Development of Dirofilaria immitis and Dirofilaria repens in Aedes japonicus and Aedes geniculatus

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Abstract: Background: The mosquito-borne filarial nematodes Dirofilaria immitis and Dirofilaria repens primarily affect dogs but also cats, causing heartworm disease or subcutaneous dirofilariosis, respectively, and both may also cause zoonotic diseases in humans. Several mosquito species have been reported as competent vectors for these nematodes, but no data are available for the invasive mosquito species Aedes japonicus (Theobald, 1901). The objective of this study was to describe the development of both D. immitis and D. repens under standardised experimental laboratory conditions in mosquitoes. Methods: For this purpose, both a laboratory strain and field-collected individuals of the invasive mosquito species Ae. japonicus and, for comparative purposes, a laboratory strain of Aedes geniculatus, a rare indigenous species sharing habitats with Ae. japonicus, and of the tropical species Aedes aegypti were used. Anticoagulated microfilariaemic blood was fed at a density of 3000 mf/ml to mosquitoes with a hemotek system. Blood-fed mosquitoes were incubated at 27 °C and 85% relative humidity, and specimens were dissected under the microscope at pre-set time points to observe developmental stages of both Dirofilaria species. Additionally, real-time PCRs were carried out in some microscopically negative samples to determine the infection rates. Results: In field-collected Ae. japonicus infectious L3 larvae of both D. immitis and D. repens developed, rendering this mosquito species an efficient vector for both filarial species. Additionally, Ae. geniculatus was shown to be an equally efficient vector for both filarial species. Aedes japonicus mosquitoes from a laboratory colony were refractory to D. immitis but susceptible to D. repens, whereas Ae. aegypti was refractory to both filarial species. Conclusions: To our knowledge, Aedes japonicus was the first time shown to be an efficient vector for both D. immitis and D. repens, indicating that this invasive and locally highly abundant species may contribute to a transmission of filarial worms. The data emphasize the necessity to perform vector competence studies with local mosquito populations as basis for risk assessments. We further demonstrated that detection of filarial DNA in a mosquito species alone does not allow to draw reliable conclusions with regard to its vector competence.

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Development of *Dirofilaria immitis* and *Dirofilaria repens* in *Aedes japonicus* and *Aedes geniculatus*

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**Abstract**

**Background:** The mosquito-borne filarial nematodes *Dirofilaria immitis* and *Dirofilaria repens* primarily affect dogs but also cats, causing heartworm disease or subcutaneous dirofilariasis, respectively, and both may also cause zoonotic diseases in humans. Several mosquito species have been reported as competent vectors for these nematodes, but no data are available for the invasive mosquito species *Aedes japonicus* (Theobald, 1901). The objective of this study was to describe the development of both *D. immitis* and *D. repens* under standardised experimental laboratory conditions in mosquitoes.

**Methods:** For this purpose, both a laboratory strain and field-collected individuals of the invasive mosquito species *Ae. japonicus* and, for comparative purposes, a laboratory strain of *Aedes geniculatus*, a rare indigenous species sharing habitats with *Ae. japonicus*, and of the tropical species *Aedes aegypti* were used. Anticoagulated microfilariaemic blood was fed at a density of 3000 mf/ml to mosquitoes with a hemotek system. Blood-fed mosquitoes were incubated at 27 °C and 85% relative humidity, and specimens were dissected under the microscope at pre-set time points to observe developmental stages of both *Dirofilaria* species. Additionally, real-time PCRs were carried out in some microscopically negative samples to determine the infection rates.

**Results:** In field-collected *Ae. japonicus* infectious L3 larvae of both *D. immitis* and *D. repens* developed, rendering this mosquito species an efficient vector for both filarial species. Additionally, *Ae. geniculatus* was shown to be an equally efficient vector for both filarial species. *Aedes japonicus* mosquitoes from a laboratory colony were refractory to *D. immitis* but susceptible to *D. repens*, whereas *Ae. aegypti* was refractory to both filarial species.

**Conclusions:** To our knowledge, *Aedes japonicus* was for the first time shown to be an efficient vector for both *D. immitis* and *D. repens*, indicating that this invasive and locally highly abundant species may contribute to a transmission of filarial worms. The data emphasize the necessity to perform vector competence studies with local mosquito populations as basis for risk assessments. We further demonstrated that detection of filarial DNA in a mosquito species alone does not allow to draw reliable conclusions with regard to its vector competence.

