Phenology of *Hyalomma marginatum* in a site in southern French

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Abstract

Hyalomma marginatum tick establishment has been recorded in southern mainland France for around ten years. This two-host species is one of the main vectors of the Crimean-Congo haemorrhagic fever (CCHF) virus. Knowing the phenology of the tick is a prerequisite for a better understanding of its spread and of CCHF virus dispersal. A six-year study was carried out in a horse herd to determine the seasonal variations of *H. marginatum* infestation. Adult ticks started infesting their hosts from the beginning of March. Infestation peaked during the first weeks of May and then steadily decreased until the end of August. This represented an average annual infestation of more than 300 *H. marginatum* per horse. Birds are parasitised by *H. marginatum* immatures (larvae and nymphs) in summer, between mid/late June to the end of September. The larvae are therefore not yet active when the migratory birds pass through in spring. Blackbirds, *Turdus merula*, were the most infested species: sixty percent of those examined in summer were parasitised by *H. marginatum*. This bird species could be a good sentinel host for monitoring the establishment of the species in currently *H. marginatum*-free areas. To complete this multi-year survey, an experimental study was carried out in quasi-natural conditions, with engorged ticks placed in cages in the *garrigue*. It was shown that the nymphs moulted into adults within 3-4 weeks in July-August. However, they remain completely immobile in the litter, undergoing a behavioural diapause, until the following spring. On the other hand, almost all the nymphs deposited later, in September or October, died during the cold and rainy season. It was also observed that engorged females are able to survive

the cold conditions up to 6-8 months before laying eggs. The study also suggested that there were predators of *H. marginatum*, likely ants and spiders, in the study area. The hosts monitored over all these years were not only infested by *H. marginatum*. The phenology of other tick species parasitizing horses (*Dermacentor marginatus*, *Haemaphysalis punctata*, *Rhipicephalus bursa*) and birds (*Ixodes frontalis*, *I. ricinus*, *Hae. punctata*) was also highlighted for this study area.

Introduction

The two-host tick *Hyalomma marginatum* is one of the main vectors of Crimean-Congo Haemorrhagic Fever virus (CCFHv) (Bakheit et al., 2012). Larvae and nymphs infest mainly lagomorphs and birds, whereas adult ticks are known to parasitize domestic and wild ungulates (Morel, 2003; Walker et al., 2003). This tick species is established in northern Africa, Asia and southern Europe, from Portugal and Spain to Turkey (Santos-Silva and Vatansever, 2017). In Corsica, it has been present for at least 60 years (Morel, 1959) and its wide distribution on the island has been confirmed (Grech-Angelini et al., 2016). Until recently, there was no evidence of *H. marginatum* establishment in mainland France (Vial et al., 2016). However, during the past few years, such populations have been identified in at least seven French departments characterized by a Mediterranean climate. Between 2016 and 2022, thousands of *H. marginatum* adults have been collected, mainly on horses and cows (Bah et al., 2022; Bernard et al., 2024).

Hyalomma marginatum spreads from already infested areas to other favourable, yet uninfested, regions through the movement of infested hosts. These movements can involve horses (horse shows, trade, breeding, long distance horse riding, transfer from one to another livery stable etc.), cattle (movement between pastures or farms, trade), sheep (summer transhumance, trade) or wildlife such as wild boars – all of which infested by adult ticks. Larvae and nymphs are frequently observed on migrating birds captured in spring in various European countries, whether it be in the south, north or east of the continent (Jaenson et al., 1994; Jameson et al., 2012; Lindeborg et al., 2012; Capek et al., 2014; Nowak-Chmura, 2014; Vial et al., 2016; De Liberato et al., 2018; Pascucci et al., 2019; Portillo et al., 2021; Sormunen et al., 2021). This attests that larvae are active, at least in some of the infested areas, while birds are migrating towards Northern Europe. Birds therefore disseminate engorged nymphs, for which some will be able to moult into adults. Such adults have been recorded in many European countries where no established population of *H. marginatum* has been observed, like Germany, Luxembourg, Netherlands, Sweden (Chitimia-Dobler et al., 2019; Grandi et al., 2020; Weigand et al., 2020; Uiterwijk et al., 2021).

The establishment of *H. marginatum* in mainland France and the recent identification of CCHFv in some of these ticks collected close to the Spanish border (Bernard et al., 2024) suggest that the

emergence of CCHF in the country may only be a matter of time. Preventing and controlling the disease first requires monitoring populations of the main tick vector. Therefore, it is essential to identify the temporal dynamics of *H. marginatum* to determine when the ticks are active and, ultimately, to assess their spreading potential. The purpose of this article is to describe various studies that led to the determination of (i) the seasonal variation of horse infestation by adult ticks, (ii) the seasonal variations of bird infestation by immature ticks, and (iii) the moulting kinetics of nymphs in quasi-natural conditions. The results provide an overview of the *H. marginatum* lifecycle in southern France.

Material and methods

Study site and animals

In April 2016, a horse herd highly infested by *H. marginatum* was identified 25 km north of Montpellier (Vial et al., 2016). This herd was chosen to study the seasonal variations of adult tick infestation. The monitored animals grazed freely 24 hours a day on a 200-ha clay-limestone land in the *garrigue* (scrubland), Mediterranean forest and formerly cultivated fields now used as pastures. During the 6-year-period of the survey, the herd size varied from 10 to 14 horses depending on births, deaths, introductions, departures or the temporary movement of mares for covering. These variations had to be considered for data analysis.

Leporidae, insectivores and ground-dwelling birds are the hosts of *H. marginatum* immature ticks (Santos-Silva and Vatansever, 2017). Hares have rarely been observed on the land, and no rabbit warren was located during the survey. To assess the phenology of immature stages, it was therefore decided to examine birds.

The development and survival of *H. marginatum* during the winter period were also studied on this land. Engorged nymphs and engorged females were placed in cages protected by an enclosure set up on one of the property's non-wooded areas. These ticks were obtained from breeding established in 2018 at the Baillarguet[1] insectarium from engorged females collected on horses within the monitored herd. The adult ticks were fed on goats and the immatures were fed on rabbits to in accordance with the procedures described in the ministerial authorisation for "feeding ticks on vertebrates for laboratory rearing" n° APAFIS#6304-2016080315594914 v4. Engorged ticks were kept in climatic chambers at low temperature and 85 % humidity to prevent them from developing in the laboratory before being released in the field.

Study periods

Tick collection on horses was performed from April 2016 to April 2022. Horse examination was

[1] https://doi.org/10.18167/infrastructure/00001

carried out every 6-14 days during the *H. marginatum* infestation peak in spring and summer (Grech-Angelini et al., 2016), and every 15-30 days during the rest of the year. The survey was interrupted on different occasions: between May 2017 and April 2018 (funding interruption), from February to mid-August in 2020 (suspension of field activities during the Covid-19 pandemic), from May to September in 2021 (treatment of the animals with different experimental acaricide preparations). Overall, tick infestation on horses was monitored for 43 months over the six years, with an emphasis in March-April, at the onset of infestation.

