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Development and lifespan of *Culicoides obsoletus* s.s. (Meigen) and other livestock-associated species reared at different temperatures under laboratory conditions

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Abstract. *Culicoides* Latreille (Diptera: Ceratopogonidae) transmit arboviruses affecting wild and domestic ruminants such as bluetongue (BTV) and Schmallenberg virus (SBV). The sub-adult development and lifespan of *Culicoides obsoletus* s.s. (Meigen), *Culicoides circumscriptus* Kieffer and *Culicoides paolae* Boorman were examined at three different temperatures under laboratory conditions. Insects were collected from field between spring and autumn 2015 in two livestock farms located in Majorca (Spain). Gravid females were held individually at 18, 25 or 30 °C. Low temperatures increased the adult lifespan, time to oviposit and rate of development, whereas high temperatures increased the number of eggs, successful pupation and adult emergence as well as the larvae growth rate. The results showed that *C. obsoletus* s.s. have optimum development at 18 °C, whereas the optimal rearing temperature for *C. circumscriptus* and *C. paolae* was under warmer conditions of 25–30 °C. Variations in temperature/humidity and assays with different materials and substrates for oviposition should be considered in future studies. Understanding the requirements of the different species of *Culicoides* optimizing the results should be of special interest for predicting environmental change effects on these species, in addition to determining the rearing conditions for candidate *Culicoides* vectors.

Key words. Arbovirus, Balearic Islands, bluetongue, Ceratopogonidae, eggs, larvae, life-cycle, lifespan, oviposition, rearing.

Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) can transmit globally important viral pathogens of domestic livestock such as African horse sickness (AHSV) to equines, Schmallenberg (SBV) and bluetongue virus (BTV) that causes infectious diseases of ruminants (Mellor *et al.*, 2000; Purse *et al.*, 2015; Wernike & Beer, 2017). Several *Culicoides* species, e.g. *C. obsoletus* s.s. (Meigen) and *Culicoides imicola* Kieffer, feed on domestic livestock and breed in suitable habitats in the surrounding areas near livestock (González *et al.*, 2013; Martínez-de la Puente *et al.*, 2015); while other species such as *Culicoides pulicaris* (Linnaeus) are not closely linked to livestock (Harrup *et al.*, 2013, 2016). Moreover, the majority of the females of these insects are mammophilic or ornithophilic blood feeders (Viennet *et al.*, 2012), suggesting that host availability could determine the *Culicoides* species composition.

Temperature and humidity are the major environmental factors affecting development, activity and distribution of *Culicoides* populations in Europe (Murray, 1991; Wittmann *et al.*, 2002; Carpenter *et al.*, 2008). In fact, adult activity and reproduction occur year round in tropical areas (Sellers, 1980; Verhoef *et al.*, 2014), but in temperate and cool regions they overwinter as larvae and the sub-adults enter a diapause period when daylight hours and/or temperature declines below a threshold level (Kettle, 1962; Rawlings & Mellor, 1994; Lysyk & Danyk, 2007). Moreover, the length of the adult vector-free period (SVFP) determines whether BTV will persist between

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years (EFSA, 2017). European Regulation (EC) 1266/20072 defines the SVFP as the absence of adult *C. imicola* collected in light traps and less than five parous females of other *Culicoides* vector species. In Spain, the SVFP is established in winter but could differ between years and latitudes (Barceló *et al.*, 2020).

To date, assays with different temperatures to record bionomic data of many European *Culicoides* populations remain unexplored. Several studies under laboratory conditions, such as Veronesi *et al.* (2009), revealed that temperature affects the development of sub-adult stages of *C. imicola* from South African populations. Also, in case of the Obsoletus complex species, temperature and composition of the breeding substrate plays an important role in larvae development (Lühken *et al.*, 2014, 2015a, 2015b; Steinke *et al.*, 2015). Other studies showed the effects of temperature on early stages, adult size and seasonality on *Culicoides sonorensis* Wirth & Jones (Akey *et al.*, 1978; Lysyk & Danyk, 2007; McDermott *et al.*, 2017); *Culicoides arakawae* (Arakawa) and *Culicoides maculatus* Shiraki (Kitaoka, 1982); as well as *C. imicola* and *Culicoides bolitinos* Meiswinkel (Verhoef *et al.*, 2014).

Purse *et al.* (2015) summarized the data available about the relationship between temperature and *Culicoides* development. This study showed that development time of *Culicoides* immature stages decreased with temperature but percentages of survival were different within species. However, only one out of the five species included in that study (*Culicoides nubeculosus* (Meigen)) were European origin. The present study shows detailed information about the basic bionomics of two different *Culicoides* populations reared at different temperatures in laboratory conditions. Except *C. imicola* (Veronesi *et al.*, 2009), this is the first study comparing the effect of temperatures among all *Culicoides* life stages from two populations of field-collected gravid females. In fact, results obtained in this study included information from species never reared before in a laboratory, such as *C. paolae*.

Material and methods

Adult *Culicoides* were collected alive from two livestock farms located in Majorca (Balearic Islands, Spain) separated 55 km

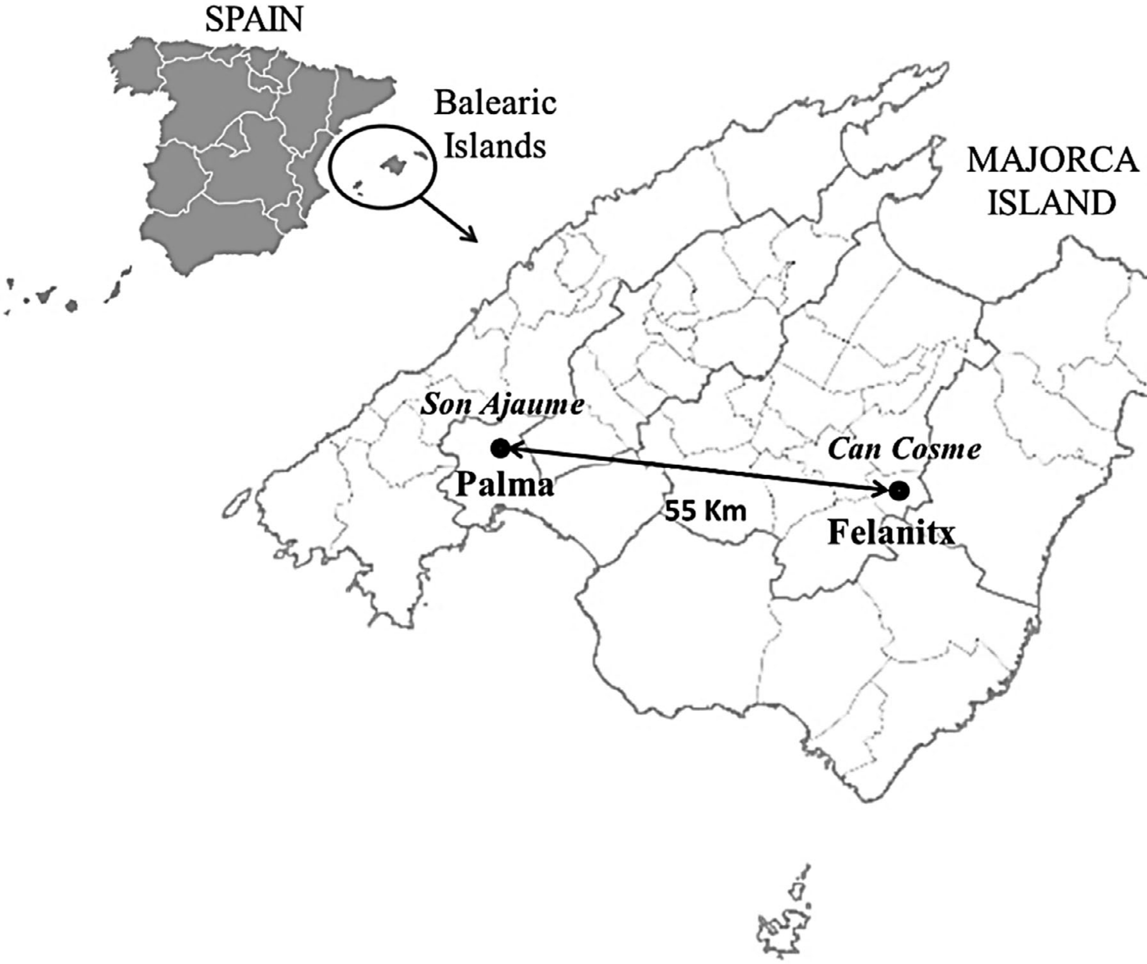

Fig. 1. Location of the two sampled livestock farms in Majorca Island, Spain.

