Development, Spatial Distribution, and Presence on Grapevine of Nymphs of Orientus ishidae (Hemiptera: Cicadellidae), a New Vector of Flavescence Dorée Phytoplasmas.

(Article begins on next page)

Development, spatial distribution and presence on grapevine of nymphs of *Orientus ishidae* (Matsumura), a new vector of Flavescence dorée phytoplasmas

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Abstract

Orientus ishidae (Matsumura) (Cicadellidae: Deltocephalinae) is an Asian species now widespread in Europe, and a vector of 16SrV phytoplasmas agents of grapevine Flavescence dorée (FDP). Embryonic and post-embryonic development, spatial distribution, and relationships with grapevine of nymphs were studied under field and laboratory conditions. Egg hatching dynamics and post-embryonic development of nymphs were studied by collecting grapevine wood from managed and unmanaged vineyards (including bot European *Vitis vinifera* L., and wild American rootstocks) and storing it inside rearing cages at T=21-23°C. Field sampling of nymphs were made on both grapevine and two elective host plants of *O. ishidae:* hazelnut and hornbeam. Taylor's Power Law was applied to assess the aggregation coefficient of early (first and second) and late (third to fifth) life instars on leaves and shoots of host plants. More nymphs were obtained from wood collected in unmanaged rather than managed vineyards. Under lab conditions, the embryonic development lasted 34 - 48 days, whereas the whole post-embryonic development averaged 27 days. Under field conditions, early instars peaked at the end of May, and late instars peaked 2-4 weeks later. The aggregation patterns decreased from early to late instars, and from leaves to shoots. Very few nymphs were observed on unmanaged grapevine, either European or American, and none on managed European grapevine. Some behavioral and FDP epidemiological consequences of the results obtained are discussed.

Keywords: grapevine, leafhopper, hatching, molt, Taylor's Power Law

Introduction

The Mosaic leafhopper *Orientus ishidae* (Matsumura) (Hemiptera: Cicadellidae: Deltocephalinae) is a new acknowledged vector of Flavescence dorée (FDP), a severe disease of grapevine caused by phytoplasmas in the 16SrV ribosomal group (Alma et al. 2015). The first clue occurred in 2010, when some adults have been found bearing 16SrV phytoplasmas in Slovenia (Mehle et al. 2010). The same results were then obtained in Northern Italy (Gaffuri et al. 2011). Meanwhile, the interest about this species increased progressively, and its presence throughout Europe was confirmed (Koczor et al. 2013, Chireceanu et al. 2017, Klejdysz et al. 2017).

Its vector ability was proved by successful inoculation of 16SrV phytoplasmas to grapevines by adults, after an acquisition at the nymphal stage from infected broad beans (*Vicia faba* L.) and a latency period on hazelnut, under laboratory conditions (Lessio et al. 2016). Until then, FDP were thought being transmitted only by *Scaphoideus titanus* Ball (Cicadellidae: Deltocephalinae), a specialist of grapevine which is still acknowledged as the main vector (Chuche and Thiery 2014, Alma et al. 2015), and the European lantern fly *Dictyophara europaea* (L.) (Hemiptera: Dictyopharidae), an occasional vector (Lessio and Alma 2008, Filippin et al. 2009, Alma et al. 2015). *O. ishidae* has a single generation per year and overwinters in the egg stage (Nickel, 2010; Lessio et al., 2016). Adults are aggregated at the edges of vineyards, depending on host plants such as hazelnut, hornbeam, willow, and others (Lessio et al. 2016; Alma et al., 2019). Previously, some seasonal dynamics of adults and nymphs, along with the description of fifth-instars, were given in a survey on ornamental honey locust, *Gleditsia triacanthos* L**.** (Valley and Wheeler Jr 1985). However, many aspects of its biology are still unknown, especially concerning the embryonic and post-embryonic development of nymphs. Moreover, its relationships with grapevine are unclear, especially concerning egg-laying and population density of nymphs.

Given the lack of basic knowledge about this emerging pest, this research deals with several aspects of the biology of *O. ishidae* nymphs: presence of eggs in wood of grapevine, depending also on the

management of vineyards; embryonic and post-embryonic dynamics under laboratory conditions; seasonal occurrence of nymphs; presence of nymphs on wild or cultivated grapevine, compared to other elective host plants; spatial distribution of nymphs on elective host plants, at leaf and shoot levels.