Birds were captured and examined in 2016 (from the end of June to the end of October), in 2017 (July and August) and in 2019 (from mid-March to mid-July). Nets were installed for 2-3 consecutive days approximately every 3-4 weeks, except when rain prevented bird captures were therefore cancelled.

Finally, the development of engorged nymphs and females was studied in quasi-natural conditions between July 2019 and June 2020.

Tick collection

All ticks attached to the examined horses were collected and identified back in the laboratory. Some of them were kept alive until identification and then deep-frozen at -80°C for further analysis (Bernard et al., 2024; Joly-Kukla et al., 2024). On other occasions, ticks were stored in 70% ethanol and discarded after identification.

Birds were caught in the study site using 210 m of nets (5 sets of 25-70 m). These were installed in narrow forest paths and opened about one hour before sunrise for4-5 hours, being checked every 30 minutes. Captured birds were identified, ringed, weighed, examined for tick infestation and then released. All attached ticks were collected and kept alive until identification in the laboratory. Bird capture was authorised by the "Centre de recherche sur la biologie des populations d'oiseaux (CRBPO)" (Research Centre on Bird Populations Biology) and the Museum national d'Histoire naturelle of Paris, and carried out by an experienced ornithologist and bird bander.

Ticks were identified by experienced acarologists using the keys and descriptions of Walker et al. (2003) and Pérez-Eid (2007).

***Hyalomma marginatum* collection analysis**

It was impossible to maintain a constant interval between two successive tick counts as the horses could not always be found on the scheduled examination date. Moreover, the number of examined animals constantly varied. All these variations had to be considered for data analysis. In order to homogenize the results and consider these constraints, it was decided to calculate the daily number of *H. marginatum* ticks attached per horse between two successive tick collections (DNT).

The total number of *H. marginatum* collected during a tick collection was divided by the number of animals examined and by the number of days separating this collection from the previous one.

Development of engorged stages in quasi-natural conditions

The cages placed in the field with engorged ticks were the same as those previously used in Burkina Faso and Madagascar for similar experiments with the hard tick *Amblyomma variegatum* (Stachurski et al., 2010; Rahajarison et al., 2014): they are 50cm-side metal frames cubes, covered with mosquito nets on all sides except the bottom. The lower frame was buried 3-5 cm into the ground to prevent ticks from escaping. The cages were placed on shrubs like thymus, rosemary, *Genista scorpius* or large tufts of grass and contained natural plant litter to ensure that the ticks could find micro-habitats favourable to their survival and development.

Four cohorts of 4 or 6 cages, each containing 26 to 30 engorged nymphs and one or two engorged females (according to the number of ticks available at the time of installation), were studied. The four cohorts were respectively installed in the field on 10/07, 14/08, 18/09 and 24/10 of 2019. The first three dates correspond to the period during which the hosts were infested by *H. marginatum* larvae and nymphs, as observed during the bird survey. Every 3 weeks, unless poor weather conditions prevented the examination from taking place, one of the cages of each cohort was examined destructively: plants were removed and the ticks were searched for, first at the base of the shrubs or grass tufts, then in the litter, and finally in the first centimetres of soil, once the plant debris had been removed. The soil removed from the cage was carefully examined. Finally, the cage itself was removed, and the ticks were searched for in the hem of the mosquito nets covering it. The collected ticks were brought back to the insectarium where the unmoulted nymphs and females which had not started to lay eggs were maintained in a climatic chamber (25°C, 85% RH) to verify whether they were alive and still able to develop or lay eggs.

Results

Horse infestation

More than 13,800 ticks were collected from the horses during the study period (Table I). *Hyalomma marginatum* was the most abundant species (85% of the collected ticks), mostly attached to the perineal area, inguinal region, udder and sheath. *Rhipicephalus bursa* (6% of the collected ticks) were collected from the same anatomical regions. Adult *R. bursa* parasitized the horses from March to August, with a peak in June-July, while nymphs were collected from December to April. The number of collected *R. bursa* decreased drastically between 2016 and 2021. *Dermacentor marginatus* (8%) and *Haemaphysalis punctata* (1%) were found during the cool season, between September-October and April-May (Supplementary Material S1). The former species was found

Year	Period	*Hyalomma marginatum*		*Dermacentor marginatus*		*Haemaphysalis punctata*			*Rhipicephalus bursa*			*Rhipicephalus* of the *sanguineus* group		*Ixodes ricinus*	
		M	F	M	F	M	F	N	M	F	N	M	F	M	F
2016	April-Dec	957	910	92	73	14	15	0	188	254	25	6	6	0	1
2017	Jan-April	475	295	199	207	8	39	0	29	37	37	5	2	0	1
2018	April-Nov	1427	1609	13	9	2	8	0	71	111	2	0	1	0	0
2019	Jan-Dec	1629	1727	99	142	5	33	1	31	50	6	0	0	0	0
2020	Jan-Feb; Aug-Dec	23	37	57	86	2	9	0	0	0	0	0	0	0	0
2021	Jan-May; Sep-Dec	1180	1015	72	78	4	14	0	2	4	0	2	0	0	0
2022	Jan-april	265	145	10	17	3	7	0	1	0	0	0	0	0	0

Table I. Number and species of the ticks collected from the monitored horses during the various study periods.

almost exclusively on the horses' mane whereas the latter was mainly attached to the chest. Finally, a few *Rhipicephalus* of the *sanguineus* group and two *Ixodes ricinus* females were also found, mainly during the first years of monitoring.

Focusing on *H. marginatum*, few ticks were collected in January and February. However, adults of this species infested horses mainly between March and the end of August (Figure 1). Generally, male ticks started to attach a slightly earlier than females, but the latter were more numerous during the second part of the infestation period (S1). During the first week of March, an average of 0.18 ticks attached daily to each horse (DNT). This pattern was almost similar over the course of the monitored years: 0.16 in 2017, 0.24 in 2019, 0.14 in 2021, 0.18 in 2022. As the infestation observed in the first survey year (April 2016 to April 2017) appeared lower than those recorded in subsequent years before levelling off, the mean DNT (black curve of Figure 1) was calculated with the data of the last five years (2018-2022). This mean DNT reached 0.5 ticks on 15th March, 1.0 on 25th March and 2.0 on 9th April. It remained higher than 2.0 until 15th June and higher than 1.0 until 20th July. The mean DNT peak (4.0 ticks attached daily) was observed during the first three weeks of May.