Table 1. Species and number of animals in the sampled farms *Can Cosme* and *Son Ajaume*

Species	*Can Cosme*	*Son Ajaume*
Bos taurus	26	27
Ovis aries	535	147
Capra hircus	7	55
Equus caballus	1	2
Equus asinus	—	4
Equus caballus x asinus	—	1
Sus scrofa domestica	16	206
Gallus gallus	25	50
Numida meleagris	—	30
Meleagris gallopavo	—	25
Anser anser	—	5
Anas platyrhynchos domesticus	—	40
Columba palumbus	15	150
Total	625	742

from each other (Fig. 1); one in Felanitx municipality (*Can Cosme*, 39° 31′ 21.12″N; 3° 6′ 21.55″E; 80 m.a.s.l.) and the other in Palma municipality (*Son Ajaume*, 39° 38′ 41.85″N; 2° 39′ 8.79″E; 89 m.a.s.l.). Mean annual temperature and precipitation between municipalities are similar, with 16.3 °C / 619 mm in Felanitx and 16.8 °C / 453 mm in Palma (AEMET, 2020). Vegetation in both farms is represented by the typical Mediterranean flora, dominated by wild olive (*Olea europaea* var. *sylvestris*), pines (*Pinus halepensis*), oaks (*Quercus ilex*) and mastic trees (*Pistacia lentiscus*). However, livestock production differed between farms (Table 1). In *Can Cosme* sheep was the main production, followed by chicken, one horse kept outdoors and pigsty located indoors. *Son Ajaume* farm had sheep, pig and poultry production but also included more species such as doves, turkeys, ducks, geese and helmed guineafowls (*N. meleagris*). The animals in *Son Ajaume* were all located outdoors. Collections were conducted using two UV light-suction traps per farm (Onderstepoort model; OVI-ARC, Onderstepoort, South Africa) during 24 non-consecutive nights (12 nights in each farm) from dusk to dawn between April 30th and November 17th 2015. No BTV was circulating during that period (RASVE, 2016). Traps were separated 30 m from each other avoiding light interference between them (Venter *et al.*, 2012; Elbers & Meiswinkel, 2015).

Captures were collected early in the morning of the following day to avoid high mortality of adults, for this, trap collection pots were filled with moistened paper to provide humid shelter for insects.

Live *Culicoides* were separated into a gauze cage from dead individuals and the non-targeted insects using a manual insect aspirator. Alive *Culicoides* were anaesthetised with cold following the procedures of Venter *et al.* (1991). Gravid females were selected on a chill table (Dyce, 1969) and kept individually in 64 × 50 mm cardboard boxes (Watkins & Doncaster®). Boxes were provided with 5 cm plastic Petri dish at the bottom with moistened cotton wool and filter paper as substrate for oviposition (Barceló & Miranda, 2017) and paper pads with 10% sucrose solution were used to provide a sugar meal, mixed with 0.2% of Nipagin (Methyl *p*-hydroxybenzoate) [AppliChem GmbH (Darmstadt, Germany) & Panreac Química SLU (Barcelona, Spain)] as an antibiotic to prevent fungal proliferation (Veronesi *et al.*, 2009). After oviposition, eggs from each single gravid female were counted and transferred using a small brush to 100 mm Petri dishes filled with 10 mL of 2% European Bacteriological Agar (CONDA® Laboratories, Pronadisa, Spain) gel medium and humidified daily to prevent desiccation (Boorman, 1985). Adults and eggs/larvae were all kept separated inside climatic chambers (Climas® Grow chamber, Barcelona, Spain) at three different temperatures and humidity: 18.5 ± 0.9 °C with 56.3 ± 4.1% relative humidity (RH) (abbreviated as 18°C), 24.3 ± 0.5 °C with 63.9 ± 2.2% RH (abbreviated as 25 °C) and 30.4 ± 0.5 °C with 82.6 ± 4.5% RH (abbreviated as 30 °C).

For larvae feeding, we provided *Panagrellus redivivus* species (Nematoda: Panagrolaimidae) grown in cereal powder (Kettle *et al.*, 1975; Boorman, 1985; Erram & Burkett-Cadena, 2020) and delivered directly in the Petri dish by a brush. Larvae were daily measured from each Petri dish using a Stereomicroscope (Nikon® SMZ 645) to record the larval growth according to rank size of the total length of larvae estimated by several authors (Kettle & Lawson, 1952; Becker, 1960b; Murphree & Mullen, 1991; Slama *et al.*, 2013). Pupae were counted and transferred from the agar medium again to the cardboard boxes where F1 adults emerged and were fed daily with 10% sucrose solution.

Culicoides females were identified using keys based on wing pattern (Rawlings, 1996; Mathieu *et al.*, 2012). The species belonging to the Obsoletus complex include *C. obsoletus* s.s., *Culicoides scoticus* Downes and Kettle, *Culicoides montanus* Shakirzjanova (Mignotte *et al.*, 2020); however, *C. obsoletus* s.s. and *C. scoticus* are the most common species in Spain (Pagès & Sarto I Monteys, 2005). Due to the difficulty in discriminating females exhibiting a wing pattern Obsoletus group-like, males from F1 were identified according to the keys of Campbell & Pelham-Clinton (1960), Delécolle (1985) and Nielsen & Kristensen (2011).

The Shannon's diversity index (H') (Shannon & Weaver, 1949) was calculated in order to measure the diversity of total *Culicoides* females collected from both farms. Results were analysed using the R® Development Core Team 4.0.1 software. Data of gravid females were subjected to One-way analysis of variance (ANOVA) followed by post hoc Tukey HSD and Bonferroni comparisons among groups, where applicable. Data of sub-adult stages obtained from gravid females of both farms were combined before analysis. Differences between sub-adult stages were assessed using a non-parametric Kolmogórov-Smirnov two-sample test (K-S).

Results

Species composition and survival in sampling

A total of 7657 *Culicoides* (males and females), included in six different subgenera, were collected from both farms. From *Can Cosme* 4862 individuals, with 16.5% (801) gravid females and from *Son Ajaume* 2795, with 48.9% (1366) gravid females. Total survival rates in light traps were 34.5% (Avg.

Table 2. Number of gravid females and total alive *Culicoides* species collected in both livestock farms with sampling survival rates. In brackets: total individuals.

	Can Cosme			*Son Ajaume*		
Species	GF	Total	Survival %	GF	Total	Survival %
Subgenus *Avaritia*						
Obsoletus complex	106 (257)	1230 (3432)	35.8%	6 (33)	43 (179)	24.0%
C. imicola	3 (14)	11 (48)	22.9%	45 (162)	177 (954)	18.6%
Subgenus *Beltranmyia*						
C. circumscriptus	108 (296)	219 (684)	32.0%	218 (750)	274 (992)	27.6%
Subgenus *Culicoides*						
C. newsteadi	13 (30)	76 (229)	33.2%	7 (20)	52 (163)	31.9%
Subgenus *Monoculicoides*						
C. puncticollis	1 (6)	3 (13)	23.1%	3 (6)	5 (23)	21.7%
C. parroti	0	0	—	2 (4)	6 (8)	75.0%
Subgenus *Oecacta*						
C. brunnicans	0	0	—	1 (1)	1 (1)	100%
Subgenus *Sensiculicoides*						
C. comosioculatus	0	0 (1)	0%	4 (6)	4 (6)	66.7%
C. cataneii	40 (146)	60 (204)	29.4%	47 (230)	49 (251)	19.5%
C. jumineri	3 (11)	4 (12)	33.2%	1 (1)	3 (3)	100%
Incertae sedis						
C. paolae	10 (41)	73 (239)	30.5%	25 (153)	37 (215)	17.2%
Total	284 (801)	1676 (4862)	34.5%	359 (1366)	651 (2795)	23.3%
	$H' = 1.475$			$H' = 2.265$		

Subgeneric classification according to Borkent & Dominiak (2020). GF, gravid females; H', Shannon's diversity index.