**Keywords:** *Dirofilaria immitis*, *Dirofilaria repens*, *Aedes japonicus*, *Aedes geniculatus*, *Aedes aegypti*, Microfilariae, Dog, Vector, Intermediate host

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Background
The mosquito-borne filarial nematodes *Dirofilaria immitis* (Leidy, 1856) and *Dirofilaria repens* (Railliet & Henry, 1911) primarily affect dogs but also cats, causing cardio-pulmonary (heartworm disease) or subcutaneous dirofilariosis, respectively. Both filarial worms may also cause zoonotic diseases in humans, in the form of pulmonary (*D. immitis*) or subcutaneous/ocular (*D. repens*) dirofilariosis [1, 2]. Both species have expanded their distribution range in the recent past [1, 3]. *Dirofilaria immitis* is endemic globally in regions with tropical or subtropical climates, whereas *D. repens* is restricted to the Old World. Recent detections of DNA of the canine heartworm in mosquitoes [4–6] in temperate climate areas in central Europe as well as climate analyses [4, 7–9] suggest a northward spread. *Dirofilaria repens* seems to be already endemic in central Europe, based on several DNA detections in mosquito populations [5, 10–12] and a growing number of autochthonous cases in both dogs and humans [13–20].

The transmission of the filarial worms is dependent on the availability of microfilariaemic hosts, competent mosquito vectors and suitable temperatures for the development of the infectious stages in the mosquito [8]. A major driver for the range expansion of canine dirofilarioses is the transport of infected animals from endemic to new areas, e.g. via the import of dogs from Mediterranean countries to central Europe where several mosquito species occur which have a demonstrated vector competence for *Dirofilaria* spp. [21]. Transmission is feasible in regions where suitable temperatures allow the development of the microfilariae (mf) to the infectious third larval stage (L3) which migrate to the proboscis of the mosquito. This extrinsic development is possible above 14 °C and is completed when the sum of the daily average degrees above this threshold value has reached at least 130. This value was initially termed 'heartworm development unit' (HDU) but later adapted to 'Dirofilaria development unit' (DDU) due to similar temperature requirements for both *Dirofilaria* species [22, 23]. Thus, the extrinsic development takes e.g. 10–12 days at 24–26 °C but as long as 29 days at 18 °C. Successful transmission of filariae to a host requires an infected mosquito to survive longer than the duration of the extrinsic incubation period. Assuming a maximal life span of 30 days for a mosquito, models revealed that summer temperatures allow the development of L3 at latitudes of 50° N in Europe [23]. Indeed, a canine autochthonous case of *D. immitis* dirofilariosis was observed at 54° N [24].

In addition, during recent decades, invasive container-breeding aedine mosquito species have been recorded in areas of Europe [25], and they might contribute to an increased transmission risk of *Dirofilaria* spp. as evidenced for the Asian tiger mosquito *Aedes albopictus* (Skuse, 1894) (= *Stegomyia albopicta*) in Italy [26, 27]. Local populations of another invasive species, *Aedes koreicus* (Edwards, 1917) (= *Hulecoetomyia koreica*), have been recorded in few instances [28–30], and this species was shown in an experimental study to allow the development of the infectious L3 larvae of *D. immitis* [31].

No data with regard to vector competence for *Dirofilaria* spp. are available for a third invasive mosquito species, *Aedes japonicus* (Theobald, 1901) (= *Hulecoetomyia japonica*). This East Asian native mosquito has in recent years invaded large parts of North America and many countries in Europe [32, 33], and it is further expanding [34, 35] (for updated European maps see www.ecdc.europa.eu).

The objectives of this study were to describe the development of both *D. immitis* and *D. repens* under standardised experimental laboratory conditions in both a laboratory strain and in field-collected individuals of the invasive mosquito species *Ae. japonicus*. For comparative purposes, these experiments were also done with each a laboratory strain of *Aedes geniculatus* (Olivier, 1791) (= *Dahlia geniculata*), a rare species in the Palaearctic Region sharing habitats with *Ae. japonicus*, and of the tropical species *Aedes aegypti* (L.) (= *Stegomyia aegypti*).

Methods
Microfilariae inoculum
Presence, vitality and number of microfilariae in all samples obtained from dogs (see below) were confirmed by microscopy. Briefly, 20 μl of blood were mixed with 40 μl of distilled water, covered with a cover slide, and microfilariae were counted by examination with a microscope under 100× magnification. Microfilaraemiae of the dogs were determined as average from three counts. Blood was anticoagulated with EDTA or heparin.

*Dirofilaria immitis*
Microfilaraemic blood samples from experimentally infected dogs (field isolate from northern Italy) as well as blood from uninfected dogs were kindly provided by Christian Epe (Elanco, St. Aubin, Switzerland).