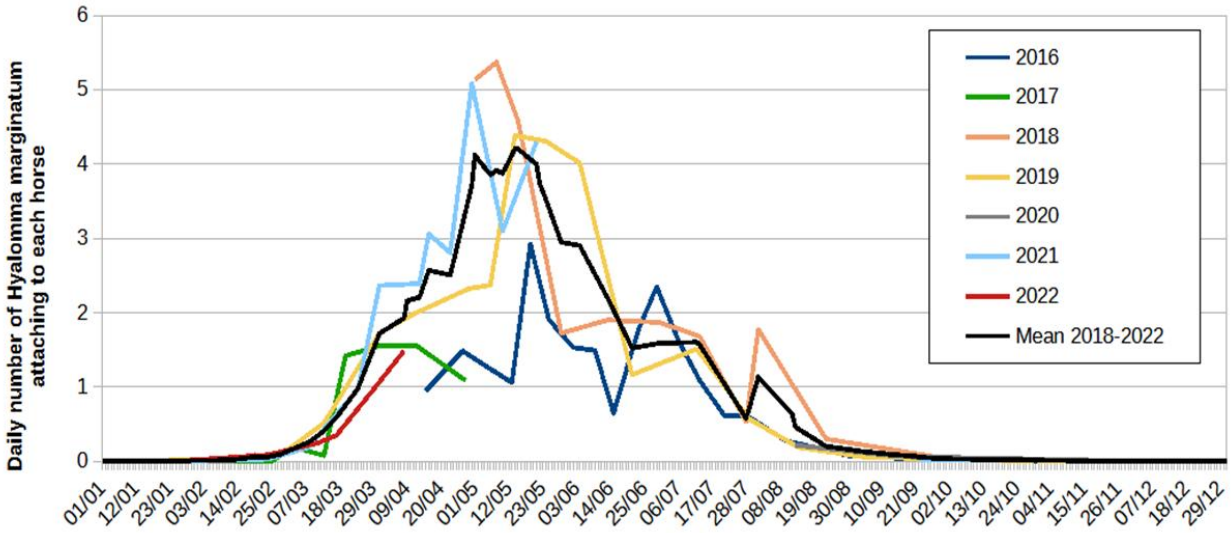

Figure 1. Number of *Hyalomma marginatum* adults attached daily to the monitored horses during the various study periods.

This daily infestation represented a tick burden of approximately 330 *H. marginatum* per year and per horse. During the three years in which the horses were regularly examined throughout summer (2016, 2018 and 2019), an additional minor increase in infestation was observed in July or August after an initial reduction of the DNT.

Bird infestation

Birds were captured during 30 non-consecutive days: 15 in 2016, 6 in 2017 and 9 in 2019. The data were aggregated per week over a fictional year (Figure 2). A total of 317 birds (1-25 per day of capture) of 25 different species were examined (Table II). Those most frequently found in the nets were the European Robin, the Common Blackbird, the Eurasian Blackcap and the Great Tit. These four species were observed on site throughout the year. Others (Flycatcher, Nightingale) were

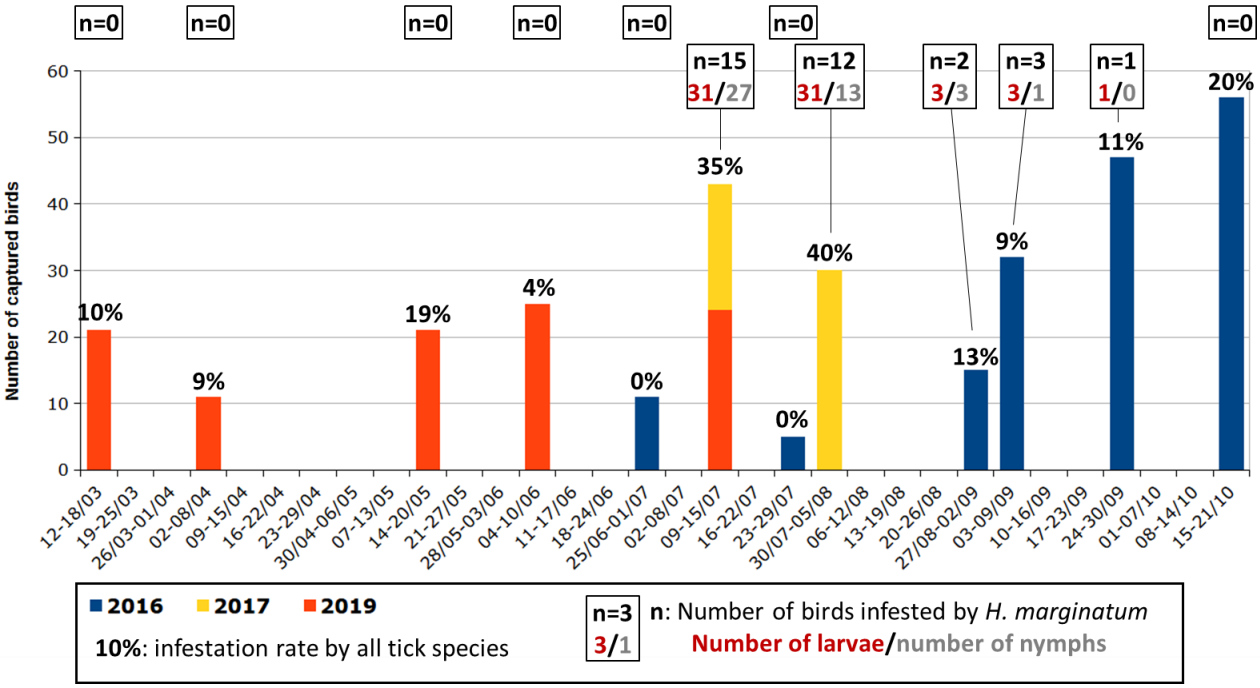

Figure 2. Number of birds examined during the surveys. Tick infestation rate of these birds with special emphasis on *Hyalomma marginatum immature* infestation.

only captured during the nesting period, from mid-May to mid-September. Several species were very rare and caught only once or twice (Song Thrush, Eurasian Wren).

Ticks were found on 56 birds of eight species (Table II). The overall infestation rate was 18%, and varied over the year (0-40%). In total, 167 ticks of four species were identified, as well as two Ixodidae larvae too damaged to be identifiable (Supplementary Material S2). The most abundant tick species was *H. marginatum* with 44 nymphs (NN) and 69 larvae (LL), collected from seven bird species (all the infested bird species except the Dunnock). *Ixodes frontalis*, *I. ricinus* and *Hae. punctata* were also collected. There were generally less than five ticks per bird (mean: 3; median: 2). The only exceptions were *Hae. punctata* larvae (14 were found on a Blackbird), and *H.*

Name of the bird	Number		Name of the bird	Number		Name of the bird	Number	
	examined	infested		examined	infested		examined	infested
European Robin (*Erithacus rubecula*)	81	15	Eurasian Blackcap (*Sylvia atricapilla*)	31	0	Cirl Bunting (*Emberiza cirlus*)	2	0
Common Blackbird (*Turdus merula*)	56	29	Common Firecrest (*Regulus ignicapilla*)	19	0	Rock Sparrow (*Petronia petronia*)	2	0
Great Tit (*Parus major*)	23	3	Long-tailed Tit (*Aegithalos caudatus*)	13	0	Garden Warbler (*Sylvia borin*)	2	0
Common Nightingale (*Luscinia megarhynchos*)	21	4	Sardinian Warbler (*Curruca melanocephala*)	10	0	Eurasian Jay (*Garrulus glandarius*)	1	0
Western Subalpine Warbler (*Curruca iberiae*)	12	1	Short-toed Treecreeper (*Certhia brachydactyla*)	9	0	Melodious Warbler (*Hippolais polyglotta*)	1	0
Eurasian Chaffinch (*Fringilla coelebs*)	11	2	European Pied Flycatcher (*Ficedula hypoleuca*)	6	0	Eurasian Blue Tit (*Cyanistes caeruleus*)	1	0
Dunnock (*Prunella modularis*)	3	1	Common Chiffchaff (*Phylloscopus collybita*)	4	0	Curruca hortensis (*Western Orphean Warbler*)	1	0
Song Thrush (*Turdus philomelos*)	1	1	Great Spotted Woodpecker (*Dendrocopos major*)	4	0	Common Redstart (*Phoenicurus phoenicurus*)	1	0
			Eurasian Wren (*Troglodytes troglodytes*)	2	0			

