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The use of path analysis to determine effects of environmental factors on the adult seasonality of *Culicoides* (Diptera: Ceratopogonidae) vector species in Spain

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Abstract

Culicoides biting midges (Diptera: Ceratopogonidae) are the main vectors of livestock diseases such as bluetongue (BT) which mainly affect sheep and cattle. In Spain, bluetongue virus (BTV) is transmitted by several *Culicoides* taxa, including *Culicoides imicola*, Obsoletus complex, *Culicoides newsteadi* and *Culicoides pulicaris* that vary in seasonality and distribution, affecting the distribution and dynamics of BT outbreaks. Path analysis is useful for separating direct and indirect, biotic and abiotic determinants of species' population performance and is ideal for understanding the sensitivity of adult *Culicoides* dynamics to multiple environmental drivers. Start, end of season and length of overwintering of adult *Culicoides* were analysed across 329 sites in Spain sampled from 2005 to 2010 during the National Entomosurveillance Program for BTV with path analysis, to determine the direct and indirect effects of land use, climate and host factor variables. *Culicoides* taxa had species-specific responses to environmental variables. While the seasonality of adult *C. imicola* was strongly affected by topography, temperature, cover of agro-forestry and sclerophyllous vegetation, rainfall, livestock density, photoperiod in autumn and the abundance of *Culicoides* females, Obsoletus complex species seasonality was affected by land-use variables such as cover of natural grassland and broad-leaved forest. *Culicoides* female abundance was the most explanatory variable for the seasonality of *C. newsteadi*, while *C. pulicaris* showed that temperature during winter and the photoperiod in November had a strong effect on the start of the season and the length of overwinter period of this species. These results indicate that the seasonal vector-free period (SVFP) in Spain will vary between competent vector taxa and geographic locations, dependent on the different responses of each taxa to environmental conditions.

Introduction

Culicoides Latreille (Diptera; Ceratopogonidae) midges are a genus of Nematocera insects with medical and veterinary relevance due to their role in pathogen transmission. Bluetongue (BT) is an economically important disease in Europe with direct effects on farms due to animal losses and indirect effects related to disease prevention and control, including trade restrictions (Coetzer *et al.*, 1995; Holbrook, 1996; Meiswinkel *et al.*, 2004; Gibbens, 2012). In Spain, bluetongue virus (BTV) is transmitted by the species from the *Culicoides imicola*, Obsoletus complex, *Culicoides newsteadi* and *Culicoides pulicaris* (Purse *et al.*, 2007; Wilson and Mellor, 2009; Ducheyne *et al.*, 2013). The measures to stop the spread of the disease and the mortality and morbidity of livestock account for losses of several millions of euros (Rasve, 2012; Pérez de Diego *et al.*, 2014). Sheep develop the most severe clinical signs of the disease (Taylor, 1986; MacLachlan *et al.*, 2009); however, other domestic species such as cattle as well as wild ruminants may be sub-clinically infected with BTV and act as silent reservoirs (Barnard, 1997) making outbreaks difficult to predict and hindering containment measures.

BT was detected for the first time in Europe in 1943 in Cyprus (Gambles, 1949). However, it was not until 1998 that the virus has been detected regularly in the southern Europe (Calvete *et al.*, 2006; Barros *et al.*, 2007), and not until 2006 in northern Europe (Saegerman *et al.*, 2008). When planning control strategies to limit BT disease in a region, it is critical to understand the basic ecology and behaviour of locally competent *Culicoides* vectors, such as their seasonality, breeding site requirements and environmental requirements for development (reviewed in Purse *et al.*, 2015).

Geographical and temporal variations in adult *Culicoides* vector abundance are a key component of the basic reproduction number (i.e. the seasonal vector–host ratios) that measures the likelihood of establishment of arbovirus transmission (Mellor *et al.*, 2000). This transmission can be calculated by the number of new cases generated from a single case when a pathogen is introduced into a naïve population (Gubbins *et al.*, 2008; Brugger and Rubel, 2013). In addition, seasonality of *Culicoides* also determines the probability that *Culicoides*-borne virus transmission and disease epidemics will persist between years, and is thus of key interest to health authorities (Bessell *et al.*, 2014).

A technique called path analysis is frequently used to explore the indirect and direct effects of interacting predictor variables on dependent variables (Norman and Streiner, 2003). Path analysis has been used in other studies with large herbivores in Europe and the USA (Mysterud *et al.*, 2008; Searle *et al.*, 2015) and insects like ants in Argentina (Fergnani *et al.*, 2008) to interpret the relationship between abundance and direct and indirect environmental drivers. Since vector populations can be highly sensitive to wide ranging biotic and abiotic drivers (Purse *et al.*, 2007, 2015), path analysis could also be useful to understand direct and indirect effects of environmental variables on vector populations, but has rarely been applied in this way.