*Dirofilaria repens*
Blood samples were from a dog naturally infected with *D. repens* (selected by one of the authors, RB). The infected dog was a mixed breed, 4.5 year-old and had never left the Daruvar region in north-eastern Croatia. The dog had first been diagnosed positive for microfilariae 2 years prior to the experiments. He was regularly checked by a local veterinarian and was always clinically unremarkable (nice fur, healthy skin, no nodules observed). The infection with *D. repens* was confirmed on DNA from the blood samples by a conventional PCR [36]. No co-infections with other filariae that are covered...
with this diagnostic approach (*Acanthocheilonema reconditum*, *Acanthocheilonema*, Dracunculoides spp., *Brugia pahangi*, *Brugia malayi*, *D. immitis*) were detected.

Mosquitoes and inoculation

**Field-collected *Aedes japonicus***

Host-seeking female *Ae. japonicus* were collected in a forest area within the urban borders of the city of Zurich, using mouth aspirators with four persons (“human baits”). Dry ice was additionally used as attractant. Mosquitoes were transferred into a cylindrical 500 ml plastic cage with moist cotton wool and were taken to the laboratory within 1 h of collection in the field for oral inoculation.

**Laboratory strains**

Laboratory colonies of *Ae. japonicus* (Pennsylvania strain, PEN) and *Ae. aegypti* (IPNC) were reared and maintained in a climate chamber in an insectarium under standard laboratory conditions at a temperature of 24 °C (*Ae. japonicus*) or 27 °C (*Ae. aegypti*), a relative humidity (rh) of 85% and a light-dark cycle of 16:8 h including dusk/dawn phases of 1 h. A recently established colony of *Ae. geniculatus* (IPZ) [37] was maintained at room temperature as described. Mosquitoes were provided with 5% glucose solution and water ad libitum. For reproduction they were either provided a mouse as blood source once a week (*Ae. japonicus*, *Ae. aegypti*; approved by the Cantonal Veterinary Office of Zurich, permission number ZH064/15) or sheep blood (approved by the Cantonal Veterinary Office of Zurich, permission number ZH008/15) using a standard artificial feeding system (Hemotek®, Hemotek Ltd, Lancashire, UK). For the inoculation experiments, females at an age of 5–7 days were chosen.

**Oral inoculation of mosquitoes**

Microfilariaeemic counts were adjusted to 3000 mf/ml with blood from uninfected dogs. Sugar was removed and the mosquitoes were allowed to feed through Parafilm® membranes for at least 2 h on 2 ml blood at 37 °C in a Hemotek® system. In order to boost the blood feeding rates of the field-collected mosquitoes, adenosine triphosphate (ATP, final concentration 5 mM) was added to the blood [38], and iGu® lure disks (Combi FRC 3003, Silva GmbH & Co. KG, Lübeck, Germany) were displayed. Mosquitoes that did not take a full blood meal were discarded.

**Molecular analysis**

A real-time PCR targeting the mitochondrial COX 1 gene [41] of *D. immitis* and *D. repens* was designed using GenScript (www.genscript.com/ssl-bin/app/primer). Forward and reverse primers and the Taqman® probe were as follows: Diro-f: 5′-GGT GTT TGG GAT TGT TAG TGA A-3′; Diro-r: 5′-CAG CAA TCC AAA TAG AAG CAA-3′; Diro-p: 5′-FAM-TCT GGCCAA TAG AAG CAA-3′; Diro-p: 5′-FAM-TCT GGC CAA
ACA AAC GAT CCT TAT CA-TAMRA-3’. The target size is 98 bp. The PCR assay was not evaluated for its diagnostic value.

Real-time PCR was performed in addition to microscopical investigations with microscopically negative samples and with dead mosquitoes as follows: up to day 5 post-infection (dpi) with abdomens only, after 7 dpi on all negative abdomens, thoraces and heads. Pools were used if there were more than three samples of the same kind on the same day of infection.

Data analysis
For each trial, feeding rates as well as mortality rates at 1 and 5 dpi and at 14 dpi were calculated. Differences in mortality rates within and between species were calculated in a 2 × 2 contingency table using Fisher’s exact test with two-tailed P-values on GraphPad Software (www.graphpad.com); P-values below 0.05 were considered as statistically significant.

The positivity rate of the mosquitoes was calculated as the percentage of blood-fed mosquitoes that had any developmental stage and/or positive PCR result out of all blood-fed mosquitoes.