Table II. Species and number of birds examined and infested by ticks of all species during the study.

marginatum immatures with six birds (five Blackbirds and one Robin) infested by six to 18 ticks. Blackbird was the most infested bird species, harbouring 83 of the 113 *H. marginatum* (73%), as well as 95% of the 19 *Hae. punctata* and 48% of the 25 *I. frontalis*. However, no *Ixodes ricinus* was found on a Blackbird.

Co-infestations (*I. ricinus* and *I. frontalis* or *H. marginatum* and *Hae. punctata*) were occasionally observed. Each tick species displayed a particular phenology. *Haemaphysalis punctata* (2 NN and 17 LL) were collected mainly in July and August, except for one nymph collected in May. *Ixodes ricinus* (2 NN and 8 LL) were observed in September and October. Larvae of *I. frontalis* (n = 13) were only collected in October while nymphs of this species (n = 9) were collected in October and from March to June, as well as 3 females in September and May (S2).

No immature *H. marginatum* were collected in May (21 birds examined) or in June (36 birds examined). They were collected from the beginning of July to the end of September, mainly during the first month (Figure 2). The first larvae and nymphs of *H. marginatum*, often observed on the same birds as this species is a two-host tick, were observed on 10th July in 2019, on 11th July in 2017 but only on 31st August in 2016. That year, only five birds were captured in July, and none of them were Blackbirds, whereas 21 of the 34 birds (62%) found infested by *H. marginatum* during the study were Blackbirds. More precisely, 19 of the 32 (59%) Blackbirds examined from July to September were infested by *H. marginatum* immatures. This proportion reached 40% (2 of 5) for the Eurasian Chaffinch, 27% (3 of 11) for the Common Nightingale, 22% (2 of 9) for the Great Tit, 13% for the Western Subalpine Warbler and 9% (4 of 45) for the European Robin. The only Song Thrush caught was also infested by *H. marginatum*.

Moulting of *Hyalomma marginatum* nymphs in semi-natural conditions

The number of ticks found in the cages was highly variable from one cage to another, with a

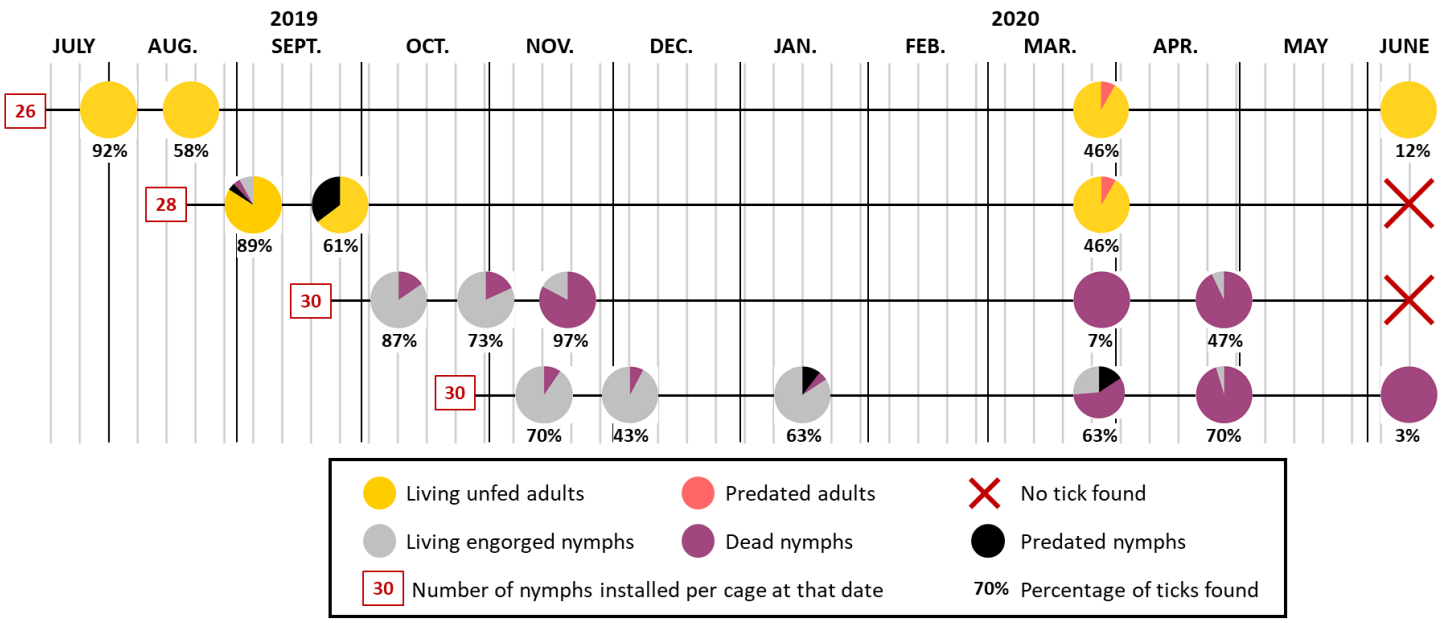

Figure 3. Graphic representation of the status of the ticks from the 4 cohorts found in the cages where batches of 26-30 engorged nymphs had been placed. The not predated nymphs were placed at 25°C and 85% RH in a climatic chamber at the CIRAD insectarium to verify whether they were still able to moult, and therefore alive.

general (but not systematic) decrease over the time (Figure 3). Similarly, the fate of the engorged nymphs varied greatly from a cohort to another. Predated ticks (mainly nymphs, but also two unfed adults) were sometimes collected in the cages. In one cage of cohort 2 in particular, almost a third of the nymphs had been predated. In these cases, the cuticle was pierced and the tick was empty (nymph), or only part of the scutum and legs were found (adult) (S3).

Three weeks after the engorged nymphs of cohort 1 had been placed in the field, all the ticks found in the first cage (24 of the 26 installed ticks) were adults. However, the metamorphosis was recent, as the legs of some ticks were still light-coloured and the pale rings at the outer ends of the segments were not yet visible. An additional three weeks later, all the ticks found were adults with clearly bicoloured legs. All the adults in these two cages were completely immobile when they were found.