= 30.7 ± 4.2%) in *Can Cosme* and 23.3% (Avg. = 26.1 ± 9.5%) in *Son Ajaume*. Obsoletus complex was more abundant in *Can Cosme* while *C. imicola* more abundant in *Son Ajaume*. All the species from the Obsoletus complex were identified as *C. obsoletus* s.s. based on their progeny. Not only had the number of total and gravid females differed within farms but also the species composition. *Son Ajaume* exhibited higher biodiversity than *Can Cosme* ($H' = 2.265$, Table 2) including two species not present at *Can Cosme*: *Culicoides parroti* Kieffer and *Culicoides brunnicans* Edwards.

Laboratory survival of field-collected gravid females

The average total lifespan of field gravid females was 23.4 ± 19.8 days for the individuals kept at 18 °C, 16.8 ± 13.7 days at 25 °C and 13.1 ± 9.2 days at 30 °C (Table 3). The highest longevity recorded was 80 days for one individual of Obsoletus complex from *Can Cosme* as well as one individual of *C. circumscriptus* Kieffer from *Son Ajaume*. In addition, Obsoletus complex, *C. circumscriptus* and *C. paolae* Boorman showed significant longer lifespan at 18 °C than the other temperatures ($P < 0.05$).

Culicoides imicola showed similar lifespan at 18 and 25 °C; *C. jumineri* Callot & Kremer lived longer at 30 °C and one individual of *Culicoides cataneii* Clastrier recorded the highest lifespan at 30 °C in *Can Cosme*, surviving 41 days, however, no statistical differences were observed among temperatures. Regarding the lifespan after oviposition, *Culicoides* parous females also showed longest lifespan at 18 °C (Table 4).

Oviposition of field-collected gravid females

A total of 387 *Culicoides* gravid females (214 from *Can Cosme* and 173 from *Son Ajaume*) were introduced in the cardboard boxes, of these 387 specimens, 49 from four species oviposited (12.7%). The total percentage of females that oviposited among all species was 15.7% at 18°C, 10.4% at 25 °C and 11.6% at 30 °C. The Obsoletus complex species and *C. circumscriptus* laid eggs at the three different temperatures. The Obsoletus complex showed the highest percentage of oviposition at 18 °C (Fig. 2) whereas *C. circumscriptus* exhibited higher percentages of oviposition at 18 and 30 °C. *Culicoides paolae* from *Can Cosme* only oviposited at 18 °C, whereas the one from *Son Ajaume* at 30 °C. *Culicoides cataneii* from *Can Cosme* only oviposited at 18 °C (Fig. 2 A). The other collected species did not lay eggs at any temperature.

The average time to oviposit among all species was 20.0 ± 17.2 days at 18 °C, 12.5 ± 9.7 days at 25 °C and 5.5 ± 4.8 at 30 °C (Table 5). *Culicoides circumscriptus* at 18°C exhibited significant longer time to oviposit than the individuals kept at 30°C ($P < 0.05$). In fact, one individual of *C. circumscriptus* showed the highest time to oviposit (59 days) at 18 °*C. Obsoletus* complex showed short oviposition time at all three temperatures from both farms.

The average number of eggs laid among *Culicoides* gravid females was 62.9 ± 38.4 eggs / female at 18 °C; 82.9 ± 55.0 eggs / female at 25 °C and 69.5 ± 49.9 eggs / female at 30°C. *Culicoides circumscriptus* showed the highest number of eggs from individuals collected in *Can Cosme* at 30 °C and *Son Ajaume* at 25 °C; however, no statistical differences were recorded between temperatures (Fig. 3).

Table 3. Lifespan of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures ± SD (Standard deviation).

		18 °C		25 °C		30 °C	
Species	F	Avg. days ± SD	Max/Min. days	Avg. days ± SD	Max/Min. days	Avg. days ± SD	Max/Min. days
Obsoletus complex	**A**	29.7 ± 24.6 (27) a	80/2	14.5 ± 14.6 (34) b	54/1	16.1 ± 9.8 (35) b	40/1
	B	19.3 ± 15.0 (3)	35/5	—	—	10.5 ± 10.6 (2)	18/3
C. circumscriptus	**A**	22.9 ± 20.2 (21)	65/1	13.4 ± 11.6 (18)	36/1	14.2 ± 10.2 (25)	34/1
	B	24.8 ± 19.7 (26) a	80/1	21.5 ± 14.3 (28) ab	52/1	11.7 ± 7.9 (22) b	22/1
C. imicola	**A**	29 (1)	29/29	28 (1)	28/28	—	—
	B	21.5 ± 14.6 (13)	42/2	22.3 ± 11.5 (11)	46/5	8.6 ± 6.5	18/1
C. cataneii	**A**	23.4 ± 18.8 (10)	62/1	19.0 ± 18.4 (9)	46/1	16.7 ± 12.6 (10)	41/1
	B	14.4 ± 15.5 (8)	41/1	7.7 ± 9.8 (3)	19/2	13.6 ± 7.1 (8)	26/1
C. newsteadi	**A**	30.8 ± 27.6 (4)	65/3	15.5 ± 11.5 (4)	32/6	13.4 ± 9.3 (5)	23/3
	B	21.3 ± 17.2 (3)	35/2	—	—	11.0 ± 7.4 (4)	18/1
C. paolae	**A**	24.8 ± 17.7 (5) a	49/8	—	—	2.5 ± 2.4 (4) b	6/1
	B	11.7 ± 13.9 (9)	43/1	9.0 ± 5.4 (6)	17/14	11.7 ± 6.1 (9)	22/2
C. jumineri	**A**	3 (1)	3/3	—	—	11 (1)	11/11
	B	—	—	—	—	26 (1)	26/26
C. puncticollis	**A**	—	—	—	—	—	—
	B	30 (1)	30/30	—	—	—	—
C. parroti	**A**	—	—	—	—	—	—
	B	22–0 ± 25.5 (2)	40/4	—	—	19 (1)	19/19
C. brunnicans	**A**	—	—	—	—	—	—
	B	—	—	17 (1)	17/17	—	—
Total average	**A**	26.0 ± 21.8 (69)	80/1	15.1 ± 14.0 (66)	54/1	14.1 ± 10.2 (79)	41/1
	B	20.5 ± 17.0 (64)	80/1	19.2 ± 13.2 (49)	52/1	11.6 ± 7.3 (56)	26/1
	T	23.4 ± 19.8 (133)	80/1	16.8 ± 13.7 (116)	54/1	13.1 ± 9.2 (135)	41/1

In brackets: Sample size. (T): Total. The different symbols ‘a’ and ‘b’ indicate significant differences between temperatures, while ‘ab’ indicates similar values to both ‘a’ and ‘b’ values (Turkey HDS test, $P < 0.05$).

Table 4. Lifespan after oviposition of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures ± SD (Standard deviation).

		18 °C			25 °C			30 °C		
Species	F	Avg. days ± SD	Max. days	Min. days	Avg. days ± SD	Max. days	Min. days	Avg. days ± SD	Max. days	Min. days
Obsoletus complex	**A**	23.9 ± 24.4 (8)	70	1	6.7 ± 4.3 (5)	13	1	12.7 ± 10.0 (3)	24	5
	B	18.0 ± 21.2 (2)	33	3	—	—	—	1 (1)	0	0
C. circumscriptus	**A**	19.5 ± 23.3 (2)	36	3	3 (1)	3	3	4.1 ± 1.2 (4)	5	2
	B	16.9 ± 10.4 (7)	32	5	6.9 ± 4.5 (6)	13	2	5.0 ± 5.6 (6)	11	1
C. cataneii	**A**	40* (1)	40	40	—	—	—	—	—	—
	B	—	—	—	—	—	—	—	—	—
C. paolae	**A**	32* (1)	32	32	—	—	—	—	—	—
	B	—	—	—	—	—	—	0.5$^{\#}$ ± 0.7 (2)	1	1
Total average	**A**	25.4 ± 21.2 (12)	70	1	6.1 ± 4.1 (6)	13	1	7.8 ± 7.4 (7)	24	2
	B	17.2 ± 11.9 (9)	33	3	6.9 ± 4.5 (6)	13	2	2.7 ± 4.3 (7)	11	1
	T	22.1 ± 18.1 (21)	70	1	6.5 ± 4.1 (12)	13	1	5.4 ± 6.5 (14)	24	1

In brackets: Sample size. (T): Total. (*): The species only oviposit at 18 °C. (#): The species only oviposit at 30 °C.