The following indices of experimental filarial infections were calculated based on previous publications [31, 42]

\[
\text{Infection rate IR} = \frac{\text{number of blood-fed mosquitoes with L3 in body}}{\text{surviving mosquitoes at end of incubation period}} \times 100
\]

\[
\text{Vector efficiency index VEI} = \frac{\text{average number of L3 in mosquitoes from end of incubation period to end of study}}{\text{average number of ingested microfilariae}} \times 100
\]

Results
Infection trial
Mosquitoes of laboratory strains of *Ae. japonicus* (*n* = 65), *Ae. geniculatus* (*n* = 35) and *Ae. aegypti* (*n* = 105) as well as field-collected *Ae. japonicus* (*n* = 151) were allowed to feed through Parafilm® membranes on blood containing microfilariae of *D. immitis* or *D. repens*, or on negative control blood (*Ae. japonicus* groups only) (Table 1). The feeding rates were around 50% for all *Ae. aegypti* and *Ae. japonicus* except in one treatment (24%, field-collected *Ae. japonicus* feeding on *D. repens* blood, Table 1), and around 70% for the *Ae. geniculatus* groups.

Cumulative mortality rates of naturally dead mosquitoes were calculated from the total number of blood-fed mosquitoes that died naturally; calculated without day 1 samples; numbers are cumulative from day 1

The end of the incubation period was set at 10 dpi, as this equals the theoretical extrinsic incubation time at 27 °C according to DDUs [22]. If L3 larvae appeared before 10 dpi, calculations were based on the first appearance of L3.

Additionally to the above mentioned calculations, the first appearance of motile L3 in the proboscis was taken into account to assess the vector competence.

**Table 1** Feeding and mortality rates of *Aedes japonicus* (laboratory strain PEN; field-collected specimens from Switzerland (CH), *Ae. geniculatus* (laboratory strain IPZ) and *Ae. aegypti* (laboratory strain IPNC) during infection trials with *Dirofilaria immitis* and *D. repens*

| Mosquito species       | Inoculation          | No. total | No. feeding (%) | Mortality at 1 dpi No. (%) | Mortality at 5 dpi No. (%) | Mortality at 14 dpi No. (%) |
|------------------------|----------------------|-----------|-----------------|----------------------------|---------------------------|----------------------------|
| *Ae. japonicus* (PEN)  | *D. repens*          | 19        | 8 (42.1)        | 0                          | 0                         | 2 (28.6)                   |
| *Ae. japonicus* (PEN)  | *D. immitis*         | 28        | 12 (42.3)       | 0                          | 5 (50.0)                  | 8 (80.0)                   |
| *Ae. japonicus* (PEN)  | Negative control     | 18        | 9 (50.0)        | 0                          | 3 (33.3)                  | 4 (44.4)                   |
| *Ae. japonicus* (CH)   | *D. repens*          | 72        | 17 (23.6)       | 0                          | 0                         | 0                          |
| *Ae. japonicus* (CH)   | *D. immitis*         | 60        | 31 (51.6)       | 0                          | 10 (35.7)                 | 13 (46.4)                  |
| *Ae. japonicus* (CH)   | Negative control     | 19        | 8 (42.1)        | 0                          | 1 (12.5)                  | 5 (62.5)                   |
| *Ae. geniculatus* IPZ  | *D. repens*          | 17        | 12 (70.1)       | 0                          | 0                         | 4 (44.4)                   |
| *Ae. geniculatus* IPZ  | *D. immitis*         | 18        | 13 (72.2)       | 0                          | 2 (18.2)                  | 4 (36.4)                   |
| *Ae. aegypti* IPNC     | *D. repens*          | 46        | 21 (45.7)       | 4 (22.2)                   | 8 (44.4)                  | 8 (44.4)                   |
| *Ae. aegypti* IPNC     | *D. immitis*         | 41        | 24 (58.5)       | 6 (28.6)                   | 11 (52.4)                 | 11 (52.4)                  |
mosquitoes in comparison to those that died until 1 and 5 dpi and until 14 dpi. Mosquitoes taken alive for dissection were not considered as dead mosquitoes but were assumed to have lived until 14 dpi (Table 1). At 1 dpi, mortality only occurred in the *Ae. aegypti* IPNC group; this was statistically significantly different to the mortality in field-collected *Ae. japonicus infected with D. immitis* (*P* = 0.046). No other statistically significant differences were observed within the *D. immitis* experiments between the different species at 5 and at 14 dpi.