Twenty-one of the 25 ticks (out of 28 installed) found in the first cage of cohort 2 were adults, while three had not yet completed their metamorphosis. Two of which moulted in less than a day when placed in the insectarium, while the last one died. As in cohort 1, all living ticks found three more weeks later (many nymphs had been predated) were adults, but they remained completely inactive in the cages.

The following spring, at the end of March, unfed adult ticks moved into the cages as soon as the cages were checked and climbed up the mosquito nets on the side where the human was. No living ticks were found immobile on the ground on which only predated adults were found. At this time, the adults were 200-240 days old. At the beginning of June, only a few active ticks (around

310 days old) were still found in the cage from cohort 1. The mosquito nets were badly damaged by that time, as a result of the adverse weather conditions, particularly sun exposure, which had been affecting them for almost a year.

Interestingly, the pattern was completely different for ticks belonging to both cohorts 3 and 4. All the ticks found in the cages were nymphs. In the first four cages of cohort 4, some of the nymphs were still mobile when found. Placed in a climatic chamber, only some were able to moult. The median duration of the metamorphosis was 10, 6 and 5 days, respectively, for the nymphs in the first three cages of cohort 3, and 18-21 days for those in the first four cages of cohort 4.

A large proportion (83%) of the nymphs of cohort 3 collected on 19/11 were dead while only 6-10% of the nymphs in cohort 4, collected on 13/11, 04/12 and 15/01, were dead. However, the mortality rate of the nymphs from these two cohorts was high the following spring: 87% of those collected between March and June were dead or unable to develop when put in the climatic chamber. In April, only one of the 14 nymphs from cohort 3 succeeded to moult within 24 hours when put at 25°C and 85% RH. This was also the case for only one of the 21 nymphs of cohort 4 collected in April. Metamorphosis occurred in 8 days.

The ticks of these two cohorts often appeared to be in good condition when placed in the climatic chamber, but many were unable to emerge from the nymph's cuticle, even if they had completed their metamorphosis. This exuvia was much thicker than that observed during normal metamorphosis. When they were voluntarily removed from the exuviae after 20-30 days, it was observed that the adults were poorly formed. In particular, the legs were twisted, the articles shorter than normal and the joints were deformed (S4). As for the few adults that were able to emerge from the exuvia in these cohorts, nearly all died within a few days, without ever showing any ability to respond to stimuli (breathing, light). No adult *H. marginatum* was found in the last cages of these two cohorts.

Egg-laying by *Hyalomma marginatum* females in semi-natural conditions

Only one of the 12 engorged females placed in the cages of cohorts 1 and 2 was found. It had begun to lay eggs; however, it was not in the litter nor on the ground but in the hem of the mosquito net. The situation was completely different for females belonging to the cohort 4: all were found, and only one, being 155 days old, was dead (in March). Those found in November-January needed 6-17 days in the climatic chamber to start laying eggs, whereas those found in March-April needed 1-2 days. The two females observed in the cage in June (230 days old) had started laying eggs in the field. The situation for cohort 3 was intermediate: all the females were found in October-November and some had started to lay eggs, while others needed 2-11 days in climatic chamber to start oviposition. Only two females (one predated and one alive, which quickly started to lay eggs in the climatic chamber) were found in spring.

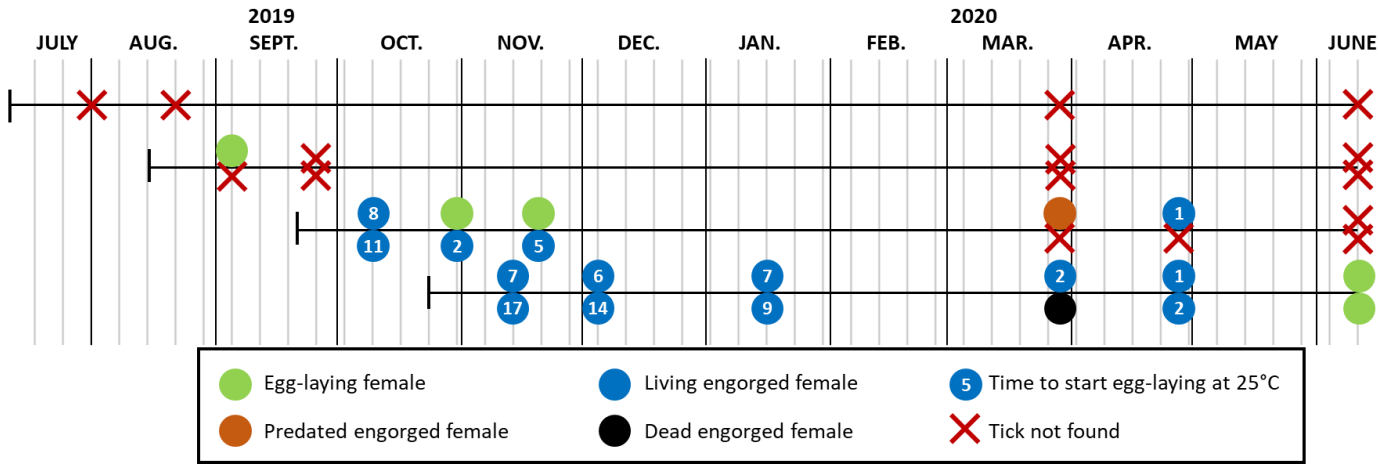

Figure 4. Graphic representation of the status of the engorged females from the 4 cohorts found in the cages where they had been placed. The females which had not started to lay eggs were placed at 25°C and 85% RH in a climatic chamber at the CIRAD insectarium to verify whether they were still alive and able to lay eggs.

Discussion

Before discussing the main results obtained from this multi-year survey on *Hyalomma marginatum* in southern France, it should be mentioned that some limitations were identified in the study. For different reasons, the ticks collected from the monitored horses do not represent all those that infested them during the study period. First, the horses selected for this study were only minimally handled because of their semi-wild lifestyle. Consequently, some individuals were sometimes reluctant to undergo a thorough examination and tick collection. Secondly, the interval between two tick samplings sometimes exceeded the duration of the female tick's blood meal. Some of those females were thus able to attach, engorge and drop off between two tick collections. Thirdly, when tick collection was suspended for too long and infestation became high, the owner treated the most infested animals with an acaricide, particularly from 2019 onwards, preventing new ticks from attaching. The acaricide was occasionally applied to the manes of the highly parasitized horses to prevent wounds and extensive scabs due to high infestation by *D. marginatus*, with a potential effect on other parts of the animal. Finally, as the objective of the study was to ascertain the seasonal variations of *Hyalomma marginatum* infestation, tick sampling was carried out with reduced frequency during the cool season, between September and February. As a result, even if these results do not represent the core of this study, the number of active ticks in autumn and winter, mainly *D. marginatus* and *Hae. punctata*, was probably underestimated compared to their actual proportion in the tick population present in the site.

