterthwaite’s method (Schabenberger and Pierce 2002). To investigate the influence of pollination treatment (ambient pollination, pollinator exclusion, and cross-pollinated) on reproductive success the same mixed model was used, but treatment was also included as a fixed effect. Model simplification was performed by deleting interactions that were not significant (*P* > 0.05), then individual factors (Westbury et al. 2017). If a factor was significant and not part of a significant interaction, Tukey (*P* = 0.05) post-hoc pairwise comparison tests were used to investigate underlying differences. Prior to all analyses, values were ln+1 transformed. To investigate differences in the abundance of *L. marginatum* according to habitat type and year of study, the non-parametric Kruskal–Wallis Test was performed using SPSS (Version 23 2015).

The relationship between the reproductive success of *O. galilaea* and bee abundance was investigated using non-parametric correlation (Spearman’s rho index) in SPSS. Values of total bee abundance, female abundance, and male abundance were investigated in relation to the average reproductive success of *O. galilaea* per population in the thirteen populations studied over two years.

To investigate the variability in *L. marginatum* abundance in relation to cover values of potential forage plants and those directly observed to be visited by *L. marginatum*, analyses were performed using CANOCO for Windows 4.5 (Ter Braak and Šmilauer 2002). Initially, a Detrended Canonical
Analysis (DCA) was performed to check if the linear or unimodal method should be used. As the longest gradient value was < 4, a constrained ordination RDA (linear method) was used (Lepš and Šmilauer 2003). The analysis was done for both years separately and combined.

Results

Visitation rates of Lasioglossum marginatum

The only insects observed to pollinate O. galilaea during 75 h of video footage over two field seasons (2011 and 2012), were male L. marginatum bees. A total of 117 approaches and 68 visits were recorded. Only nine visits led to pollinia attachment. Visits were infrequent and on average lasted 14.1 s (± 2.5) per flower (Table 1). Irrespective of habitat type and year, the average visitation rate of L. marginatum was 0.84 (± 0.42) visits per hour of video recorded. There was no significant difference in visitation rates (F2, 5,6 = 0.8, P = 0.492) or the number of approaches to O. galilaea flowers (F2, 5,9 = 1.2, P = 0.366) between habitat types, but there was a tendency for a greater bee activity in mixed woodland habitats (Table 1). The number of pollinia attachments observed per hour were also not influenced by habitat type (F2, 6,6 = 0.6, P = 0.581). Responses were consistent between years, with no significant difference in the number of visits (F1, 7,4 = 0.8, P = 0.389) and approaches (F1, 7,3 = 0.9, P = 0.386), although there was generally a greater number of observations in 2011 compared to 2012. There was also no significant effect of year on the number of pollinia attachments observed per hour (F1, 6,7 = 1.0, P = 0.355), although values tended to be greater in 2011 compared to 2012. Interactions between habitat type and year for all types of observations were not significant.

Reproductive success

Pollination limitation

Across habitat types and between years there was no significant difference in the reproductive success of O. galilaea. The mean reproductive success of O. galilaea under ambient pollination was 23.5% (± 0.5%) in the garrigue habitats, 35.2% (± 1.9%) in oak woodland, and 29.2% (± 4.2%) in mixed woodland. However, there was a significant effect of pollination treatment on reproductive success (F2, 3,8 = 65.98, P < 0.001). Post hoc multiple comparisons indicated that reproductive success was significantly greater in flowers ‘cross-pollinated’ by hand (Tukey test, P < 0.05), compared to the ambient and pollinator exclusion treatments. The reproductive success of O. galilaea subjected to ambient pollination was also significantly greater than flowers that had pollinators excluded (Tukey, P < 0.05) (Figure 1). Irrespective of year and habitat type, average fruit set of flowers cross-pollinated by hand was 89.0% (± 2.1%) (n = 5 flowers × 38 plants), compared to 29.3% (± 2.4%) (n = 5 flowers × 46 plants), with ambient pollination, and 2.5% (± 2.5%) (n = 5 flowers × 36 plants) for flowers bagged. Fruit set for this treatment was 0% in 35 individuals, but 100% for one individual. There were no significant interactions between habitat type and pollination treatment, habitat type and year, or treatment and year.

Lasioglossum marginatum abundance and reproductive success of Orchis galilaea

From a total of 120 pan traps across two years, 13 male and 107 female L. marginatum bees were caught from all sites. Irrespective of habitat type, the number of female L. marginatum bees caught ranged from zero to 33, compared with up to four male bees. Across both years, the abundance of male L. marginatum bees was not significantly different between habitat types (Kruskal–Wallis H = 5.90, df = 2, P = 0.052), although there was a tendency for a greater number in association with garrigue and mixed woodland habitats compared to oak woodlands. The total number of L. marginatum caught according to habitat ranged from 67 (57 females and 10 males) in garrigue, 23 (22 females and one male) in oak woodland, and 30 (28 females and two males) in mixed woodlands. No significant difference in abundance was found between years according to habitat type. However, irrespective of habitat type the abundance of male bees was positively correlated to the reproductive success of O. galilaea (r = 0.43, df = 22, P < 0.05).

Lasioglossum marginatum abundance in relation to forage abundance

Across all habitat types, a total of 25 co-flowering plant species (Appendix Table 2) were recorded as potential forage
plants for L. marginatum. However, the Monte Carlo permutation test revealed no significant relationship between bee abundance and forage availability according to habitat type ($P > 0.05$).