Materials and methods

Study area

Samplings took place in 2017 and 2018, in vine growing areas settled within the following districts of Piedmont, North-western Italy: Caluso (45.31796 °N; 7.88061 °E), Mazzé (45.309539 °N; 7.934383 °E), Borgiallo (45.399622 °N; 7.667674 °E), Portacomaro (44.962311 °N; 8.258457 °E), Mombercelli (44.820415 °N; 8.302749 °E), and Vesime (44.644452 °N; 8.226433 °E). Within these areas, we chose eight experimental sites: four managed (organic) vineyards; two abandoned vineyards (that is, let unpruned and unmanaged for a period of 2-5 years, with many weeds but without overgrown trees or vine rootstocks) (Camerano and Terzuolo, 2015); and two woods mainly consisting in wild hazelnut (*Corylus avellana* L.) and hornbeam (*Carpinus betulus* L.) trees, with overgrowing American rootstocks of *Vitis berlandieri* x *riparia* (e.g. Kober 5bb, SO4) and *Vitis berlandieri* x *rupestris* (e.g. 1003 Paulsen) (Camerano and Terzuolo, 2015), which are also a source of 16SrV phytoplasmas although often symptomless (Lessio et al. 2007). All of the sites were small-sized (1500-4000 m^2). The vineyards were settled within an heterogeneous landscape consisting of many patches of broadleaf woods and edges of spontaneous hazelnut and hornbeam trees, which are two elective host plants of *O. ishidae* (Lessio et al. 2016). In organic vineyards, two insecticidal sprays were made by farmers with natural pyrethrum at the middle and end of June in both years, according to Regional Phytosanitary Service guidelines.

Egg-laying and hatching dynamics

To quantify egg-laying by *O. ishidae* on grapevine (*Vitis* spp.), and to study egg-hatching dynamics, wood was randomly collected in winter time from the each of the experimental sites (Table 1). Two-year (or more) old grapevine canes and branches were cut into pieces (15-20 cm), stored into a cool chamber (+5°C), and periodically sprinkled with water to avoid dehydration of eggs.

At the end of January, the wood was placed indoors at an average temperature of T=22±1°C, to start egg hatching. Each field-collected sample was weighted and placed inside a separate insect-proof cage (cm 110 x 110 x 80) made of mesh and aluminium frame, along with potted hazelnut and broad bean plants to provide food for nymphs. A layer of vermiculite was placed on the bottom of the cage to preserve humidity, and the cages were periodically sprinkled with water. The cages were inspected daily, and the emerged nymphs of *O. ishidae* were counted, removed and kept alive for the post-embryonic development experiments.

<u>Post-embryonic development</u>

The post-embryonic development of *O. ishidae* was studied under the same conditions of the previous experiment. Newly hatched nymphs were retrieved from the rearing cage, and placed singularly inside Plexiglas cylinders (h=20 cm; diameter: 12 cm) closed with a fine mesh on the top. Two circular holes (diameter: 2 cm) were made on the walls of each cylinder, and covered with the same mesh to allow a better circulation of air. Each cylinder was placed onto a potted broad bean plant, and a disk of filter paper was placed on the soil to decrease humidity and for a better detection of any dead insect. Cylinders were inspected daily to observe moults up to the adult stage, and after each moult the corresponding *exuvia* was removed from inside.

<u>Sampling of nymphs on host plants</u>

Nymphs of *O. ishidae* were sampled on European grapevine, on wild American rootstocks, and on surrounding spontaneous hazelnut and hornbeam plants, in all of the experimental sites. On the whole, N=79 plants were observed (European grapevine: 20; American rootstocks: 18; hazelnut: 33;

hornbeam: 8). Samplings took place from the middle of May to the end of July (2017-2018). On hazelnut and hornbeam, for each plant, we observed 50 leaves distributed on 10 shoots (5 leaves per shoot). On grapevine species, we inspected 50 leaves close to the trunk or to wooden canes. Leaves were gently turned upside down, and nymphs were counted under a lens without being removed, assigning them to I-II instar (N1 and N2: winglets absent), and III-V instar (N3, N4 and N5: winglets present).