Percentage of egg hatching, pupation success and adult emergence

The total average of egg hatch among all species from both farms was 26.7 ± 31.3% for the individuals kept at 18 °C, 31.8 ± 34.6% at 25 °C and 13.8 ± 20.4% at 30 °C. *Culicoides obsoletus* s.s. showed the highest percentage of egg hatching at 18°C (35.0 ± 36.1%), while for *C. circumscriptus* and *C. paolae* they were at 25 and 30 °C, respectively (Fig. 4).

Larvae reared at 25 °C showed higher growing rates over all larval stages than the other temperatures (Fig. 5). The larvae of *C. obsoletus* s.s. and *C. circumscriptus* growing at 18 °C exhibited significantly lower growth rates than at the other temperatures ($P < 0.05$). The maximum values were 0.5 ± 0.2 mm/day recorded by *C. circumscriptus* at 25°C. *Culicoides paolae* showed similar growth rates to *C. circumscriptus* at 30 °C.

Total averages of percentage of pupation were 13.9 ± 19% at 18 °C, 33.5 ± 30% at 25 °C and 25.3 ± 33.6% at 30 °C. *Culicoides obsoletus* s.s. and *C. circumscriptus* showed higher

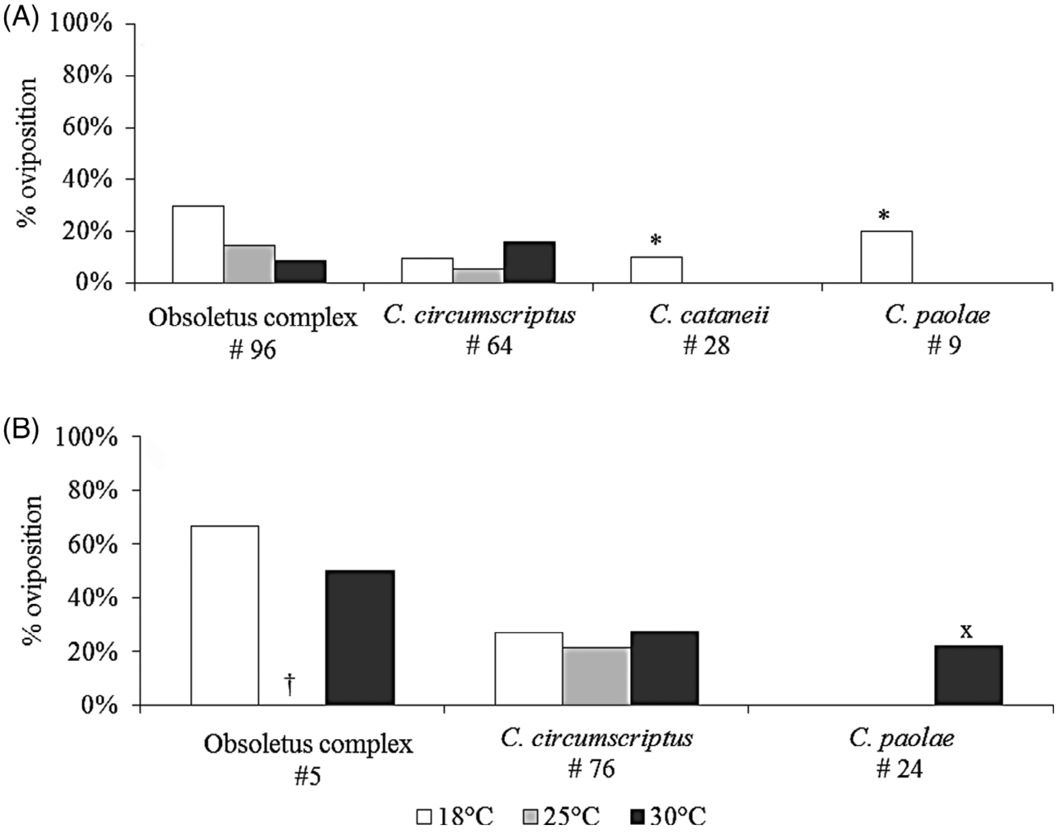

Fig. 2. Percentage of *Culicoides* field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) that oviposited at different temperatures under laboratory conditions. (#): Sample size. (*): The species only oviposited at 18 °C. (x): The species only oviposited at 30 °C. (†): The individuals at 25 °C did not survive.

Table 5. Time to oviposit of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures ± SD (Standard deviation). In brackets: Sample size.

		18 °C		25 °C		30 °C	
Species	F	Avg. days ± SD	Max/min. days	Avg. days ± SD	Max/min. days	Avg. days ± SD.	Max/min. days
Obsoletus complex	**A**	14.3 ± 17.0 (8)	54/1	8.1 ± 6.9 (5)	20/3	2.3 ± 1.2 (3)	3/1
	B	2 (2)	2/2	—	—	3 (1)	3/3
*C. circumscriptus**	**A**	44.0 ± 21.2 (2)	59/29	1 (1)	1/1	4.4 ± 5.1 (4)	12/1
	B	25.1 ± 13.9 (7)	48/10	18.1 ± 9.3 (6)	30/8	7.8 ± 5.1 (6)	17/2
C. cataneii	**A**	22 (1)	22/22	—	—	—	—
	B	—	—	—	—	—	—
C. paolae	**A**	17 (1)	17/17	—	—	—	—
	B	—	—	—	—	7.0 ± 7.1 (2)	12/2
Total average	**A**	20.0 ± 18.9 (12)	59/1	6.9 ± 6.8 (6)	20/1	3.5 ± 3.8 (7)	12/1
	B	19.9 ± 15.7 (9)	48/2	18.1 ± 9.3 (6)	30/8	7.1 ± 5.0 (9)	17/2
	T	20.0 ± 17.2 (21)	59/1	12.5 ± 9.7 (12)	30/1	5.5 ± 4.8 (16)	17/1

(*): Significant differences between 18 and 30 °C (data from both farms combined) (Bonferroni test $P < 0.05$).

percentages of pupation at 25 °C (Fig. 4) while *C. paolae* showed the highest percentage of pupation among all taxa at 30 °C (65.3%), however, no statistical differences were observed between temperatures. *Culicoides cataneii* and *C. paolae* held at 18 °C died just after egg hatching.

Pupation of *C. obsoletus* s.s. occurred between the 5th and the 80th day (a period of 75 days) at 18 °C (Fig. 6), whereas L4 of this species finished pupating earlier (less than 50 days) at 25 °C. However, number of larvae that pupate at 25 °C was lower at 18 °C.