Until 5 dpi, there was no mortality in the *D. repens* inoculated groups, except in *Ae. aegypti* IPNC. The mortality on 5 dpi in the *D. repens* inoculated groups was significantly different between *Ae. aegypti* IPNC and *Ae. geniculatus* (*P* = 0.0299) and field collected *Ae. japonicus* (*P* = 0.0047). Mortality rates varied between 0 and 80%, and were usually around 40% or higher at 14 dpi, including the control mosquitoes. The exception was the field-collected *Ae. japonicus* population inoculated with *D. repens* with an overall mortality rate of 0%. This was significantly different to the mortality in other *D. repens* infected groups (*P* = 0.0361 for *Ae. japonicus* PEN, *P* = 0.0211 for *Ae. geniculatus* and *P* = 0.01 for *Ae. aegypti*) and significantly different in comparison to this field population infected with *D. immitis* at 5 dpi and at 14 dpi (*P* = 0.0086 and 0.0035, respectively). Only in *Ae. aegypti* IPNC was the overall mortality rate observed already at 5 dpi, whereas in all other mosquito species mortality increased until at 14 dpi (Table 1).

The calculated infectious dose per mosquito in the trials was 12 mf, considering the microfilarial density of 3000/ml blood and an average blood meal volume of 4 μl. The observed infectious doses as determined by microscopy at 1 dpi differed considerably (Tables 2, 3, 4, 5, 6, 7, 8 and 9). For *D. repens* they varied from 0 to 22 and for *D. immitis* from 0 to 7.

### *Aedes japonicus* (PEN) inoculated with *D. immitis* or *D. repens*

Altogether 12 blood-fed mosquitoes were in the trial with *Ae. japonicus* PEN inoculated with microfilariae of *D. immitis*. Out of these, alive mosquitoes (*n* = 4) or freshly dead mosquitoes (*n* = 6) were dissected (Table 2), and a further 2 dead mosquitoes (desiccated) were analysed by PCR only. Four of the 10 dissected mosquitoes were positive for *D. immitis* in microscopy, and another 5 were positive by PCR only. The 2 dead and desiccated mosquitoes were PCR positive [at 2 dpi (abdomen) and 12 dpi (abdomen and head)]. Thus, 11 out of the total 12 mosquitoes (91.7%) were positive for *D. immitis*. Mosquitoes were positive in microscopy until at 5 dpi, but by PCR the last positive was found at 12 dpi. Figure 1

### Table 2 Specimens of laboratory colony *Aedes japonicus* PEN (*n* = 10) examined microscopically for larval stages of *Dirofilaria immitis*

| Dpi | Total no. | No. positive | mf | L1 | L2 | L3 | Location (total no. in all dissected mosquitoes) |
|-----|-----------|--------------|----|----|----|----|---------------------------------------------|
| 1   | 2         | 1*           | 2  | –  | –  | –  | Midgut (2)                                  |
| 3   | 3         | 2*           | –  | 1,3| –  | –  | Malpighian tubules (4)                      |
| 4   | 1         | 0*           | –  | –  | –  | –  | Malpighian tubules (11, of which 3 melanized) |
| 5   | 2         | 1*           | 1  | 11 | –  | –  |                                            |
| 6   | 1         | 0*           | –  | –  | –  | –  |                                            |
| 14  | 1         | 0            | –  | –  | –  | –  |                                            |

**Abbreviations:** dpi day post-inoculation, mf microfilariae, L1 first-stage larva, L2 second-stage larva, L3 third-stage larva (infectious stage)

*a* One additional mosquito positive in PCR only  
*b* Data given only when localisation was clearly assignable after dissection

### Table 3 Specimens of laboratory colony *Aedes japonicus* PEN (*n* = 8) examined for larval stages of *Dirofilaria repens*

| Dpi | Total no. | No. positive | mf | L1 | L2 | L3 | Location (total no. in all dissected mosquitoes) |
|-----|-----------|--------------|----|----|----|----|---------------------------------------------|
| 1   | 1         | 1            | 22 | –  | –  | –  |                                            |
| 3   | 1         | 1            | 1  | –  | –  | –  |                                            |
| 5   | 1         | 1            | –  | 1  | –  | –  |                                            |
| 6   | 2         | 1            | –  | 1  | –  | –  |                                            |
| 12  | 1         | 0            | –  | –  | –  | –  |                                            |
| 14  | 1         | 1            | –  | –  | –  | 8  | Proboscis (6), head (1), Malpighian tubules (1) |
| 16  | 1         | 1            | –  | –  | –  | 4  | Head (1), abdomen (3)                       |

**Abbreviations:** dpi day post-inoculation, mf microfilariae, L1 first-stage larva, L2 second-stage larva, L3 third-stage larva (infectious stage)

*a* Data given only when localisation was clearly assignable after dissection
shows L1 larvae (alive and melanized) in the Malpighian tubules and a melanized microfilaria at 5 dpi. As no L3 of *D. immitis* were observed, both IR and VEI for *D. immitis* in *Ae. japonicus* PEN were 0%. However, the head of a dead mosquito was PCR-positive at 12 dpi. Nonetheless, *Ae. japonicus* PEN may be refractory to infection with *D. immitis*.