Data analysis

Nymphs emerging from wood collected in different types of vineyards (cultivated or wild) in different years were compared with a GLM procedure. The number of nymphs was used as the dependent variable; the type of vineyard (either managed or unmanaged), the species of *Vitis* (either *V. vinifera* or American rootstocks) and the year of collection were the factors; and the weight of collected wood was the offset variable. To overcome problems due to zero values, we used a Tweedie mixed distribution, which manages properly both over-dispersion and zero-inflation, with a *Log* link function. A cumulative distribution function was used instead to describe egg hatching dynamics. Finally, we calculated the descriptive statistics (mean, standard deviation, and confidence limits) of the duration for both the embryonic development of nymphs emerged from different wood lots and post-embryonic development of each life instar.

The seasonal distributions of early (N1-2) and late (N3-5) instar of field-collected nymphs between the two sampling years were compared with a chi-square test with four degrees of freedom (five sampling dates, from the middle of May to the middle of July, at a bi-weekly step).

Counts of nymphs on plants were analyzed with a GLM procedure testing the effect of plant species (European grapevine, American rootstocks, hazelnut, hornbeam). In this case, we did not separate early from late instars nor we considered sampling date. The number of sample units per date and plant species was considered as an offset variable. A Poisson distribution of data was assumed, and the *Log* link function was used.

Taylor's Power Law (TPL) (Taylor 1984) was used to analyze data of nymphs distribution on leaves and shoots of hazelnut and hornbeam. The general equation of TPL is:

$$\log_{10} S^2 = \log_{10} a + b \cdot \log_{10} m$$

where, having counted a given object (e.g. nymphs) on a given number of sampling units, S^2 and m are the sample variance and mean, respectively. While the coefficient a (intercept) depends just on the sampling method, the coefficient b (slope) is typical for the species considered: $b<1$ indicates randomness, $b=1$ uniformity, and $b>1$ aggregation. In this case, we considered first the leaves and then the shoots as a sampling unit, calculating therefore the mean and variance for each tree. In this case, we excluded counts on grapevines (no or too few nymphs detected, see results): therefore, a dataset of N=41 was available. On the other hand, given the similar plant architecture (small broadleaf trees or bushes, with a relatively similar leaf size and distribution of leaves on the branches), we did not distinguish between counts on hornbeam or hazelnut. Data were analyzed separately depending on life instar (N1 and N2; N3, N4 and N5), and were previously Log10 transformed according to Taylor (1984) after adding a 0.1 constant to avoid transformation problems in zero values. Finally, a linear regression was run between the sample variance (dependent variable) and the sample mean (independent variable).

All of the statistical analyses listed were performed with SPSS 25.0® statistical package.

Results

<u>Egg hatching and post-embryonic development</u>

Collectively, 142 nymphs of *O. ishidae* were obtained from wood of grapevine, ranging 0.33 - 14.8 per kg of wood in single collections. Overall, significant differences were found in the number of nymphs hatched from different kinds of wood collected in differently managed sites and during different years (GLM: χ^2= 40.01; df=3; P<0.001). When considering single factors, the type of vineyard had a significant influence on the number of nymphs hatched (χ^2= 16.86; df=2; P<0.001): organic vineyards were significantly lower than the other two kinds, whereas no differences were

detected between abandoned vineyards and woods with American rootstocks (Figure 1). No differences were found between the two years of wood collection (χ^2= 0.81; df=1; P=0.37).

The wood collected in 2017 in the sites of Caluso (abandoned vineyard; nymphs/kg wood: 14.78; total: 17), Mazzé (wild American grapevine; nymphs/kg wood: 7.53; total: 29), Borgiallo (abandoned vineyard; nymphs/kg wood: 8.83; total: 72), and Portacomaro (wild American grapevine; nymphs/kg wood: 3.38; total: 13) provided enough nymphs to describe egg-hatching dynamics. Hatching started from 2 to 4 weeks (13-30 d) after the beginning of incubation, and lasted 2-7 weeks (14-51 d). The mean hatching time was minimum in Portacomaro (34 d) and maximum in Mazzé (48 d) (Table 2; Figure 2).

Data of post-embryonic development under laboratory conditions were obtained from a set of N=20 newly hatched nymphs, which were successfully reared up to the adult stage (other specimens died before becoming adults and were therefore excluded from analysis). Developmental times increased along with life stages: N1 lasted approx. 4-5 days, whereas N5 lasted 6-7 days. Overall, the post-embryonic development lasted 27-29 days (Table 3).