The development, demographic and activity rates and distribution of *Culicoides* populations, are not only sensitive to temperature, humidity and rainfall but also to land cover and host availability (Harrup *et al.*, 2013; Zimmer *et al.*, 2014; Purse *et al.*, 2015). Aside from the impacts on development and survival and breeding site availability, environmental factors have wide ranging impacts on *Culicoides* populations, including on the presence, abundance and seasonality of adults (Barceló *et al.*, 2021). Therefore, understanding the underlying mechanisms and drivers of these interactions is of great importance for surveillance of *Culicoides* populations and defining the period of risk for arbovirus transmission (Sanders *et al.*, 2011). When interpreting relationships between adult seasonality of *Culicoides* and local environmental conditions, it is important to account for the fact that adults are more likely to be trapped overall and for longer during the season in a large midge population compared to a small population, due to both sampling and environmental effects (Sanders *et al.*, 2019). Cold winters or heavy rain, for example, exert a direct effect on the adult activity period of these insects (Rawlings *et al.*, 1998; Alekseev *et al.*, 2007), and also an indirect effect via female abundance. Barceló *et al.* (2021) already demonstrated through more traditional mixed modelling approaches, that in more abundant *Culicoides* populations, adult females emerge earlier, and the season lasts longer.

In the current study, we use path analysis for the first time to link changes in seasonal environmental variables and variation in phenology of adult females of *Culicoides* populations across sites. This is performed separately for different metrics of adult seasonality, namely the seasonal appearance, disappearance and overwinter period of the nulliparous and parous adults of the species from Obsoletus complex, *C. imicola*, *C. newsteadi* and *C. pulicaris*, in order to understand variation in environmental responses between these potential vector species, and between the northern species Obsoletus complex/*C. pulicaris* and the south-eastern species *C. imicola*/*C. newsteadi*.

Materials and methods

To explore the direct and indirect effects of environmental factors on adult *Culicoides* female seasonality, we selected the following

seasonal metrics: start of the season, end of the season and the length of overwinter period. We implemented hierarchical Bayesian structural equation modelling (Searle *et al.*, 2016) using data from 329 *Culicoides* sites in Spain, spanning the whole Iberian Peninsula and the Balearic Islands, sampled over the period 2005–2010 during the National Entomosurveillance Program for BTV, sponsored by the Spanish Ministry of Rural and Marine Environment.

As described in Barceló *et al.* (2021), Miniature Downdraft Black light (UV) traps (John W. Hock®) were set to collect *Culicoides* from dusk to dawn in a weekly basis (at least 45 weeks per year). The traps were located at 1.7–2.0 m from the floor and between 1 and 30 m from the livestock of domestic animals. The trap collectors were provided with antifreeze and alcohol to prevent the samples from decaying. Samples were transported to the laboratory where they were classified by species according to Mathieu *et al.* (2012) taxonomic key and by gonotrophic stage (Dyce, 1969).

We consider the four BT-vector taxa present in Spain: *C. imicola*, Obsoletus complex species, *C. newsteadi* and *C. pulicaris* (Purse *et al.*, 2007; Vanbinst *et al.*, 2009; Wilson and Mellor, 2009; Goffredo *et al.*, 2015; Foxi *et al.*, 2016). The female species from Obsoletus complex, *Culicoides obsoletus* and *Culicoides scoticus* are two of the most common species in Spain (Pagès and Sarto I Monteys, 2005) and are usually identified through molecular assays (Garros *et al.*, 2014; Harrup *et al.*, 2015). Since molecular methods were not included in the National Surveillance Programme, *C. obsoletus* and *C. scoticus* are modelled together here as the Obsoletus complex.

A threshold of sampling effort required in particular sites each year was established to ensure only well sampled sites were included in the calculated phenology metrics and models. Site-years with at least 45 trapping weeks/year and no more than 3 consecutive weeks with no-sampling were included. Moreover, site-by-year combinations were only included in analyses if at least 2 weeks of trapping had occurred prior to the week identified as 'start of season' and after the week identified as 'end of season'. We also excluded sites where species occurred at low average abundance for those taxa during one or more 2-month periods of the year. These 2-month periods were: January–February (period 1), March–April (period 2), May–June (period 3), July–August (period 4), September–October (period 5) and November–December (period 6).

The abundance thresholds that were used to define 'low abundance', and the start and end of seasonal activity were taxa-specific following the seasonal vector-free period (SVFP) criteria defined in Annexe V of Commission Regulation (EC) No. 1266/2007 by the European Union council: for the Obsoletus complex, *C. newsteadi* and *C. pulicaris* – using a number more than five individuals per trap catch; in contrast, for *C. imicola*, we used a number of more than one individual per trap catch.

To examine correlations between adult seasonality and environmental factors, we calculated the following metrics of annual abundance and seasonality for each site-by-year combination in which the above criteria had been found (Searle *et al.*, 2014):

- Start of the season: the first week of the year (Julian days) in which more than five (Obsoletus complex, *C. newsteadi* and *C. pulicaris*) or one (*C. imicola*) females were collected.
- End of the season: the last week of the year (Julian days) in which more than five (Obsoletus complex, *C. newsteadi* and *C. pulicaris*) or one (*C. imicola*) females were caught.