It appeared that *H. marginatum* was the most abundant tick on the monitored horses. Its abundance even increased during the survey. While each animal was estimated to be parasitized by approximately 200 ticks the first year of the study, between April 2016 and April 2017, the number

of ticks infesting each horse annually reached 300-330 ticks during the final years. This rapid increase suggests that the establishment of *H. marginatum* on the farm was relatively recent. Over the same period, the number of *R. bursa* collected from horses dramatically decreased for an unknown reason. It is also remarkable that very few *Rhipicephalus* of the *sanguineus* group were collected from the horses (19 found on the herd from April 2016 to April 2017), whereas 34 of them were found on a single wild boar that was shot by a hunter on the land in the summer of 2016. This wild boar was also infested by six *H. marginatum*, whereas 1.5 *H. marginatum* attached daily to each horse during this period. This is an indication that these two tick species probably do not have the same predilection host.

According to the owner, who could not be more precise, *H. marginatum* was observed on the horses 3-5 years before the beginning of the survey. It has not been possible to determine how the tick was introduced in the farm. No horse was purchased in the years prior to the start of the survey, but a few mares have been sent to other farms each year for 2-3 months in spring to be covered. They may have come back infested with *H. marginatum* from one of these farms. Migratory birds (Nightingale, Song Thrush) coming from southern regions (Spain, North Africa) nest on the study site every year. Other migratory birds (Dunnock, Robin, Eurasian Blackcap), infested by *H. marginatum* nymphs, were observed in spring near a pond 35-40 km further south. The proportion of birds infested by the ticks was very low at this pond (unpublished data), as was the number of *H. marginatum* collected from these birds (Vial et al., 2016). Given the very small number of migratory birds caught in spring on the site where the present study was carried out (no Nightingale was examined before mid-May, for example), it is not possible to know whether the introduction of *H. marginatum* nymphs was likely to occur through this route. Would this be enough to allow the establishment of a *H. marginatum* population on the study site? When discussing the spread of *H. marginatum* in Eastern Europe, Gray et al. (2009) wrote that "it is debatable (...) because very small numbers of ticks, all immature stages, would be involved." They rather considered that "establishment of *H. marginatum* (*would*) mainly result from the introduction of adult females". Whatever the case, the conditions prevailing on the 200-ha of land studied seem to be particularly favourable for *H. marginatum* (presence of hosts of the different tick instars, presence of Mediterranean scrubland, temperature very warm in summer), and the tick appears to have rapidly multiplied on the site to reach its current abundance.

Infestation of adult *Hyalomma marginatum* on the horses started slowly at the end of February or at the beginning of March. The number of ticks which attached daily increased until the beginning of May to reach roughly four ticks per host per day, *i.e.*, about 30 *H. marginatum* per week in the final years. From June, the number of active adult ticks in the environment and infesting animals decreased, dropping back to nearly zero by early September. Interestingly, this temporal pattern was globally redundant over the six years, suggesting predictable temporal variations in *H. marginatum*

dynamics. In 2016, 2018 and 2019, a new infestation peak was observed in late summer (Figure 1), when the animals were free to graze on a pasture that had been deferred a few months earlier to preserve the grass resource. This points out that adult ticks, which could not previously attach, were still alive and active in the late summer. In autumn and winter, a few *H. marginatum* adults, mainly males, had been found on the hosts, but this was rather unusual. Only 0.5% of the 11,694 *H. marginatum* collected from the horses during the study were found between mid-September and mid-February.

Hyalomma marginatum males have never been reported to produce attraction-aggregation-attachment pheromone similar to that emitted by *Amblyomma hebraeum* males (Rechav et al., 1977) or *A. variegatum* males (Norval and Rechav, 1979) after 3-4 days of attachment. The fact that the males attach slightly earlier than the females is more likely due to differences in the level of stimuli that trigger host-seeking activity at the end of winter. The data collected did not permit determination of the main stimulus (daylength, temperature exceeding a given threshold, another phenomenon) inducing the host-seeking activity of *H. marginatum*.

Hyalomma marginatum nymphs were observed on birds from 10th July. As this species is a two-host tick whose immature stages infest their host for about 3 weeks (Gargili et al., 2013), it is assumed that larvae are active from the second half of June. However, by the end of August, the number of *H. marginatum* found on the birds dropped drastically, suggesting that there were no longer active/living larvae in the environment. The infestation period by *H. marginatum* larvae lasts about 3 months (mid-June to mid-September), shorter than the period estimated by Estrada-Peña et al. (2011) for whom "larval recruitment may be feasible from March to September". Larvae are therefore not yet active in March-May, when the migratory birds arrive from Africa or Spain. Birds parasitised by *H. marginatum* immatures flying to northern Europe could not have been infested during their passage through southern France. They were thus infested by larvae further south, for example in Morocco, where birds were found to be infested by *H. marginatum* immatures as early as April (Palomar et al., 2013). To the best of our knowledge, the period of activity of *H. marginatum* larvae in Spain has not been identified. It may also be different in southern regions (Andalusia, Extremadura, Castilla–La Mancha) and in those close to France, such as Catalonia.

Birds infested by *H. marginatum* were mainly ground-dwelling birds, spending a large part of their life on soil: Blackbird and probably Song Thrush (although only one was examined), Nightingale, Robin, Chaffinch. Other ground-dwelling birds, such as Partridge and Quail, which were not caught in this study, are also likely to be good hosts for *H. marginatum* larvae (Hoogstraal et al., 1963; Hosseini-Chegeni et al., 2019). Blackbird seems to be a good sentinel for determining whether *H. marginatum* is established in a region and whether larvae are active in the area. They are generally abundant and widespread, and they are heavily parasitised by this tick species.

While the core information of this study concerns *H. marginatum*, it is interesting to note that

none of the Blackbirds examined in September and October, when the migratory birds arrive in the Mediterranean region from the north, were infested by *Ixodes ricinus* larvae or nymphs. It suggests that the vast majority of those captured at that time were birds that had grown up in the area. Conversely, the number of Robins caught increased dramatically from the end of September (28 in 27-29/09 and 26 in 19-21/10 instead of 1-3 per day at the other periods). Six of them (11%) were infested by *I. ricinus* larvae or nymphs. However, only two *I. ricinus* females were found on the monitored horses (one in April 2016 and another in March 2017). This indicates that despite the autumnal introduction of *I. ricinus* larvae and nymphs, the species does not seem to be able to establish in this site, where the climate is most likely unsuitable. Moreover, during surveys carried out between 2017 and 2022 to determine the distribution of *H. marginatum* (Bah et al., 2022), it was found that the two species rarely coexisted: *H. marginatum* was found in hot, dry areas, and *I. ricinus* in wetter, temperate sites. In Corsica, *I. ricinus* was hardly found, uniquely at altitudes of over 600 m in the coolest areas (Grech-Angelini et al., 2016).

The infestation rate of Blackbird (52%) or Robin (19%), as well as the overall infestation rate of the birds captured on the site (18%), even taking into account bird species that have never carried ticks, was much higher than the rates generally observed in studies focusing on migratory birds. Hoogstraal et al. (1963) observed ticks on 3% of the birds captured in 1959-1961 "in Egypt while en route from Asia and eastern Europe to tropical Africa". Pascucci et al. (2019) found that 7% of the 3444 birds examined during spring migration in Ventotene island, in the central Tyrrhenian Sea, were infested by ticks. De Liberato et al. (2018) found ticks on 0.3% of the birds examined in March-May 2013 and 2014 in various western coastal sites of central Italy. Bacak et al. (2023) found ticks on 6% of the 10,650 birds examined in the Turkish Thrace during the migrating periods of 2020-2022. This was very probably because almost 60% of the birds examined in the study presented here were ground-dwelling birds, which was generally not the case.