Discussion

The main aim of this study was to investigate the interactions between O. galilaea and its pollinator L. marginatum, and to investigate the relationship between L. marginatum abundance and the reproductive success of O. galilaea.

Field observations confirmed that O. galilaea is pollinated exclusively by male L. marginatum bees, and that female L. marginatum did not visit O. galilaea despite their greater abundance across sites. This further confirms the role of sexual deception in O. galilaea to increase the likelihood of pollination (Bino et al. 1982). Male bees visiting flowers of O. galilaea followed the same mating behavior as previously observed by Barrows (1975) for male Lasioglossum zephyrum bees before, during and after copulation with a female of the same species. The observed behavior of male L. marginatum bees on O. galilaea was likely to be in response to gyne odor, a sexual stimulant (Ayasse et al. 1999). Overall, due to it being nectarless, it is apparent that O. galilaea exploits L. marginatum as a pollinator (Vereecken 2009). However, it is not known whether male L. marginatum consumes O. galilaea pollen, although no pollen is collected for the nest (Pla-teaux-Quénu 1962).

Despite the dependence of O. galilaea on pollination services from L. marginatum, low visitation rates were observed and pollinia attachments were rare. This finding is consistent with Tremblay et al. (2005) who concluded that flower visits and pollination events to sexually deceptive orchids are rare under natural conditions. A key concern is that low visitation rates can lead to pollen limitation in orchids, affecting reproductive success (Jersáková et al. 2006). Across all habitat types and years, it was evident that O. galilaea was pollen limited as hand cross-pollination resulted in significantly greater values of reproductive success (89.0%) compared with 29.3% under ambient pollination. Low rates of reproductive success are typical in deceptive orchids, which is usually attributed to pollinator and/or pollen limitation (Nilsson 1992; Wilcock and Neilland 2002; Vandewoestijne et al. 2009). A reliance on L. marginatum for pollination was also demonstrated through negligible reproductive success (2.5%) in flowers where all insects were excluded, although this result suggests that pollen transfer did occur after exclusion (bagging) as O. galilaea is believed to be allogamous (not self-fertile) (Bino et al. 1982). Similar benefits of hand-pollination have been obtained by Willems and Lahtinen (1997) for Spiranthes spiralis, and by Kropf and Renner (2005) for Dactylorhiza sambucina. Despite the low numbers of male L. marginatum recorded across sites, the positive correlation between their abundance and the reproductive success of O. galilaea indicates to the importance of this plant-pollinator relationship. Furthermore, it also stresses the importance of protecting habitat for L. marginatum for the continued existence of O. galilaea. The lack of significant difference in L. marginatum abundance between habitat types indicates that it has broad habitat requirements. Lasioglossum marginatum has also been reported to be one of the most abundant bee species in Mediterranean landscapes that consist of garrigue, pine, oak and pine/oak woodlands habitats (Potts et al. 2003; Potts et al. 2006). These are all habitats frequently inhabited by O. galilaea. In addition, Potts et al. (2006) also found a positive correlation between the abundance of L. marginatum and overall floral abundance, but this is in contrast to the current study.

The specific and delicate relationship of O. galilaea with its only pollinator L. marginatum makes it particularly prone to extinction (Vereecken et al. 2010). This issue is further exemplified by the endangered sexually deceptive orchid, Caladenia hastate, for which the number of potential reintroduction sites is restricted due to the absence of its sole pollinator (Reiter et al. 2017). However, despite the importance of pollination by L. marginatum for the reproductive success of O. galilaea, this orchid species is a long-lived perennial and unlike annual species it is not dependent on producing seed regularly to maintain its long-term existence (Grime 1979). Following seed germination, it can take between two and 15 years of growth before O. galilaea first flowers. As a geophyte, O. galilaea can remain dormant below ground, or in a vegetative state above ground for a number of years until climatic conditions favor the development of reproductive structures (Shefferson et al. 2014). Orchis galilaea is unable to reproduce vegetatively and so seed production is vital for its long-term existence, especially with regards to its ability to adapt to change (Grime 1979). Continued habitat loss and degradation leading to reduced population sizes will be confounded with increased fragmentation of populations, which in turn could lead to a reduced genetic diversity (Faast et al. 2011). Coupled with low rates of reproductive success and limited seed dispersal, characteristic of many Orchis species (Helsen et al. 2016), O. galilaea is highly vulnerable to extinction. If Lebanon is to remain a stronghold for O. galilaea, protection and appropriate management of suitable habitat is required.

Acknowledgements

The authors would like to thank Salma Talhouk, Ken Norris, Stuart Roberts, Eleanor Allan, Mohammad Al-Zein, Mariana Yazbeck, Nour Abdel Samad, Houry Zournajian, Farah Abed Ali, Jessica El Asmar, Aveer Mahfouz, Mohammad Kanso, Anis Kadado, Hibah Ismail, Iman Dbaibo, Marwa Jammal, Sarah Sadek, Ghina Kobotan, Ryan Khar-rat, Hanin Shehab and Fady Daw, for their help in various aspects of this project.

Declaration of authorship

NMH designed the study, collected data, conducted experiments, and co-wrote the manuscript with DBW. DBW also designed the study, supervised field work, and performed the data analysis. AH helped with data collection, KK contributed to the study design and insect identification.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The study received no external funding and was funded solely by Nisrine Machaka-Houri.
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