<u>Seasonal dynamics of nymphs on host plants</u>

Collectively, 507 nymphs were observed on hazelnut and hornbeam plants throughout different sampling periods (N1-N2: 285; N3-N5: 222). The first nymphs (N1-2) appeared at the middle of May, whereas N3-5 appeared between the end of May and the middle of June. The peak of N1-2 occurred at the end of May in both years, whereas N3-5 peaked at the end of June in 2017 and at the middle of June in 2018. At the end of July, no nymphs were found. Data are shown in Figure 3. Referring to the distribution among five sampling dates (from the middle of May to the middle of July), no differences were found between years, neither for early stages (χ^2 = 0.22; df=4; P=0.05), nor for late ones (χ^2 =0.42; df=4; P=0.09). On the other hand, only three specimens were observed on grapevine (abandoned grapevines: 1 early instar on May 31, 2017, and 1 late instar on June 14, 2017; wild grapevine: 1 late instar on June 16, 2018). A significant difference was observed in the number of

nymphs detected on different plant species (GLM: χ^2= 3971.05; df=3; P<0.001). The number of nymphs per plant was significantly higher on hazelnut with respect to all other. As well, it was significantly higher on hornbeam with respect to both species of grapevine, whereas no significant differences were found between European and American grapevine (Table 4).

Aggregation patterns of nymphs

The aggregation patterns of *O. ishidae* nymphs, calculated with TPL, resulted highly significant and changed depending on the instar and the plant organ considered as sample unit (Table 5). When considering leaves, early life instars were indicating a moderately aggregated (*b*=1.29), whereas late ones resulted less crowded (*b*=1.16). The same trend was observed for spatial distribution at a shoot level, however both early and late instars were less aggregated with respect to leaves. In particular, late instars on shoots had an almost uniform spatial distribution (*b*=1.07).

Discussion

The present research confirmed that *O. ishidae* is capable of laying eggs on grapevine. This behavior may be promoted by the proximity of trees to vineyards or by the co-habitat of wild grapevine and other plants in woods. In fact, more nymphs hatched from wood collected from abandoned vineyards and/or wild rootstocks, where hazelnut and hornbeam plants were generally closer to the edges or, in the case of woods, mingled with overgrowing grapevine rootstocks. Another reason may be the greater presence of older wood (2 years, or more) due to no pruning. Although we did not take into account 1-year old wood for the presence of eggs, it is likely that *O. ishidae* prefers older grapevine wood just like *S. titanus* (Lessio and Alma 2013, Chuche and Thiery 2014). Therefore, females might have fed and developed on other plants and only afterwards exploited grapevine wood for egg-laying. However, while this species was very abundant on hornbeam and hazelnut, very few nymphs were found on grapevine leaves. Although in organic vineyards pyrethrum was sprayed on grapevines after the middle of June, nymphs were not found even before treatments. Therefore, there must be another

reason to explain such a discrepancy. Perhaps, grapevine is used just for egg-laying, and nymphs move elsewhere after hatching. Among leafhoppers in the same subfamily (Deltocephalinae), *Anoplotettix fuscovenosus* (Ferrari) exhibits a similar strategy: eggs are laid under the bark of grapevine, but nymphs move to the weeds in the inter-row (Alma 1995). Sometimes, *Phlogotettix cyclops* (Mulsant & Rey) lays eggs under the bark of grapevine canes too (Chuche et al. 2010). It is not surprising therefore that females of *O. ishidae* exploit grapevines for egg-laying, given also their frequent drifting behaviour in vineyards (Lessio et al. 2016). However, it is not clear if egg-laying on grapevines represents a biological strategy or just a casual occurrence. In any case, although nymphs of *O. ishidae* are capable of acquiring 16SrV phytoplasmas from grapevines (Lessio et al. 2016), this aspect does not seem important from an epidemiological point of view, as very few specimens have been found on grapevines.