Except for the cages examined in June and one cage from cohort 3, 43-97% (mean: 67%) of the ticks placed in the cages were found from three weeks to eight months after installation in the field. The disappearance of some ticks may be due to an imperfect examination of the litter, especially when the ground was muddy and sticky, just after a storm. This may also be due to ticks escaping through the holes in the mosquito netting, especially during the final months of the study, when the unfed adults were very active and the nets were badly damaged from sun exposure.

However, some ticks had also been predated. This was confirmed by the discovery of partially devoured engorged female and engorged nymphs, as well as the remains of unfed adults in the cages. The most often mentioned predators, which were frequently found in the cages, were ants. In certain regions of Australia favourable to *Rhipicephalus microplus*, the absence of the tick has been attributed to the predatory action of ants (Wilkinson, 1970). Ants of the genus *Solenopsis* have also been observed feeding on engorged female *R. (Boophilus)* or *Amblyomma* (Burns and Melancon,

1977; Butler et al., 1979; Petney et al., 1987; Barré et al., 1991). More recently, it was shown that the number of questing ticks (mainly *I. ricinus*) was negatively correlated with the size of red wood ant nests (Zingg et al., 2018). In any case, when ants are involved, it is likely that they do not leave the corpse of the predated ticks in the cage but take it to their anthill, as observed by Mwangi et al. (1991). This could explain why some of the ticks had disappeared from the cages. To date, no data has been reported on tick-predating ant species in France and future experimental studies should be carried out to fill this information gap. As for the ticks found pierced and empty, they were probably preyed by other predators such as spiders or beetles (Samish and Alekseev, 2001; Vinogradov et al., 2023), which were also found in the cages.

The predatory activity is extremely variable and unpredictable in the field (Wilkinson, 1970; Petney et al., 1987; Stachurski et al., 2010). This was also observed in this study. Predated nymphs were observed in only four of the 20 examined cages. In addition, only one of the six cages examined in March-April contained less than 10% of the ticks installed 5-8 months earlier (46-70% in the other cages). There are thus predators of *Hyalomma marginatum* in the environment, but their importance in terms of reducing tick populations is unknown. Predators seem to be more active in hot weather because engorged females installed in July and August, as well as ticks that should have been found the following June were the most affected.

The cage experiment showed that *H. marginatum* nymphs moult very quickly into adults in summer. In the first two cages of cohorts 1 and 2, examined between 31st July and 25th September, almost all the live ticks were inactive adults. At this time, the number of *H. marginatum* attached to horses was very low, decreasing from one tick per day per animal at the beginning of the period to 0.2 tick on 20th August, and to 0.03 tick at the end of September. It indicates that *H. marginatum* adults, present in the environment from the end of July, do not attach to their hosts. They undergo a behavioural diapause, similar to that observed with *Amblyomma variegatum* adults in Burkina Faso (Stachurski et al., 2010). In this latter case, the end of the diapause was observed with the onset of the rainy season. The situation with *H. marginatum* is likely to be different as there is no significant increase in rainfall in early March, when the adults become active. Behavioural diapause, characterised by the suppression of host-seeking activity in unfed ticks, has been observed in adults *H. marginatum* for a long time (Belozerov, 1982). For this author, diapause is a "pre-adaptive behaviour which precedes the actual onset of unfavourable environmental conditions", mainly regulated by the photoperiod. It enables thus the synchronisation of the life-cycle with favourable seasons of the year. The host-seeking activity of unfed *H. marginatum* adults in March would be triggered by the increase in daylight hours, which announces the rise in temperature, which in turn favours eggs incubation.

When placed at 25°C immediately after detachment, *H. marginatum* engorged nymphs moult in 18.6 days (median time; unpublished results). The living nymphs in the first four cages of cohort

4 needed 18-21 days to moult: they had virtually not begun their development when they were recovered, and some were even still moving, although they had been in the field for 20-155 days. In contrast, the living nymphs in cages 1-3 of cohort 3 needed only 5-10 days at 25°C to moult. Metamorphosis had thus already begun during the weeks spent in the field between mid-September and the end of October. However, a large proportion of the nymphs in cage 3 of cohort 3, examined on 19th November, were dead, unlike those in cohort 4 collected before and after that day (Figure 3). Most of the nymphs in cohort 4 were still alive in January (6 weeks later) at the coldest period of the year. Something happened between the end of October and mid-November that prevented the nymphs in cohort 3 from moulting and even killed most of them, without affecting those in cohort 4 which were in the field at the same time. In autumn, there are frequent thunderstorms in the region. It is hypothesised that heavy rainfall may hamper the successful moulting of the nymphs once the development had begun. At that moment, the ticks are completely immobile, hidden in the litter, unable to escape temporary submersion in water or mud. Conversely, if ticks are still moving, they can escape when the ground is temporarily unfavourable for their metamorphosis. This would explain why most of the nymphs in cohort 4 were still alive in November and December, unlike the nymphs in cohort 3 which were collected at the same time but installed 5 weeks earlier. The survival rate of cohort 4 nymphs decreased to 30% in March, and then to 5% in April. At that time, temperatures had started to rise again, which should have allowed nymphs to begin their development. However, rain remained heavy and could have led to the death of these nymphs, as the autumn rains did for the nymphs in cohort 3. The metamorphosis of nymphs subjected to adverse weather conditions (rains, cold) was thus deeply disrupted: the nymphs died or the moulted adults, very deformed with twisted legs, could not extricate from the very thick cuticle.

Hyalomma marginatum unfed adults can survive the winter in behavioural diapause, but engorged nymphs do not show a morphogenic diapause. The situation is different from that observed with the tropical tick *A. variegatum*. In Burkina Faso, unfed adults of this species can survive the cool dry season via a behavioural diapause (Stachurski et al., 2010). In the Malagasy highlands, engorged nymphs of the species can survive the dry and sometimes very cold season. Development resumes when the temperature rises again, up to 20 weeks later (Rahajarison et al., 2014). In both cases, the cold period unfavourable to nymph metamorphosis is also the dry season. This may explain why *A. variegatum* engorged nymphs are able to survive the unfavourable cold season. This would be another indication that it is probably rainfall, or a combination of rainfall and low temperatures, that prevents engorged *H. marginatum* nymphs from surviving the autumn and winter months in continental France. This should be verified experimentally.