- Length of the overwinter: the difference in weeks between the end of the season in 1 year and the start of the season of the following year.
- Mean annual female *Culicoides* abundance for each taxa, site and year (hereafter *Culicoides* female abundance).

We used hierarchical structural equation models within a Bayesian framework (Clark and Gelfand, 2006). This modelling is considered suitable for this kind of analysis because it allows a series of hypothesized cause and effect relationships to be captured within a single model, estimating the magnitude of both direct and indirect (via *Culicoides* female abundance) effects of the independent variables on dependent variables (seasonal metrics), accounting for the random effects of site and year (Shipley, 2016). The independent variables were: climate (temperature and precipitation), topography (elevation and slope), land cover, host density (cattle and sheep), photoperiod in March, April, September and November and *Culicoides* female abundance obtained from different sources (Supp. table S1).

In the current study, these models were used to examine links between variation in the seasonal metrics of *Culicoides* nulliparous (NF) and parous females (PF), *Culicoides* female abundances and environment variables. NF are adults that have emerged from the pupal stage, but not yet taken a blood meal. PF are those that have emerged from the pupal stage and have already taken a blood meal. We distinguish between NF and PF since PF are the proportion of the population that may carry transmissible infections of BTV. Based on our understanding of the system, we developed a path analysis model for how environmental variables and female abundance directly and indirectly affect the seasonal metric for each four analysed taxa (*C. imicola*, Obsoletus complex, *C. newsteadi* and *C. pulicaris*) incorporating knowledge on drivers of these individual taxa from literature. The models quantified the direct effects of climate, land cover, hosts, topography and photoperiod on the seasonal metrics, and the indirect effects of all of these variables on the seasonal metrics via *Culicoides* female abundance (Supp. fig. S1). We did not look for indirect effects of photoperiod through female abundance because we considered that availability of daylight hours may only cause an effect on the seasonality of *Culicoides* species through an overcoming of insect diapause (Tauber and Tauber, 1976; Isaev, 1985). In fact, some significant effects of photoperiod on variation in seasonality of *Culicoides* across sites were found in previous studies using surveillance data such as Searle *et al.* (2013, 2014) while in another laboratory study this variable was not significant (Lühken *et al.*, 2015).

All models were fitted using WinBUGS (Spiegelhalter *et al.*, 2004) software and a Markov chain Monte Carlo procedure for each model run for 10,000 iterations after an initial burn in of 10,000 iterations to ensure convergence of all model parameters.

Results

A total of 12,321 *C. imicola* PF and 4226 *C. imicola* NF were included in the path analysis, being the most abundant and observed taxa included in the path analysis, followed by the species from the Obsoletus complex (table 1). Among all taxa, PF abundance was higher than NF and *C. pulicaris* was the less abundant and observed species.

C. imicola and the species from the Obsoletus complex were the ones with most significant environmental effects on seasonality, including topography, temperature, rainfall, agro-forestry and

Table 1. Total and average (av.) number of *Culicoides* caught by site from 2005 to 2010 used in the path analysis

Taxa	Stage	Obs.	Sites	Total (av. ± SD)
C. imicola	NF	86	46	4226 (48.02 ± 132.13)
	PF	92	48	12,321 (48.02 ± 132.13)
Obsoletus complex	NF	61	41	2407 (39.45 ± 97.59)
	PF	67	45	3389 (50.58 ± 76.28)
C. newsteadi	NF	53	34	623 (11.75 ± 20.42)
	PF	58	38	1869 (32.21 ± 55.27)
C. pulicaris	NF	39	25	436 (11.18 ± 16.71)
	PF	40	25	1366 (34.17 ± 42.05)

NF, nulliparous females; PF, parous females; Obs, number of observations; SD, standard deviation.

sclerophyllous vegetation areas, livestock density and photoperiod in autumn. Except for *C. pulicaris*, the timing metrics of all taxa studied showed significant effect by *Culicoides* female abundance. In addition, only *C. imicola* and Obsoletus complex species showed indirect significant effects through *Culicoides* female abundance.

Effects on start of the season

Accumulated degree days over 10°C in winter, the percentage of agro-forestry areas and the *Culicoides* female abundance showed a strong negative effect on the start of season of *C. imicola* NF (more than the 95% of credible intervals (CIs) for the fixed effects lower than zero) (table 2 and Supp. fig. S1A). Conversely, precipitation in spring and cattle density had a strong positive effect on the start of the season (more than the 95% of the CI for the fixed effects greater than zero). Therefore, the season of *C. imicola* NF tended to start earlier in sites with higher temperature during winter, higher percentage of agro-forestry areas and higher *Culicoides* abundance in addition to lower precipitation in spring and lower cattle density. For PF, two top models received similar support in the data, including a range of environmental covariates (ΔDIC < 2; Supp. table S5). The best model for PF start of season showed a strong negative effect of accumulated degree days in winter and *Culicoides* female abundance, in addition to a weak positive effect of accumulated degree days over 10°C in spring (more than the 90% of the CI for the fixed effects greater than zero) (Supp. fig. S1B). Thus, the season of *C. imicola* PF tended to start earlier in sites with higher temperature in winter, lower temperature in spring and higher abundance of *Culicoides* females. The null model received essentially no support in the data in comparison to the best models of *C. imicola* NF and PF (ΔDIC = 12.86 and 4.64 respectively, Supp. table S5).