The embryonic development of *O. ishidae* lasted 34-48 days at T=21-23°C. A similar trend has been observed in *S. titanus*, which has a mean hatching time of 30 and 45 days at constant temperatures of 20 and 22°C, respectively (Falzoi et al. 2014). The discrepancies observed in the egg-hatching dynamics among sites may be due to cold or mild winters. In fact, it has been demonstrated that *S. titanus* eggs hatch faster when exposed to cold rather than mild winter temperatures (Chuche and Thiery 2009). Concerning the post-embryonic development, late instars of *O. ishidae* take longer to develop that early ones: this is similar too to *S. titanus* (Falzoi et al. 2014), and is related to the fact that body size has an effect on developmental time (Gillooly et al. 2002).

Seasonal dynamics of nymphs observed in the field were partially in accordance with the data presented by Valley and Wheeler jr (1985), who found them from the beginning to the end of June on ornamental honey locust in Pennsylvania. In our research, nymphs were found from the middle of May up to the middle of July. These differences may be due to different temperatures between Pennsylvania and Northern Italy, to increasing temperatures over the last three decades, to differences in host plants, or simply to sampling discrepancies. With regard to the influence of host plants, it is possible that *O. ishidae* populations have adapted to differences in bud break among plant species.

This synchrony has been detected concerning grapevine and *S. titanus* (Chuche et al. 2015), which however is a monophagous species and is probably co-evolving with its own host plant. *O. ishidae* is highly polyphagous (Nickel, 2010; Lessio et al., 2016; Alma et al., 2019), and therefore it is less likely that populations specialize on one host plant: in fact, the success in this species is probably due to its plasticity.

The spatial distribution of nymphs was different considering both the life stage and the sampling unit. Dispersal increased from early to late instars, and from leaves to shoots. Late instars are less aggregated probably because they disperse due to overcrowding on leaves. This aspect is not observed in *S. titanus*, which rarely builds up great densities on grapevine leaves. In fact, the nymphs of this species are aggregated (Lessio and Alma 2006), and have also an aggregative feeding behaviour of under laboratory conditions (Chuche et al. 2009). This could be due to differences in plant architecture. In fact, grapevine (especially if row-shaped) has few shoots sprouting from the trunk: nymphs of *S. titanus* hatching from eggs laid under the bark have therefore less shoots to colonize. On the other hand, nymphs of *O. ishidae* have more possibilities of reaching sprouts when eggs are laid on the trunk and on the branches of broadleaf trees. Another reason may be the different feeding habits between these two species. *O. ishidae* causes severe stunting on leaves (Felt and Bromley 1941, Lessio et al. 2016), probably because of a cell rupture feeding behavior. In fact, damages resemble in some way marginal burning caused by *Empoasca vitis* (Goethe), a typical cell rupture feeder (Jin et al. 2012). Therefore, overcrowding may cause a decrease of food resources. On the other hand, *S. titanus* usually probes in one point producing salivary sheaths (Chuche et al. 2017), without affecting directly grapevine leaves.

The similarity of life cycles between *S. titanus* and *O. ishidae* may be the reason why both are vectors of 16SrV phytoplasmas to grapevine, although with different efficiency and therefore importance. However, phytoplasma sources for nymphs of *O. ishidae* are less certain. No nymphs collected on many host plants tested FD-positive, although they are capable of acquiring from infected grapevines (Lessio et al. 2016). Recently, some host plants (e.g. willow, hazelnut) have been found positive to

16SrV phytoplasmas in Switzerland (Casati et al. 2017). Another possibility is that adults of *O. ishidae* acquire phytoplasmas directly on grapevine. In fact, it has recently been proved that *S. titanus* is capable of acquiring 16SrV phytoplasmas in the adult stage, and transmitting them within only two weeks (Alma et al. 2018). Given the biological similarities between *S. titanus* and *O. ishidae*, this matter should be further investigated.

Acknowledgments

We are grateful to all the farmers who provided suitable sites for experiments.

Author's contribution statement

FL designed the experiments, made field samplings, conducted laboratory rearing and tests, analyzed data, and wrote the manuscript. FB made field samplings and analyzed data. AA conceived and designed the research. All authors read and approved the manuscript.

Compliance with Ethical Standards

Conflict of interest: all Authors declare that they have no conflict of interest.