The experiment carried out with cages showed that engorged females could survive the unfavourable cold and rainy conditions, contrary to the nymphs. Two females placed in the field in October (cohort 4) survived autumn and winter and were recovered in June (230 days later) when

they had started to lay eggs. Almost all the females of cohorts 3 and 4 recovered between October and April were also alive. Those which did not start to lay eggs in the field, began after a few days at 25°C in the climatic chamber. Two of the females of cohort 3, installed in September, had however started to lay eggs in the cages examined in October or November. It is thus not sure that females *H. marginatum* undergo a developmental diapause during the cold period, i.e., as quoted by Belozerov (1982), a "pre-adaptive behaviour which precedes the actual onset of the unfavourable environmental conditions" (in that case, unfavourable for egg incubation). It is more likely that egg-laying was postponed only because temperatures no longer allowed it. It is actually not known whether the eggs laid in autumn could have survived the winter and hatched the following spring. In any case, the study showed that engorged females can survive the winter and start laying eggs once favourable weather conditions return, joining those of the next generation. However, this observation probably does not reflect what is actually occurring in the field. Indeed, as shown by the horse monitoring, very few females feed after the beginning of September.

These various studies have made it possible to identify the cycle of *Hyalomma marginatum* in this site in southern France. Adult ticks emerge from their behavioural diapause from late February or early March and then infest their ungulate hosts. In the study site, these hosts are mainly horses, but the tick can also parasitise wild boars and the very few roe deer present. In the vicinity of the study site, there are also sheep flocks and cattle herds that are infested by *H. marginatum*. Tick infestation peaks in May. Afterwards, the population of available adult ticks in the environment gradually decreases as they find a host or die for different reasons, including exhaustion. Adult ticks can however resist this exhaustion for quite a long time if no host is available. The females, which engorge and drop off from March onwards, do not seem to lay eggs immediately, or the eggs do not hatch straight away. Larvae are active and can infest their hosts, in this case mainly ground-dwelling birds, from only the second half of June. Three weeks later, the first engorged nymphs are found in the environment. Quickly, in about 3-4 weeks, they moult into adults which remain inactive in the litter, undergoing a behavioural diapause. Immatures can engorge on birds until the end of September, but nymphs that detach too late are unable to moult before the onset of the cold season and most of them die during the winter. On the contrary, if engorged females detach late from their hosts, egg-laying may be delayed until the following spring. The situation in southern France is therefore similar to that described in Turkey by Vatansever (Gray et al., 2009): "If temperatures are high enough to allow molting before the cold winters, unfed adults can survive the next active season. Field observations recorded the feeding of nymphs in late summer in Turkey, with the resulting unfed flat adults commonly overwintering in the first few centimetres below the soil surface." Conversely, the phenology of *H. marginatum* may be slightly different in other regions of France where the tick is endemic. The species is now established in a vast area covering 300 km from south to north (42.50° N to 44.80° N) and 400 km from west to east (2.25° E to 6.90° E) (Bah

et al., 2022). Therefore, the period of activity of ticks, both adults and larvae, could vary by a few weeks between these different zones.

The periods and months mentioned here are those observed during the study years (thick arrows in Figure 5). A very warm spring could enable the eggs to hatch and the larvae to become active earlier, at the end of May or beginning of June (small plain blue arrow on Figure 5), while a mild autumn could allow the late engorged nymphs to moult later (small plain green arrow). On the other hand, with a colder summer and/or an early autumn (dotted arrows in Figure), the larvae will be active too late and/or the nymphs will not be able to moult before the cold months arrive. This is probably the situation in French regions where the climate is not Mediterranean and the tick is not established. This was also very likely the situation that prevailed in southern France until recently. At the end of the twentieth century, Morel (2003) pointed out that the French coast was the coolest part of the Mediterranean region which could explain why *H. marginatum* was not yet established in this area. Current global warming has probably corrected this "anomaly". It is to be feared that, in the coming decades, the climate of other French regions will become favourable to this tick which

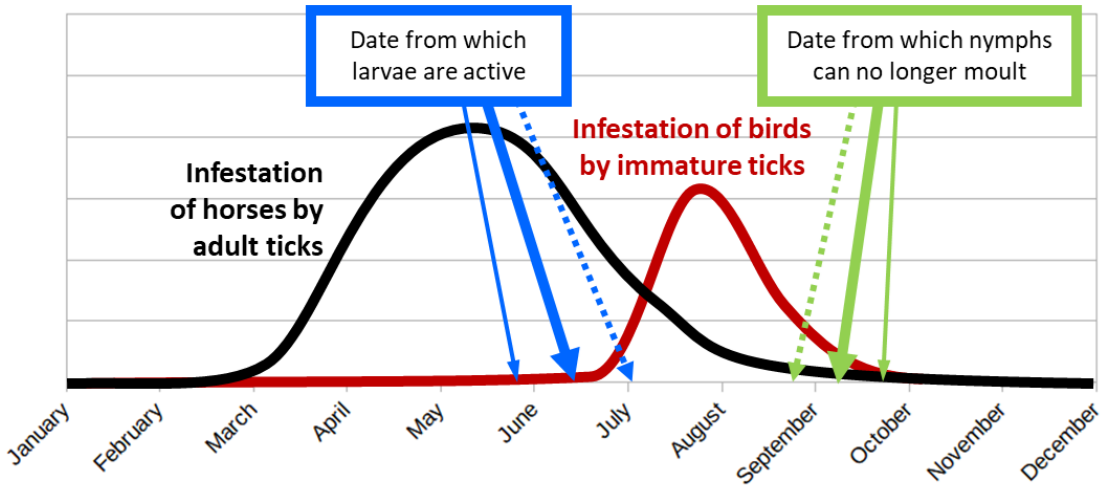

Figure 5. Stylized cycle of *Hyalomma marginatum* in southern France, based on observations made during the studies described in the text. The thick plain arrows symbolize the present situation, and the other arrows different hypotheses. See the text for explanations.

will be able to establish in these new areas due to the movement of its hosts.

Experiments were carried out in the CIRAD laboratory under controlled conditions to determine the precise temperatures allowing the development of *H. marginatum* nymphs and females. They were implemented with a tick population originating from the site where the studies presented here were performed. They are presented in other articles.

Conclusion

In this study site of southern France, *H. marginatum* adults infest their host between March and August, with a peak in May. The larvae are active from mid- or end of June. They are thus not yet available in spring to infest the migratory birds passing through the area. Engorged nymphs moult in 3-4 weeks in summer but most die if the metamorphosis cannot take place before the onset of cold conditions. Adult ticks that moulted when the weather conditions are unfavourable are not able to extricate from the exuvia and are deformed. Unfed adults that moulted in summer display a behavioural diapause until the following spring. Engorged females can survive the cold condition for up to 6 months before laying eggs. Predators are present in the environment but their impact is highly variable.

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Supplementary Material

S1. Infestation of the monitored horse herd by the tick during the whole survey

S2. Infestation of the captured birds by the ticks during the whole survey.

S3. A. Pictures of predated adult (in the top right-hand corner) and predated engorged nymphs. B. Close-up of a predated nymph. C. Predated engorged female.

S4. A. Normal moulting *Hyalomma marginatum* adults (thin exuvia and legs with straight articles) B. Adult jammed in a thick exuvia. C. The same adult after extraction from the exuvia. D. Close-up of the first pair of twisted legs. E. Different poorly formed adults after extraction from the exuvia.

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