The L4 larvae of *C. circumscriptus* started to pupate earlier than *C. obsoletus* s.s. and were generally concentrated during the first 10 days. Moreover, number of daily pupae of this species was higher than *C. obsoletus* s.s. and increased proportionally

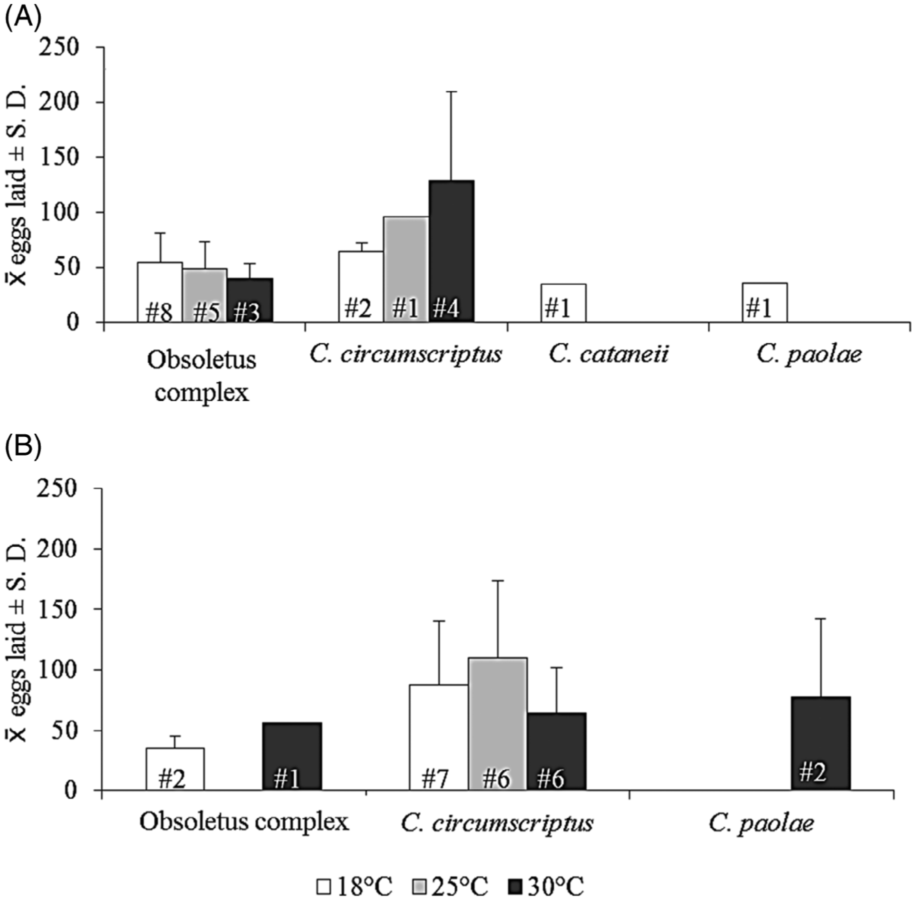

Fig. 3. Average number of eggs laid ± S. D. of the *Culicoides* field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures under laboratory conditions. (#): Sample size.

with the temperature. Although the pupation period of *C. circumscriptus* was shorter at 25 °C, number of pupae recorded per day of this species was higher and more evenly distributed at 18 °C.

Culicoides paolae larvae showed a similar pupation period as *C. circumscriptus* at 25 °C. In fact, pupation of these two species finished the 6th day after the first L4 larvae.

The percentage of adult emergence showed high values in general and was higher at high temperatures; the total average among all species was 58.3 ± 47.4% at 18 °C, 86.8 ± 26.4% at 25 °C and 95.5 ± 11.0% at 30 °C. This percentage reached 100% for *C. paolae* at 30 °C (Fig. 4). *Culicoides obsoletus* s.s. at 25 °C showed significantly higher percentage of adult emergence than the ones kept at 18 °C ($P < 0.05$) while F1 adults of *C. circumscriptus* emerged at any temperature.

Sex ratio

The species *C. circumscriptus* and *C. paolae* showed similar ratio of males to females between the three temperatures (Fig. 7). *Culicoides obsoletus* s.s. were male biased reaching a 100% of males from pupae kept at 18°C.

Total life-cycle and F1 lifespan

The duration of each life-cycle stage increased as temperatures decreased. The longest life-cycle was 83.2 days for *C. circumscriptus* followed by 75.6 for *C. obsoletus* s.s. at 18°C (Fig. 8). The longest life stage was the F1 adult's lifespan; reaching a maximum of 98 days for one individual of *C. circumscriptus* followed by 88 days for one individual of *C. obsoletus* s.s. at 18 °C. Adults of *C. obsoletus* s.s. were unable to complete its life-cycle at 30 °C, whereas *C. paolae* only finished its life-cycle at that temperature. In fact, *C. paolae* recorded the shortest life-cycle period at 30 °C among all taxa (32.6 days).

Discussion

Statistical analysis demonstrated that temperature was the only variable that recorded statistical differences among taxa. All species kept 18 °C recorded the longest lifespans and oviposition periods and Obsoletus complex species clearly showed preference for low temperatures indicating its adaption to cold temperatures. Conversely, *C. circumscriptus* and *C. paolae* performed well at higher temperatures for its development which indicates warm adaptation.

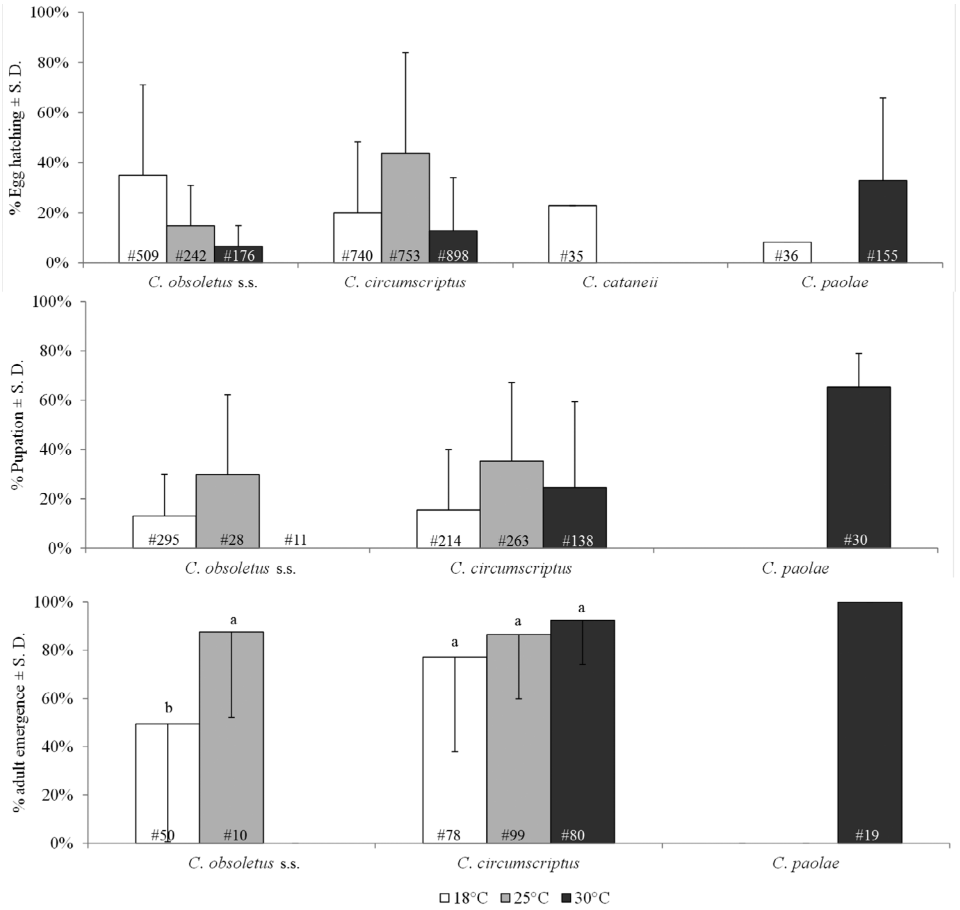

Fig. 4. Average percentage ± S. D. of egg hatching, larvae pupation and F1 adult emergence obtained from *Culicoides* field gravid females in both farms at different temperatures under laboratory conditions. The different symbols 'a' and 'b' indicate significant differences between temperatures (2-tailed K-S test, $P < 0.05$). (#): Sample size.

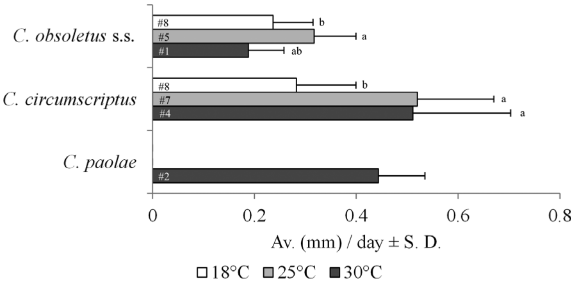

Fig. 5. Average length of larval growth (in mm per day) ± S. D. of each *Culicoides* species from at different temperatures under laboratory conditions The different symbols 'a' and 'b' indicate significant differences between temperatures, while 'ab' indicates similar values to both 'a' and 'b' values (2-tailed K-S test $P < 0.05$). (#): Sample size.