For both Obsoletus complex NF and PF, elevation and photoperiod in March showed a strong positive effect on the start of the season; conversely, *Culicoides* female abundance had a strong negative effect also for both NF and PF (table 2 and Supp. fig. S2). Accumulated degree days over 10°C and precipitation in spring showed a strong negative effect on the start of the season for NF (Supp. fig. S2A). In addition, elevation and cattle had a strong and weak positive effect respectively through female abundance on the start of the season of NF. Regarding Obsoletus complex PF, precipitation in winter and percentage of

Table 2. Summary of the significant environmental parameters for start of the season models of each taxa

Taxa	Stage	Elev	Cattle	Sheep	AgFor	NatGras	SchVeg	BrdMix	DDwin	DDspr	Pwin	Pspr	Phmarch	Fem. abund.
C. imicola	NF		(+)		(−)		(+)		(−)			(+)		(−)
	PF								(−)	(+)				(−)
Obsoletus complex	NF	(+) (−A)	(−A)							(−)		(−)	(+)	(−)
	PF	(+)				(−)					(−)		(+)	(−)
C. newsteadi	NF													
	PF			(+)					(−)		(−A)			(−)
C. pulicaris	NF								(−)					
	PF								(−)					

NF, nulliparous females; PF, parous females; light grey, variables included in the best model; dark grey, variables with weak effect (90% of CI did not include zero); black, variables with strong effect (95% of CI did not include zero); (+), positive effect; (−), negative effect, (A), indirect effect via Culicoides female abundance.

natural-grassland areas showed a strong negative effect on the start of the season (Supp. fig. S2B). The following two top models of PF received similar support in the data, including a range of environmental covariates (ΔDIC < 2; Supp. table S6). Therefore, season of Obsoletus complex NF started earlier in lower elevation sites with higher temperatures and precipitation during spring, higher abundance of *Culicoides* females, lower cattle density and low number of daylight hours during March. In addition, season of Obsoletus complex PF started earlier in low elevated natural-grassland sites with high precipitation during winter, higher abundance of *Culicoides* females and low number of daylight hours during March. The null model received essentially no support in the data in comparison to the best models of Obsoletus complex NF and PF (ΔDIC = 25.79 and 5.17 respectively, Supp. table S6).

Regarding *C. newsteadi*, female abundance showed a strong negative effect on the start of the season of PF (table 2 and Supp. fig. S3). Accumulated degree days over 10°C on winter and sheep livestock had a weak negative and positive effect respectively on the start of season of *C. newsteadi* PF. On the other hand, precipitation during winter showed a weak negative indirect effect on the season through female abundance. The following two top models of PF received similar support in the data, including a range of environmental covariates (ΔDIC < 2; Supp. table S7). Therefore, sites with higher average female population, higher number of days over 10°C in winter season and lower density of sheep showed an earlier start of *C. newsteadi* PF season. In addition, sites with higher precipitation in winter were associated with a decrease in the abundance of *C. newsteadi* females causing an indirect effect on the seasonality of this species. The null model received essentially no support in the data in comparison to the best model of *C. newsteadi* PF (ΔDIC > 7.49, Supp. table S7). No environmental variables were included in *C. newsteadi* NF best path model.

Both *C. pulicaris* NF and PF showed that accumulated degree days over 10°C in winter had a strong negative direct effect on the start of the season for this species (table 2 and Supp. fig. S4). The following top model of NF which is the null model and the following two top models of PF received similar support in the data (ΔDIC < 2; Supp. table S8). Thus, sites with higher temperatures in winter had an earlier start of *C. pulicaris* season. The null model received essentially no support in the data in comparison to the best model of *C. pulicaris* PF (ΔDIC = 14.64, Supp. table S8).