Eight blood-fed mosquitoes were in the trial with *Ae. japonicus* PEN inoculated with microfilariae of *D. repens* (Table 3), and all mosquitoes were dissected (6 alive and 2 freshly dead). Six of the 8 mosquitoes were positive for *D. repens* in microscopy (75%), and no additional one was identified positive by PCR. First L3 larvae were observed at 14 dpi in abdomen and proboscis (Table 3). The IR for *D. repens* was calculated to be 33.3% and the VEI 18.2%. Thus, *Ae. japonicus* PEN is susceptible to infection with *D. repens*.

*Aedes japonicus* CH inoculated with *D. immitis* or *D. repens*

Thirty-one blood-fed mosquitoes were in the trial with *Ae. japonicus* CH inoculated with microfilariae of *D. immitis*, and 24 mosquitoes were dissected (18 alive and 6 freshly dead; see Table 4). Nineteen were positive for *D. immitis*, and an additional 2 were identified positive by PCR. Five of 7 further dead and desiccated mosquitoes were positive for *D. immitis* DNA in their abdomens at 2, 7, 8 and 10 dpi. Thus, altogether 26 out of total 31 (83.9%) mosquitoes were positive for *D. immitis*. Various developmental stages are shown in Fig. 2.

Seventeen mosquitoes took a blood meal containing microfilariae of *D. repens* (Table 5). There were no mortalities, and all were dissected. A total of 16 (94.1%) were positive for *D. repens*. The only microscopically negative mosquito was also negative by PCR. Different developmental stages are shown in Fig. 3.

First L3 larvae of *D. immitis* were observed at 14 dpi in the proboscis and of *D. repens* at 10 dpi. L3 of both *Dirofilaria* species were found until the end of the experiments at 21 dpi.

The IR and VEI were 27.8 and 66.7% for *D. immitis*, and 47.1 and 85.9% for *D. repens*, respectively, rendering *Ae. japonicus* CH a susceptible vector for both filarial species.

*Aedes geniculatus* IPZ inoculated with *D. repens* or *D. immitis*

Thirteen blood-fed mosquitoes were in the trial with *Ae. geniculatus* IPZ inoculated with microfilariae of *D. immitis*, and 9 alive mosquitoes were dissected (Table 6).
Six of them were positive in microscopy for *D. immitis*, and an additional 3 were identified positive by PCR. Furthermore 3 of 4 dead and desiccated mosquitoes was positive for *D. immitis* DNA in their abdomens at 4, 7 and 9 dpi. Thus, altogether 12/13 (92.3%) mosquitoes were positive for *D. immitis*.

Twelve mosquitoes took a blood meal containing microfilariae of *D. repens*. Eight were dissected and were positive in microscopy (Table 7). One out of 4 dead and desiccated mosquitoes was also PCR positive. Thus, 9 out of 12 (75%) mosquitoes were positive for *D. repens*. Figure 4 shows a massive infection of Malpighian tubules with L2 and L3 larvae at 9 dpi.

First L3 larvae of *D. immitis* were observed at 10 dpi in the abdomen and at 13 dpi also in the thorax. *Dirofilaria repens* L3 first occurred at 9 dpi in Malpighian tubules and at 14 dpi in the proboscis.

The IR and VEI were 22.2 and 100% for *D. immitis*, and 37.5 and 70.8% for *D. repens*, respectively. Thus, *Ae. geniculatus* IPZ is susceptible to both filarial species.

### Table 6: Specimens of laboratory colony *Aedes geniculatus* IPZ (n = 9) examined microscopically for larval stages of *Dirofilaria immitis*

| Dpi | Total no. | No. positive | Mf | L1 | L2 | L3 | Location (total no. in all dissected mosquitoes) |
|-----|-----------|--------------|----|----|----|----|-----------------------------------------------|
| 1   | 2         | 2            | 5, 6 | –  | –  | –  | Malphighian tubules (3)                        |
| 4   | 2         | 0a           | –   | –  | –  | –  |                                                |
| 7   | 2         | 2b           | –   | 25 | –  | –  | Malphighian tubules (24), melanized in Malpighian tubules (1) |
| 9   | 0         | 0b           | –   | –  | –  | –  |                                                |
| 10  | 2         | 1b           | –   | –  | –  | 11 | Malphighian tubules (11)                      |
| 13  | 1         | 1            | –   | –  | –  | 6  | Abdomen (5), Thorax (1)                       |

*Abbreviations: dpi day post-inoculation, Mf microfilariae, L1 first-stage larva, L2 second-stage larva, L3 third-stage larva (infectious stage)*

*Two additional positive in PCR only*

*One additional positive in PCR only*

*Data given only when localisation was clearly assignable after dissection*

Therefore, a total of 17/24 (70.8%) mosquitoes were positive.