Species composition and survival in sampling

The survival rates in both farms during the sampling with UV light traps did not exceed 35%. These results were similar than values recorded by Del Río *et al.* (2014) where species such as *C. imicola* also showed survival rates lower than 35% using the same sampling method. The moistened paper set in the traps collector was insufficient to maintain the *Culicoides* alive until its transport to the laboratory. The decrease of temperature during the night and the fan drying effect were probably unfavourable for *Culicoides* individuals. Sampling improvements such as a decrease of fan power or special chambers avoiding the direct effect of the fan (e.g. chambers with shelters for the midges) should be considered.

Culicoides species composition and abundance were different between the two sampling sites. *H′* value determines the diversity in ecosystems. Values up to 2 such as in *Son Ajaume* are considered to be highly diverse (Shannon & Weaver, 1949). *Culicoides* abundance can vary over short distances. Blood feeding preferences (Ninio *et al.*, 2011; Martínez-de la Puente *et al.*, 2012, 2015; Viennet *et al.*, 2012), different substrate composition of the breeding sites (Foxi & Del Rio, 2010; Harrup *et al.*, 2013) or also the position of the traps in relation to physical structures (Venter *et al.*, 2018), could determine the observed differences in *Culicoides* diversity and abundance between farms. Only *Son Ajaume* farm recorded few individuals of C. *parroti* and *C. brunnicans*. Prior studies demonstrated that *C. parroti* showed preferences to feed on pigs (Martínez-de

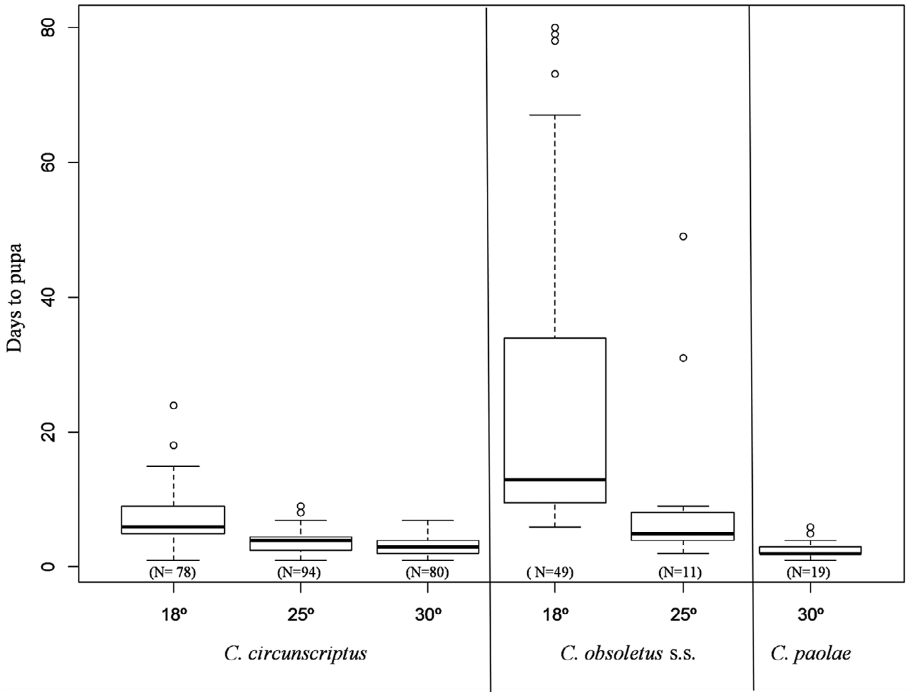

Fig. 6. Number of daily L4 larvae of each *Culicoides* species that pupated at different temperatures under laboratory conditions. (N): Sample size.

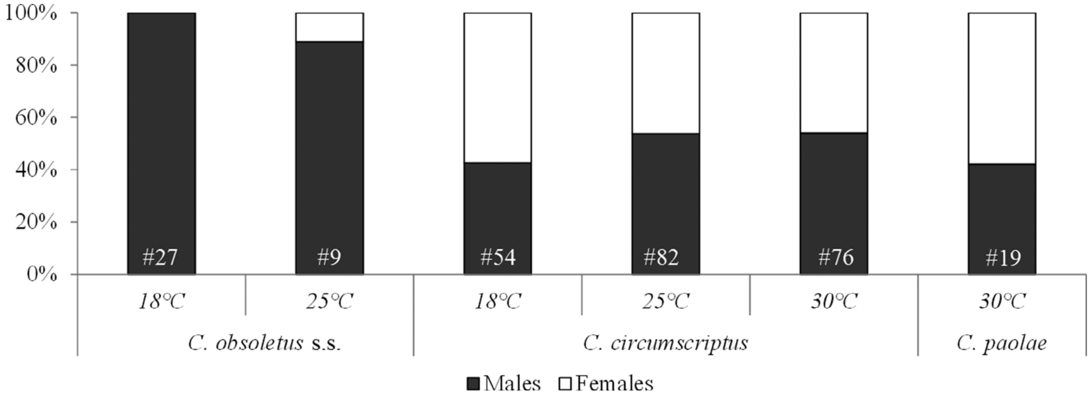

Fig. 7. Percentage of total F1 *Culicoides* adult males and females for each species that emerged at different temperatures under laboratory conditions. (#): Sample size.

la Puente *et al.*, 2015) which could be related with the high density of pigs located outdoors in *Son Ajaume*. Conversely, the exclusive presence of one individual *C. brunnicans* in *Son Ajaume* was unexpected since this species feed on cattle and sheep (Garros *et al.*, 2011; Ninio *et al.*, 2011; Calvo *et al.*, 2012; Martínez-de la Puente *et al.*, 2015), being less abundant in *Son Ajaume*. However, it is difficult to reach any conclusion due to the small sample size of these two species and we could consider them anecdotal captures.

The phenomenon of high abundance of gravid females, especially of *C. circumscriptus* collected in both farms and also in that of Barceló & Miranda (2017) remains unknown, but was probably related with long gonotrophic cycles of this species (Carpenter *et al.*, 2006).

Laboratory survival of field-collected gravid females

In the current study, individuals kept at 18°C showed the highest lifespan. In Europe members, Obsoletus complex can be active at temperatures as low as 10°C (Versteirt *et al.*, 2017) and, in laboratory conditions, *C. obsoletus* s.s. can survive more than 90 days at 17 °C and up to 10 days at 4 °C (Goffredo *et al.*, 2004). In South Africa, the adults of *C. imicola* can survive for more than 15 days at 22.2 °C (Nevill, 1971). These results are similar to the maximum of 80 days of lifespan recorded for *C. obsoletus* s.s. in our study at 18 °C and the 21.5 and 29 days for *C. imicola* at 18 and 25 °C. The minimum temperature required for viral replication (11–13 °C) inside vectors is generally consistent for different arboviruses transmitted by *Culicoides* vector species

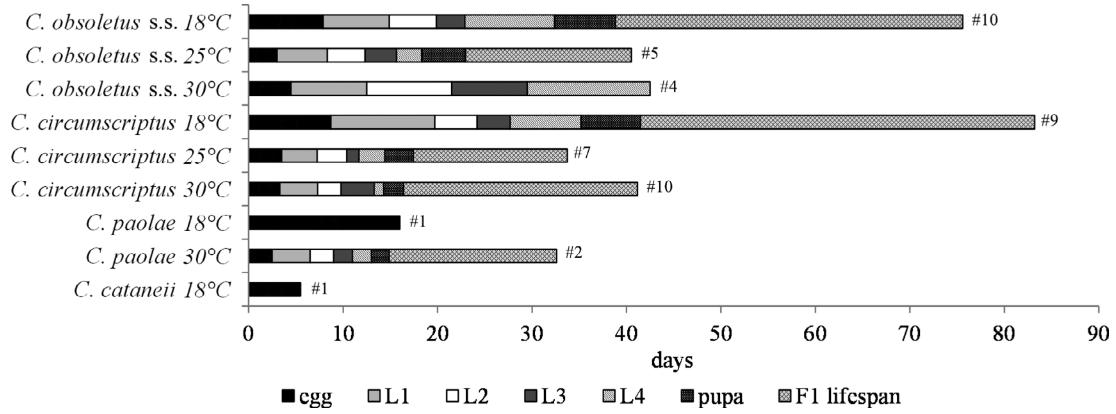

Fig. 8. Average of sub-adult stages duration in days and F1 adult survival of each *Culicoides* species at different temperatures under laboratory conditions (#): Sample size.