Ethical approval: this article does not contain any studies with human participants performed by any of the Authors; all applicable international, national and institutional guidelines for the care and use of animals were followed; no unauthorized sampling of wildlife forms was performed;

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Table 1. Wood of grapevine (*Vitis* spp.) collected in different experimental sites

Site	District	Management type	*Vitis* species	year	Kg wood collected
1	Mazzé	Wood	American rootstocks	2017	3.71
				2018	1.00
2	Caluso	Abandoned vineyard	*V. vinifera*	2017	1.50
				2018	1.00
3	Borgiallo	Abandoned vineyard	*V. vinifera*	2017	2.00
				2018	6.90
4	Portacomaro	Wood	American rootstocks	2017	4.00
				2018	1.15
5	Mongardino	Organic vineyard	*V. vinifera*	2017	2.00
				2018	6.00
6	Caluso	Organic vineyard	*V. vinifera*	2017	5.00
				2018	9.00
7	Mazzé	Organic vineyard	*V. vinifera*	2017	5.00
				2018	4.00
8	Vesime	Organic vineyard	*V. vinifera*	2017	5.00
				2018	5.00

Table 2. Time of embryonic development (TD, in days) in *O. ishidae* at T=21-23°C

Site	Year	N	TD_{min}	TD_{max}	HD	TD_{mean}	SE	Lower CI	Upper CI
1	2017	30	25	59	34	48.87	0.34	48.20	49.54
2	2017	17	30	50	20	39.76	0.30	39.17	40.36
3	2017	72	13	64	51	46.36	0.15	46.08	46.65
4	2017	13	28	42	14	34.46	0.39	33.69	35.23

N: number of hatched nymphs; TD_{min} and TD_{max}: time of development of the first and last hatched specimen; HD: hatching duration ($HD = TD_{max} - TD_{min}$); SE: standard error; CI: 95% confidence interval. Site 1: Mazzè (unmanaged, Am. grapevine); site 2: Caluso (unmanaged, Eur. grapevine); site 3: Borgiallo (unmanaged, Eur. grapevine); site 4: Portacomaro (unmanaged, Am. grapevine).

Table 3. Duration of post-embryonic development (in days) in *O. ishidae* (N=20) at T=21-23°C

Life stage	Mean	SE	Lower CI	Upper CI
N1	4.45	0.18	4.09	4.81
N2	5.30	0.46	4.40	6.20
N3	5.30	0.29	4.73	5.87
N4	5.85	0.17	5.52	6.18
N5	6.95	0.34	6.28	7.62
Total	27.85	0.55	26.76	28.94

SE: standard error; CI: 95% confidence interval.

Table 4. Nymphs of *O. ishidae* counted on different host plants. Different letters indicate significant differences (GLM, $P<0.05$).

Plant species	Nymphs (mean ± s.e.)	χ^2 (d.f.)	*P*
Corylus avellana L.	13.00 ± 1.32 a	3971.05 (3)	<0.001
Carpinus betulus L.	9.38 ± 2.68 b		
Vitis vinifera L.	0.10 ± 0.07 c		
American rootstocks	0.06 ± 0.05 c		

Table 5. Taylor's Power Law regressions on nymphs of *O. ishidae* counted on leaves and shoots of broadleaf host plants

Source of variation	R^2	ANOVA		Coefficients			
		$F_{(1, 39)}$	*P*		B	t	*P*
N1+N2, leaves	0.96	894.75	0.000	Intercept	0.52	9.72	0.000
				Slope	1.29	29.91	0.000
N3+N4+N5, leaves	0.95	726.38	0.000	Intercept	0.29	5.41	0.000
				Slope	1.16	26.95	0.000
N1+N2, shoots	0.93	510.06	0.000	Intercept	0.25	5.03	0.000
				Slope	1.16	22.58	0.000
N3+N4+N5, shoots	0.93	528.31	0.000	Intercept	0.13	2.92	0.01
				Slope	1.07	22.99	0.000

Captions to figures

Figure 1. Nymphs of *O. ishidae* (mean ± s.e. per kg of wood) hatched from different kinds of grapevine's wood. Different letters indicate significant differences in wood type (GLM, $P<0.05$)

Figure 2. Cumulative frequency distribution of egg-hatching in *O. ishidae* obtained from grapevine wood at T=21-23°C

Figure 3. Seasonal distribution of nymphs in *O. ishidae* collected on host plants (hazelnut and hornbeam). A: 2017; B: 2018