Effects on end of the season

Elevation and sclerophyllous vegetation had strong negative effects on the end of the season for both *C. imicola* NF and PF season, the latter acting through female abundance (table 3 and Supp. fig. S5). Accumulated degree days over 10°C in summer showed also a strong negative effect on the end of season for NF, while *Culicoides* female abundance had a weak positive effect on the end of season (Supp. fig. S5A). Regarding *C. imicola* PF, the photoperiod in November had a strong positive effect on the end of season. The following two top models of *C. imicola* PF received similar support in the data, including a range of environmental covariates (ΔDIC < 2; Supp. table S9). Therefore, sites with higher percentage of sclerophyllous vegetation were associated with an increase in the abundance of *C. imicola* females causing an indirect effect on the seasonality of this species. Then, the season of *C. imicola* NF were longer in lower elevation sites with sclerophyllous vegetation, lower temperatures during

Table 3. Summary of the significant environmental parameters for end of the season models of each taxa

Taxa	Stage	Elev	Slope	Cattle	SchVeg	BrdMix	DDaut	DDsum	Paut	Psum	Phsep	Phnov	Fem. abund.
C. imicola	NF	(−)			(+A)			(−)					(+)
	PF	(−)			(+A)							(+)	
Obsoletus complex	NF	(−) (−A)		(+) (−A)		(+)		(−A)	(−A)				(+)
	PF			(+)	(−)		(+)		(+)				(+)
C. newsteadi	NF												
	PF										(A+)		
C. pulicaris	NF												
	PF												

NF, nulliparous females; PF, parous females; light grey, variables included in the best model; dark grey, variables with weak effect (90% of CI did not include zero); black, variables with strong effect (95% of CI did not include zero); (+), positive effect; (−), negative effect; (A), indirect effect via *Culicoides* female abundance.

summer and high abundance of *Culicoides* females. Conversely, longer season of *C. imicola* PF were also related to lower elevation sites with sclerophyllous vegetation, and high number of daylight hours during November. The null model received essentially no support in the data in comparison to the best models of *C. imicola* PF (ΔDIC = 2.40, Supp. table S9).

Regarding the Obsoletus complex species, results showed that accumulated degree days over 10°C in summer, precipitation in autumn and elevation had a strong negative effect through *Culicoides* female abundance on the end of the season of Obsoletus complex NF (table 3 and Supp. fig. S6A); in addition, cattle density showed a weak negative effect through *Culicoides* female abundance on the end of the season of NF. Broad-leaved forest and mixed forest and *Culicoides* female abundance had a strong positive effect on the end of the season of Obsoletus complex NF, while cattle density had a weak positive affect on the same timing. Conversely, elevation showed a strong negative effect on the end of season of NF. The following two top models of NF received similar support in the data, including a range of environmental covariates (ΔDIC < 2; Supp. table S10). Regarding Obsoletus complex PF, accumulated degree days over 10°C in autumn, cattle density and *Culicoides* female abundance had a strong positive effect on the end of season while the percentage of sclerophyllous vegetation showed negative effect on the same timing metric. In addition, precipitation in autumn had a weak positive effect on the end of season of PF of Obsoletus complex (Supp. fig. S6B). Thus, the season of Obsoletus complex NF was longer in low elevated broad-leaved and mixed forest areas with lower temperatures in summer, lower precipitation in autumn, lower cattle density and higher abundance of *Culicoides* females. Conversely, the season of Obsoletus complex PF was longer in sclerophyllous vegetation sites with higher temperatures and precipitation in autumn, higher cattle density and higher *Culicoides* female abundance, extending the risk of transmission of BTV to later through the year. The null model received essentially no support in the data in comparison to the best models of Obsoletus complex NF and PF (ΔDIC = 25.18 and 12.12 respectively, Supp. table S10).

The best path models for the end of season of *C. newsteadi* NF did not include any variables (null model). Conversely, photoperiod in September showed a weak positive effect on the end of PF season (table 3 and Supp. fig. S7). The following two top models of PF received similar support in the data, including the null model and a range of environmental covariates (ΔDIC < 2; Supp. table S11). Therefore the season of *C. newsteadi* PF was longer in sites with high number of daylight hours in September. However, we must conclude from these results that our analysis was unable to detect any meaningful associations between seasonality and the measured environmental variables for this species.

No environmental variables for the end of season model for both *C. pulicaris* NF and PF were included in the best model (tables 3, S3 and S12, null model).

Effects on overwintering

The path analysis showed that accumulated degree days over 10°C in winter had a strong positive effect through *Culicoides* female abundance on the length of overwinter of both *C. imicola* NF and PF (table 4 and Supp. fig. S8). In addition, accumulated degree days over 10°C in winter and the slope of the land showed a strong negative effect on the length of overwinter of NF (Supp.

Table 4. Summary of the significant environmental parameters for length of overwinter period of each taxa

Taxa	Stage	Elev	Slope	Cattle	Sheep	NatGras	PastGras	AgFor	SchVeg	DDwin	DDaut	Pwin	Paut	Phsep	Phnov	Fem. abund
C. imicola	NF		(−)							(−) (+A)						
	PF				(−)					(+A)						(+)
Obsoletus complex	NF	(+)		■												
	PF														(+)	
C. newsteadi	NF		(+)						(−)							(−)
	PF															(−)
C. pulicaris	NF						(−A)						(+)		(+)	
	PF															

NF, nulliparous females; PF, parous females; light grey, variables included in the best model; dark grey, variables with weak effect (90% of CI did not include zero); black, variables with strong effect (95% of CI did not include zero); (+), positive effect; (−), negative effect; (A), indirect effect via *Culicoides* female abundance.