(Carpenter *et al.*, 2011). Previous studies have also demonstrated the capability of species from the Imicola group (*C. imicola* and *C. bolitinos*) to harbour BTV for more than 20 days at 10 °C (Paweska *et al.*, 2002). However, *C. bolitinos*, which is a high-altitude species, seems to be better adapted to cooler temperatures than *C. imicola* and able to survive the relatively mild winter temperatures as adults in South Africa (Verhoef *et al.*, 2014).

The maximum lifespan of *C. circumscriptus* at 18 °C was higher than that of Becker (1960b), where adults survived over 2 weeks at temperatures between 17°C and 20°C. These results could be related to the variations of temperature in the study of Becker (1960b) versus the fixed temperature of 18°C in the current study.

Adult lifespan was shorter in individuals kept at 30°C. *Culicoides* individuals at 30°C in the current study were kept at 82.6 ± 4.5% RH. This high percentage of humidity could explain the low ratio of *Culicoides* survival. At high temperatures, high humidity (up to 85%) hinders the ability of midges to eliminate excess metabolic water by evaporation and was considered detrimental for species such as *C. sonorensis* (Wittmann *et al.*, 2002). High humidity could be also related with fungi or other pathogens that can thrive in moist conditions and cause an increase of *Culicoides* mortality.

Oviposition of field-collected gravid females

The total percentage of oviposition on artificial substrate at different temperatures recorded in this study (18, 25 and 30 °C) indicates that the substrate and the technique were adequate to obtain eggs from field-collected females (Barceló & Miranda, 2017). Even still, less than 20% of total gravid females collected in both farms managed to lay eggs (Obsoletus complex and the ornithophilic species *C. circumscriptus*, *C. paolae* and *C. cataneii*) and only two of them (Obsoletus complex and *C. circumscriptus*) oviposited eggs at the three temperatures. The vector species *C. imicola* and *Culicoides newsteadi* Austen, as well as the non-vectors *C. jumineri*, *Culicoides puncticollis* (Becker), *C. parroti*, *C. brunnicans* and *Culicoides comosioculatus* Tokunaga were less abundant in samples and did not succeed in ovipositing; however, *C. imicola* and *C. newsteadi* have been previously reared under laboratory conditions (Barceló & Miranda, 2017). These low oviposition rates could be linked with the eggs absorption of some *Culicoides* females related with the unsuitability of the artificial substrate (Carpenter *et al.*, 2001). Insects in field conditions are subjected to oscillations of temperature and the accumulation of that temperature may affect the reproduction metabolism of *Culicoides* females (Lühken *et al.*, 2015b). It should be considered that this is the first study using different temperatures for *C. newsteadi*, *C. paolae*, *C. cataneii* and *C. jumineri* and poor information is available about the bioecology of these species; thus, highlighting the novelty of the current study.

Despite no statistical differences recorded, biting midges kept at 18 °C showed higher oviposition rates than the ones kept at 25 and 30 °C. In fact, obsoletus complex showed the highest oviposition rates at this temperature, being higher than the value of 7% recorded by Jamnback (1961) at 21.1 °C. These results were probably related to its aforementioned capacity to adapt to different substrates.

The time taken for females to oviposit increased at the relative low temperatures used in this trial (18 °C). Results obtained for *C. obsoletus* s.s. were similar than that of Parker (1950), where oviposition took place between the first and the second week at 17.2 °C. Conversely, individuals kept at relative high temperatures (30 °C) oviposited earlier than other temperatures. These results agree with a prior study of the life-cycle of *C. imicola* (Veronesi *et al.*, 2009), demonstrating that these insects are highly temperature dependent.

Fecundity of females was also variable between temperatures. Individuals kept at 30 °C laid more eggs than at other temperatures, except the Obsoletus complex from *Can Cosme* which laid more eggs at 18 °C. *Culicoides circumscriptus* laid the highest number of eggs at any temperature, possibly linked to the autogenous nature of this species (Kettle, 1977) and its large body size (2.2 mm; Delécolle, 1985). However, the average eggs laid by this species were lower than other studies which obtained more than 250 eggs of *C. circumscriptus* from field gravid females (Becker, 1960a, 1960b). The number of oviposited eggs by *C. obsoletus* s.s. at 18 and 25 °C was similar to previous studies (Hill, 1947; Parker, 1950; Braverman & Linley, 1994) where *C. obsoletus* s.s. laid between 30 and 57 eggs at temperatures ranging 17.2 and 21.1 °C. Conversely, Jamnback (1961) reported lower average number of eggs per female (14.7) at 21.1 °C than our results. The aforementioned eggs absorption under unfavourable conditions or also bionomic particularities related to local populations and conditions on the artificial rearing could be considered as possible interpretations. In addition, fecundity may be affected by the quality of the blood source since Hill (1947), Jamnback (1961) and Braverman & Linley (1994) used artificial blood feeding or frozen blood to obtain eggs instead of natural oviposition recorded in the current study. *Culicoides cataneii*, which only oviposited at 18 °C, recorded lower number of eggs than the value of 157.9 ± 7.4 obtained in that of Braverman & Linley (1994) at similar temperature. Our results were based on a single individual and we cannot define an accurate conclusion; however, this difference may be related with the methodology used for obtaining eggs, since the authors of Braverman & Linley (1994) dissected field gravid females to obtain the eggs. This methodology allows getting all eggs from the abdomen avoiding the eggs absorption by *C. cataneii* females.

The remaining species *C. paolae* only oviposited at 18 and 30 °C. This species showed the highest number of eggs laid at 30 °C in *Son Ajaume*, similar to the number of eggs laid by the other bird feeder and largest species *C. circumscriptus* at this temperature. Conversely, the individuals kept at 18 °C showed similar number of eggs to *C. obsoletus* s.s. at that temperature. This was an unexpected result and may be related to the unknown fecundity aspects of this ornithophilic species.

The use of mud and vegetation as oviposition substrates (Erram & Burkett-cadena, 2018) could be considered in further studies to improve the oviposition rates among all *Culicoides* taxa.

Percentage of egg hatching, pupation success and adult emergence

The percentages of egg hatching among all taxa were higher at 25 °C followed by 18 °C. In addition, *C. obsoletus* s.s. preferred low temperatures for egg hatching, whereas *C. circumscriptus* and *C. paolae* preferred higher ones. Limitations to rearing *C. cataneii* in artificial conditions included its low percentage of egg hatching, the lower percentage of oviposition by females and the lower number of eggs produced per female. This was the second attempt to our knowledge of laboratory rearing of this species (Barceló & Miranda, 2017); however, better information about its bioecology is required to improve its laboratory rearing. Conversely, *C. circumscriptus* developed best at 25 °C but the egg hatching rates were still lower than the 88.7% obtained by Becker (1960b) as found in that of Barceló & Miranda (2017).

The current results showed that the development of *Culicoides* larvae was delayed at low temperatures. *Culicoides* larvae may enter into diapause during cold and unfavourable conditions (Rawlings & Mellor, 1994; Lühken *et al.*, 2015a). In fact, the study of Lühken *et al.* (2015b) demonstrated that adult individuals from Obsoletus complex emerged later at cold temperatures from larvae developing directly in dung under laboratory conditions. In general, 25 °C was the most suitable temperature for larvae development and lower temperatures such as 18 °C reduced larvae growth among all taxa included in the current study. Species such as *C. circumscriptus* (the species of largest body size) and *C. paolae* grew faster than Obsoletus complex at any temperature, suggesting faster daily rates of development probably related to higher metabolic activity.