Nineteen blood-fed mosquitoes were in the trial with *Ae. aegypti* IPCN inoculated with microfilariae of *D. repens* (Table 9) and 18 (12 alive and 6 freshly dead) were dissected. Six were positive for *D. repens* in microscopy and an additional 3 by PCR (up to day 9). A total of 9 out of 19 (47.4%) mosquitoes were positive.

Microfilariae and L1 larvae could only be observed in microscopy until at 5 dpi, and no developmental stages were observed after day 5 for both filarial species. As no L3 developed, infection rate and vector efficiency index were 0% for both *D. immitis* and *D. repens*. Therefore, according to our study, *Ae. aegypti* IPNC is refractory to infection with both filarial species.

### Development of larval stages

Infected Malpighian tubules had sac-like appearance and developmental stages were generally found in the distal part of the tubules, as has been described previously [22]. In none of the mosquitoes were all tubules infected. The length and width of the observed larval stages in the mosquitoes are shown in Tables 10 and 11. Generally, fairly large variations in the sizes of the developmental stages were observed. Additionally, melanized larval stages were observed: for *D. immitis* in *Ae. japonicus* PEN at 5 dpi (microfilaria: 305 × 8 μm; L1: 164 × 22 μm) and in *Ae. japonicus* CH at 7 dpi (L1: 157 × 24 μm; 173 × 24 μm; 188 × 24 μm).

### Table 7: Specimens of laboratory colony *Aedes geniculatus* IPZ (n = 8) examined microscopically for larval stages of *Dirofilaria repens*

| Dpi | Total no. | No. positive | Mf | L1 | L2 | L3 | Location (total no. in all dissected mosquitoes) |
|-----|-----------|--------------|----|----|----|----|-----------------------------------------------|
| 1   | 3         | 3            | 6, 8, 20 | –  | –  | –  | Malphighian tubules (3)                        |
| 5   | 2         | 2            | –   | 3, 7 | –  | –  |                                                |
| 9   | 1         | 1            | –   | 19  | 14 | –  | Malphighian tubules (36)                      |
| 14  | 2         | 2            | –   | 1   | –  | 1, 25| L3: Proboscis (20), thorax (2), Malpighian tubules (4) |

*Abbreviations: dpi day post-inoculation, Mf microfilariae, L1 first-stage larva, L2 second-stage larva, L3 third-stage larva (infectious stage)*

*Data given only when localisation was clearly assignable after dissection*
187 × 25 μm), and for D. repens in Ae. japonicus CH at 14 dpi (L1: 177 × 26 μm).

**Discussion**

More than 60 mosquito species are incriminated vectors of Dirofilaria spp., and several species have been examined under laboratory conditions for their potential vector competence by observing the development to the infective L3 stage. For D. immitis these include for example Ae. aegypti [40, 43–45], Ae. albopictus [46–48], Ae. koreicus [31], Ae. vexans (Meigen, 1830) [22] and Ae. triseriatus Say, 1823 [49] and Ae. japonicus [32, 53–56]. Further, Ae. japonicus was shown to readily feed on mammals including humans and dogs [56]. Taken together, our findings suggest that there is an increased risk of Dirofilaria transmission in areas populated by this species. This is somewhat reminiscent to the situation in Italy where the establishment of Ae. albopictus, a suitable vector of D. immitis, changed the epidemiology of canine dirofilarioses (transmission in new areas, higher prevalences) [26, 27].

Interestingly, the laboratory colony of Ae. japonicus PEN was susceptible to D. repens but seemed refractory to D. immitis, i.e. no L3 larvae developed and reached the proboscis. Though we only had few blood-engorged

| Table 8 Specimens of laboratory colony Aedes aegypti IPNC (n = 22) examined microscopically for larval stages of Dirofilaria immitis |
| --- |
| Dpi | Total no. of individuals | No. of positive individuals | Number of larval stages per individual positive mosquito*a |
| --- | --- | --- | --- |
| 1 | 9 | 5*a | 1, 1, 5, 7, 10 – – – |
| 2 | 1 | 1 | – 1 – – |
| 3 | 4 | 1*b | 1 – – – |
| 5 | 2 | 1 | – 3 – – |
| 7 | 2 | 0*a | – – – – |
| 9 | 1 | 0*b | – – – – |
| 12 | 1 | 0*b | – – – – |
| 14 | 1 | 0*b | – – – – |
| 16 | 1 | 0*b | – – – – |