fig. S8A). Regarding *C. imicola* PF, sheep density a strong negative effect on the length of overwinter whereas the photoperiod in September showed a weak positive effect (Supp. fig. S8B). The following two top models of both NF and PF received similar support in the data, including a range of environmental covariates (ΔDIC < 2; Supp. table S13). Therefore, sites with high slope having high temperatures in winter had shorter overwintering periods of NF. In addition, sites with high temperatures, high sheep density and low number of daylight hours during September showed shorter overwintering period of *C. imicola* PF, therefore, longer period of risk of BTV transmission. The null model received essentially no support in the data in comparison to the best models of *C. imicola* NF and PF (ΔDIC = 7.34 and 19.26 respectively, Supp. table S13).

Elevation and the photoperiod in November had a significant positive effect on the length of overwinter of Obsoletus complex NF (table 4 and Supp. fig. S9) meaning that the overwinter period of NF was longer in elevated sites with higher number of daylight hours during November. The null model received essentially no support in the data in comparison to the best models of Obsoletus complex NF (ΔDIC = 16.49, Supp. table S14). For PF, the best model did not include any variables (the null model); however, the following top model which included seven variables received similar support in the data (ΔDIC < 2, table S14). From these results, we must conclude that our analysis for PF was unable to detect any significant relationships between the measured environment variables and seasonality.

Female abundance had a strong negative effect on the *C. newsteadi* NF length of overwinter season (table 4 and Supp. fig. S10A) and a weak negative effect on the length of overwinter of PF (Supp. fig. S10B). In addition, sclerophyllous vegetation and slope had a weak negative and positive effect respectively on the length of overwinter period of NF. The second top model of NF and PF received similar support in the data, including a range of environmental covariates (ΔDIC < 2; Supp. table S15). Therefore, high female abundance was associated with a decrease in the overwintering period of both NF and PF lengthening the potential BTV transmission period. Meanwhile, sites with higher sclerophyllous vegetation areas and lower slope showed a longer period of activity of *C. newsteadi* NF. The null models received essentially no support in the data in comparison to the best models of *C. newsteadi* NF and PF (ΔDIC = 7.93 and 4.89 respectively, Supp. table S15).

Regarding *C. pulicaris*, photoperiod in November in addition to the precipitation in autumn had a strong and weak positive effect respectively on the *C. pulicaris* length of overwinter period of NF (table 4 and Supp. fig. S11A). In addition, the combination of pastures and natural grassland areas had a weak negative effect on NF abundance. The following top model of NF, which is the null model, received similar support in the data, including a range of environmental covariates (ΔDIC < 2; Supp. table S16). Therefore, sites with lower precipitation in autumn and a lower number of daylight hours during November were associated with shorter overwintering period and therefore a long period of activity of NF. Also, sites with smaller areas of pastures and natural grasslands were associated with a decrease in the number of NF as an indirect effect on the overwintering period of *C. pulicaris* NF. Regarding the PF, no variables of the overwintering period model showed strong or weak effects (Supp. fig. S11B). The following top models of PF ad similar support to the data, including a similar range of environmental covariates (ΔDIC < 2; Supp. table S16). The null model received essentially no support in

the data in comparison to the best model of *C. pulicaris* PF (ΔDIC = 11.27, Supp. table S16).

To summarize, it was aforementioned that *C. imicola* and Obsoletus complex were the species for which most significant environmental effects on seasonality were detected, especially on the timing of the end of season. *Culicoides* population abundance had significant effects on seasonality of almost all of the species except *C. pulicaris*. The activity period of NF and PF was found to be longer in populations with high overall abundance for all the taxa studied. The other environmental variables had different effects on start, end and length of overwinter between life stages (NF or PF), especially for Obsoletus complex. In fact, the land cover only had a significant effect on the PF of this species (tables 2 and 3).

The environmental variables showed different effects on the overwinter periods between studied taxa (table 4). *C. imicola* over-wintering was strongly affected by the slope of the land, the density of sheep and the temperature during winter whereas Obsoletus complex was affected by the elevation and the photoperiod in November. For *C. newsteadi*, length of overwinter was only strongly affected by the abundance of females. Meanwhile, *C. pulicaris* overwinter was only strongly affected by the photoperiod in November.

The indirect effects via *Culicoides* female abundance were mainly observed in *C. imicola* and Obsoletus complex. Five different variables had strong indirect effects: sclerophyllous vegetation and temperature during winter for NF and PF of *C. imicola*, and elevation, temperature during summer and precipitation in autumn for Obsoletus complex NF (tables 2–4). In addition, cattle density showed a weak indirect effect on the season of Obsoletus complex NF via *Culicoides* female abundance (tables 2 and 3). Conversely, precipitation in winter and the photoperiod in September had a weak significant indirect effect on the start and end of season of *C. newsteadi* PF via female abundance (tables 2 and 3); whereas pastures and natural grassland areas had a weak significant indirect effect on the overwinter period of *C. pulicaris* NF (table 4).