Pupation showed also to be different among species according to temperatures and is considered the most critical point in the life-cycle of *Culicoides* species, especially for *C. newsteadi*, *C. cataneii* and *C. imicola* (Veronesi *et al.*, 2009; Barceló & Miranda, 2017). Percentage of pupation was low among all tested species at 18 °C due to the high larvae mortality compared to the other temperatures. Other variables affecting the likelihood of pupation are competition for resources or cannibalism (i.e.: larvae predating pupae and earlier instars).

The highest percentage of pupation was recorded by *C. paolae* at 30 °C, showing again its preference for high temperatures. Conversely, no larvae of *C. obsoletus* s.s. survived at 30 °C, demonstrating that this species is favoured by mild spring temperatures, as it is shown in its seasonal pattern (Ortega *et al.*, 1998; Calvete *et al.*, 2008; Barceló *et al.*, 2020).

Pupation of *C. obsoletus* s.s. was the longest and occurred during several discrete time periods, instead of being synchronized in a short time. In addition, the number of larvae pupation of *C. obsoletus* s.s. was higher and more frequent at 18 °C, demonstrating again the cold adaptation of this species. Conversely, the time for pupation obtained from *C. circumscriptus* and *C. paolae* lasted over 1 week, being similar to the results from Becker (1960b) in the case of *C. circumscriptus*. In fact, number of pupae of *C. circumscriptus* was higher at elevated temperatures. It was also observed that pupation period of *C. circumscriptus* at 18 °C was more evenly distributed than the pupae kept at 25 °C, being related with the deterrent effect of the low temperature on *Culicoides* individuals particularly for more southerly species. The other ornithophilic species *C. paolae* recorded lower number of daily pupae but exhibited a similar pupation period than *C. circumscriptus*.

The percentage of adults emerged from reared pupae was relatively high in all of the species assayed, demonstrating that is a less sensitive stage of the life-cycle of this genus. Individuals kept at 30 °C showed the highest percentage of adult emergence. F1 adults from *C. circumscriptus* were the only ones emerged at any temperature, making this species suitable for rearing in laboratory conditions. In fact, results for *C. circumscriptus* at 18 °C showed higher percentage of adult emergence than that of Becker (1960b) at 17.2 °C, which recorded a percentage of 48.3%.

Sex ratio

The ornithophilic species *C. circumscriptus* and *C. paolae* had similar sex ratio supporting the results from Becker (1960b) which obtained an average of 51% *C. circumscriptus* females. As well as other haematophagous insects such as the yellow fever mosquito *Aedes aegypti* (L.) (Mohammed &

Chadee, 2011) temperature may play also an important role in sex determination of *Culicoides*, the mechanisms for which remains unclear. Males were predominant in the case of *C. obsoletus* s.s., which reached a 100% of males at 18 °C. Low percentages of females hinder the possibilities of mating; however, these results agree with the bias of the sex ratio obtained in that of Boorman (1985) and the reason still unclear. Higher mortality among female larvae or limitations in the feeding method to support *C. obsoletus* s.s. female development compared to males are possible explanations pointed out by Veronesi *et al.* (2009) and Nevill (1967). Other studies such as that of Erram & Burkett-Cadena (2020) showed that sex ratio was 1:1 using also nematode diet for *Culicoides stellifer* Coq. larvae and male biased when used nematodes with yeast diet. That study suggested that microbes from nematode medium play an important role for midge larvae development.

Total life-cycle and F1 adult lifespan

Temperature clearly plays an important role in the larvae development inducing diapause periods during low temperatures (Rawlings & Mellor, 1994; Lühken *et al.*, 2015b).The current study showed that *C. obsoletus* s.s. only completed its life-cycle at 18 and 25 °C. These results coincided with the temperatures in spring during peak abundance of the Obsoletus complex in Spain (Ortega *et al.*, 1998; Miranda *et al.*, 2004; Cuéllar *et al.*, 2018; Barceló *et al.*, 2020). Conversely, *C. circumscriptus* completed its development at any temperature and *C. paolae* only at 30 °C. This species along with *C. cataneii* showed difficulties in developing at 18 °C in laboratory conditions. The biogeographical distributions among the species including the photoperiod (northerly vs. southerly species) as well as the long egg hatching period of *C. paolae* and *C. cataneii* were a possible indicator of these difficulties. The low temperature and the artificial substrate may cause a deterrent effect on egg hatching. Hence, variations of temperatures in the laboratory such as swapping the insects from one to other temperature (Lühken *et al.*, 2015b), could be used in future works to improve the development of immature stages of those species.

The shortest life-cycle was showed by *C. paolae* at 30 °C. This result suggests that this species prefer high temperatures and humidity for development. *Culicoides paolae* is distributed in Mediterranean climate areas (Estrada *et al.*, 2011); so, may be adapted to develop at temperatures and humidity found during summer in Mediterranean climate areas.

It must be considered that immature development times may be quite different under field conditions where immatures are found in appropriate habitat; however, the duration of each of the four larvae stages and pupa stage were similar among all the taxa studied and the effect of temperature can be observed, especially in the L4 instar larvae which were longer in individuals kept at 18 °C.

The longest life-cycle stage was recorded from the F1 *Culicoides* showing that low temperatures also increased its lifespan. This is the first study where we obtained F1 adults of the vector species *C. obsoletus* s.s. and the non-vector species *C. paolae* at 18 and 30 °C, respectively, underlying the novelty of the current study. F1 adults of *C. obsoletus* s.s. reached a maximum of 88 days alive, close to the lifespan recorded from the field-collected gravid females and the values obtained in that of Goffredo *et al.* (2004); thus, demonstrating that the methodology used in the current study was successful for rearing and maintaining this species in laboratory conditions.

Conclusions

This study has contributed to the understanding of the basic bionomics at different temperatures of farm-associated species of *Culicoides* in Spain. In fact, this is the first study where *C. obsoletus* s.s. and *C. paolae* completed their life-cycle at these temperatures in laboratory conditions; *C. obsoletus* s.s. at 18 and 25 °C and *C. paolae* at 30 °C.

Mating conditions of these insects is one of the major obstacles for understanding the rearing requirements (Jamnback, 1961; Kettle, 1977; Veronesi *et al.*, 2009; Erram & Burkett-Cadena, 2020). Some species such as *C. obsoletus* s.s. mate in swarms (Kettle, 1995; Kirkeby, 2018) but the details of this behaviour in vector *Culicoides* species remains unknown (Blackwell *et al.*, 1992; González *et al.*, 2017). Several methods were used in Becker (1960a) to persuade *C. circumscriptus* to mate in laboratory conditions but without success; so, further studies including mating of different species could be interesting.

The vector species *C. obsoletus* s.s. and the non-vector species *C. circumscriptus* and *C. paolae* are the most promising species to be reared in laboratory conditions, due to its high oviposition percentages, lifespan, percentages of egg hatching, pupation and adult emergence, in addition to temperature adaptability in the case of *C. circumscriptus*. This species along with *C. paolae* were recently found BTV positive in Sardinia, which could be considered potential vectors (Foxi *et al.*, 2019). In fact, *C. circumscriptus* and *C. paolae* can bite humans and are able to transmit pathogens such as *Plasmodium* and *Leishmania* in field conditions (Lassen *et al.*, 2012; Ferraguti *et al.*, 2013; Slama *et al.*, 2014; Veiga *et al.*, 2018) and thus are of high priority for laboratory rearing.

These results must be considered in terms of BTV prevalence, since the climatic fluctuations in temperate regions such as Spain are large and the total transmission potential of an infected vector is maximized at intermediate temperatures (Brand & Keeling, 2017). However, we are far to find the best requirements of the European BTV vector species *C. imicola* and *C. newsteadi* for laboratory rearing or even to establish a colony in the laboratory. Oviposition and larvae substrates, material of cages for keeping the gravid females, oscillations in temperatures and humidity are subjects to improve to check the preferences of *Culicoides* species for breed.

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Authors declare that there are no conflicts of interest.

Data availability statement

Research data are not shared

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