Discussion

This study develops a novel methods for understanding diverse environmental drivers of metrics of adult seasonality of *Culicoides* vector taxa in Spain that define the SVFP, used to target surveillance and animal movements in outbreak regions (Brugger and Rubel, 2013; Brugger *et al.*, 2016; Napp *et al.*, 2016). Larger populations may have longer adult seasons, either due to sampling effects or because the conditions that promote higher populations are those that also favour long adult activity periods. Path analysis helped us to separate environmental effects on female abundance, narrowing down which environmental variables affect seasonality directly opposed to indirectly via their action on population abundance.

Path analysis has shown for the first time the indirect effects of environmental variables on seasonality through the *Culicoides* female abundance. Strong indirect effects of environmental factors on seasonality, via population abundance, were detected in *C. imicola* NF and PF and Obsoletus complex NF. Elevation, livestock density, temperature and precipitation were all found to influence seasonality through *Culicoides* female abundance, highlighting the importance of using an approach like path analysis to separate direct and indirect effects of environment on overall population abundance and seasonality. Future research with richer measures of phenology (e.g. daily rather than weekly trapping), population size and environmental variability (e.g. microclimate variation) could improve the present results.

Environmental effects were more difficult to detect across taxa on the timing of the end of the adult season, compared to the start of the season or the length of the over-winter period. In fact, L4 instar larvae of *Culicoides* cease diapause and continue development when temperature increases, thus synchronizing the adult emergence in spring (White *et al.*, 2017). Thus while the timing of the start of the season may be quite tightly linked to temperature and photoperiod that was captured in our analysis, the timing of the end of the adult season is more highly variable and sensitive to local unmeasured factors such as movement of livestock away from trap sites.

For northern species from the Obsoletus complex, path analysis showed that temperature and precipitation play an important role on the seasonality of this species. Females were detected as beginning their season earlier when temperature increases in spring, coinciding with its peak of abundance in Spain (Ortega *et al.*, 1998, 1999; Miranda *et al.*, 2004) and consistent with the modelled population behaviour (White *et al.*, 2017). Higher precipitation in winter and spring may increase the moisture levels in breeding sites providing favourable development conditions for the beginning of Obsoletus complex season (Harrup *et al.*, 2013). Sclerophyllous vegetation had a negative effect on the seasonality of Obsoletus complex species, ending the adult season earlier, possibly because this species prefers natural grassland sites and broad leaved and mixed forests, which are common in the north of Spain (Conte *et al.*, 2007; Harrup *et al.*, 2013). The current results showed that the number of daylight hours in March and November decreased the active season of Obsoletus complex species. The number of daylight hours seemed to be an insignificant parameter in other studies based on results from different treatments combining photoperiod and temperature for rearing *Culicoides chiopterus* and *Culicoides dewulfi* under laboratory conditions (Lühken *et al.*, 2015) or studies using the photoperiod as a predictor in statistical models for *C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. chiopterus* (Searle *et al.*, 2014). However, the photoperiod was an important driver for other *Culicoides* species like *C. pulicaris* (Searle *et al.*, 2013). A possible explanation remains unclear but could be related to the different latitudes within the sampling sites, since Obsoletus complex species are widespread distributed across the whole country. In fact, March and November are considered cold months in Spain with lower captures of this species, probably coinciding with their overwintering period (Miranda *et al.*, 2004; Cuéllar *et al.*, 2018a, 2018b; Barceló *et al.*, 2020).

Our finding that Obsoletus complex species had longer periods of activity in lower elevation sites is consistent with prior studies (Capela *et al.*, 2003; Torina *et al.*, 2004; Conte *et al.*, 2007). Further the finding that Obsoletus complex adult activity periods were longer in sites with high cattle livestock is consistent with this species abundance in livestock farms and mammalophilic behaviour (Talavera *et al.*, 2015). The Obsoletus complex includes five different species that may have different biotic and abiotic requirements. Further studies including molecular methods to determine the species from the Obsoletus complex must be considered to link environmental parameters to species-level adult phenology (Searle *et al.*, 2014).

Consistent with prior studies, it was more difficult to detect significant environmental effects on seasonality of the other northern species *C. pulicaris*, aside from the relationship between

high winter temperature and an earlier start of the adult season and between photoperiod and the length of the overwinter period. The finding that the length of the adult activity period of NF *C. pulicaris* is longer in sites with high precipitation during autumn is consistent with previous studies that found positive effects of this variable on the abundance and seasonality of *C. pulicaris* (Purse *et al.*, 2004a; Ducheyne *et al.*, 2013; Searle *et al.*, 2013).

Regarding the southern species, our finding that the seasonality of *C. imicola* was sensitive to temperature, altitude, land cover and photoperiod is consistent with prior studies (Acevedo *et al.*, 2010). The current study indicated that higher temperatures in winter and lower temperatures during summer prolonged the activity period of this species, aligning with prior findings; *C. imicola* is more abundant in sites with lower altitudes and extensive plains (Conte *et al.*, 2003, 2007; Torina *et al.*, 2004). The path analysis indicated the potential importance of land cover for adult seasonality. Contrasting with the Obsoletus complex species, cover of sclerophyllous vegetation indirectly affected seasonality by increasing of *Culicoides* female abundance during the end of season of this species, possibly linking to this species preference for drier grassland areas in south Europe (Mellor and Pitzolis, 1979; Acevedo *et al.*, 2010; Peters *et al.*, 2014). In fact, NF seasonality of that species seems to be negatively affected when the cattle density increases, and the overwintering of PF was shorter in sites with higher sheep abundance. This phenomenon was possibly because of competition with adult traps or because blood meals will be more widely available and NF will be more likely to develop into PF. Otherwise, the period of adult activity of *C. imicola* was negatively affected by the precipitation in spring, probably due to the lower density of this species during those seasons and preference for dry substrates of the immature stages (Conte *et al.*, 2007; Foxi and Del Rio, 2010). Finally, longer PF seasons in sites with longer days in November are aligned with the peak of abundance of this species in autumn (Ortega *et al.*, 1999; Miranda *et al.*, 2004; Grimaud *et al.*, 2019; Barceló *et al.*, 2021); therefore, a longer period of risk of transmission of BTV during this season.

Regarding *C. newsteadi*, results showed that this species was strongly affected by the abundance of females and, with weak effects, the climate, topography, land cover, photoperiod and livestock. The path analysis suggested that sites with lower slopes had shorter overwinter period, similar than results recorded by Torina *et al.* (2004) where, as well as *C. imicola* and Obsoletus complex species, *C. newsteadi* was more abundant in sites with low altitude. The temperature during winter was important for PF of this species which usually appear during seasons with high minimum temperatures, consistent with previous studies that have found a positive effect of high winter temperatures on abundance (Ortega *et al.*, 1999; Purse *et al.*, 2004a). In fact, path analysis results showed that high precipitation in winter had an indirect weak negative effect via female abundance on the start of the season of *C. newsteadi* NF, coinciding with the dry preferences of the south-western species (Ducheyne *et al.*, 2013). The short overwinter period of *C. newsteadi* NF on sclerophyllous vegetation could be related to the distribution of this species. As well as *C. imicola*, higher abundance of *C. newsteadi* occurs in warmer locations of the south and east of Spain (Ortega *et al.*, 1999; Del Río *et al.*, 2013; Ducheyne *et al.*, 2013), where sclerophyllous vegetation is the aforementioned most common type of vegetation in dry and warm climates such as in the Mediterranean basin. Livestock also played an important role. The season of *C. newsteadi* PF started later in sites with higher sheep densities. This

result was unexpected since *C. newsteadi* prefers sheep as a host (Garros *et al.*, 2011; Calvo *et al.*, 2012; Martínez-de la Puente *et al.*, 2015; Slama *et al.*, 2015) and early stages of this species breed in sheep livestock holdings (Foxi and Del Rio, 2010; González *et al.*, 2013). In fact, this result was contradictory to previous studies where *C. newsteadi* total females started earlier in sites with higher density of sheep (Barceló *et al.*, 2021). A possible explanation was unclear but could probably be related to the distribution of the midges and the livestock across Spain. The number of daylight hours in September increases the abundance of *Culicoides* females during the end of season of PF of this species. This result was probably related to the coincidence of higher abundance of *C. newsteadi* PF during summer and autumn (Barceló *et al.*, 2020). Best models for both NF of *C. newsteadi* start and end of season did not include any variables, suggesting that other environmental drivers that other variables such as Land Surface Temperature (LST), Enhanced Vegetation Index (EVI) (Purse *et al.*, 2012) or also other remotely sensed imagery data e.g. the normalized difference vegetation index, the middle infrared reflectance of the land cover and the air temperature a few metres above ground (Purse *et al.*, 2004a, 2004b) must be considered in order to point out the role of the environmental effects on the phenology and abundance of PF of this species.

This study provides a generalizable path analysis framework for understanding how environmental drivers modulate the seasonality and the abundance of *Culicoides* vector species in Spain. It has been demonstrated that the different *Culicoides* species respond to different environmental variables dependent upon their biological requirements, latitudinal distribution and life stage (NF or PF). Therefore, the significant environmental drivers should be included in the determination of the SVFP of these species.

New unmeasured variables like availability of breeding sites, air temperature and moisture levels within micro-climates for *Culicoides* (Mullens *et al.*, 2004) or the aforementioned could be integrated into a more nuanced understanding of direct and indirect environmental impacts on adult *Culicoides* phenology and statistical approaches can be combined with other empirical lab and field studies (e.g. studies of *Culicoides* early stages, substrates for oviposition or adult attraction by traps) and mechanistic population modelling approaches (White *et al.*, 2017) in order to provide a holistic understanding of seasonal regulation of insect vector populations.

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