**Abbreviations:** dpi day post-inoculation, mf microfilariae, L1 first-stage larva, L2 second-stage larva, L3 third-stage larva (infectious stage)

*aTwo additional positive in PCR

*bOne additional positive in PCR only

*cLocalisation of developmental stages was not clearly assignable after dissection

| Table 9 Specimens of laboratory colony Aedes aegypti IPNC (n = 18) examined microscopically for larval stages of Dirofilaria repens |
| --- |
| Dpi | Total no. of individuals | No. of positive individuals | Number of larval stages per individual positive mosquito*c |
| --- | --- | --- | --- |
| 1 | 6 | 3*a | 1, 3, 6 – – – |
| 3 | 4 | 2 | 16, 2 – – – |
| 5 | 2 | 1 | – 1 – – |
| 7 | 2 | 0*a | – – – – |
| 9*b | 1 | 0*a | – – – – |
| 12 | 1 | 0 | – – – – |
| 14 | 1 | 0 | – – – – |
| 16 | 1 | 0 | – – – – |

**Abbreviations:** dpi day post-inoculation, mf microfilariae, L1 first-stage larva, L2 second-stage larva, L3 third-stage larva (infectious stage)

*aOne additional positive in PCR only

*bHead was lost during the dissection process

cLocalisation of developmental stages was not clearly assignable after dissection
females of this mosquito strain in this trial, this finding emphasizes again the need to carry out vector competence experiments with local and wild specimens to obtain relevant results. Unfortunately, the experiment cannot be repeated due to loss of the colony.

Additionally we could show that a laboratory colony of *Ae. geniculatus* derived from field-collected individuals [37] is an equally good vector for both *Dirofilaria* species. The univoltine *Ae. geniculatus* shares larval breeding sites such as tree holes with the invasive *Ae. japonicus* [37]. It generally occurs in low abundances but large numbers may be present in focal areas. Taking into account its aggressive mammophilic biting behaviour, *Ae. geniculatus* may contribute to local transmission cycles. Further, our established colony might be of value for further studies on host-pathogen interactions.

Constant temperatures of 27 °C are not realistic for central Europe, though the average temperature might reach this level during hot summer spells. Temperatures fluctuating over the day and between days are reality for the climate in central Europe, and further investigations will be done at more realistic fluctuating temperature regimes. Interestingly, daily temperature fluctuations accelerated pathogen development in the mosquito as compared to constant conditions with the same average temperature, as was shown with *Plasmodium* parasites, *D. immitis* and dengue viruses [57–59], and this was particularly observed under cool conditions which is of significance at the cooler margin of a suitable climate.

The developmental time from microfilariae to infectious L3 stage was in accordance with the predictions from the DDU formula. The experiments in this study were done at constant 27 °C; at this temperature, the development time is expected to be 10 days until the first observation of L3. However, even though first L3 larvae were observed within 10 days in *Ae. japonicus* and *Ae. geniculatus*, they tended to reach the proboscis only a few days later, and this has to be taken into account additionally when making a risk assessment.

In the first 2 dpi, *D. repens* L1 stages started to shorten in length, but remained at the same approximate width as microfilariae as compared to measurements described in literature [60]. After 3–6 days both *D. repens* and *D. immitis* had reached the typical sausage stage [39, 40] in

| Days post-infection | Measurement (μm) | *Ae. japonicus* PEN | *Ae. japonicus* CH | *Ae. geniculatus* IPZ | *Ae. aegypti* IPNC |
|---------------------|------------------|---------------------|-------------------|---------------------|-------------------|
| 1–2                 | Mean length (range) | 331 (309–350) | – | 237 (221–254) | – |
|                     | Mean width (range) | 8 (5–10) | – | 6 (5–6) | – |
| 3–6                 | Mean length (range) | 208 (144–272) | – | – | 345 (313–361) |
|                     | Mean width (range) | 25 (21–30) | – | – | 7 (6–9) |
| 7–9                 | Mean length (range) | 263 (na) | 602 (535–678) | 732 (518–848) | – |
|                     | Mean width (range) | 283 (na) | 30 (23–34) | 35 (10–48) | – |
| 10–14               | Mean length (range) | 954 (822–1030) | 822 (685–954) | 911 (657–1120) | – |
|                     | Mean width (range) | 26 (23–28) | 24 (22–27) | 29 (26–36) | – |
| More than 14        | Mean length (range) | 976 (na) | – | – | – |
|                     | Mean width (range) | 27 (na) | – | – | – |

Abbreviations: na not applicable (only single specimens available); –, no specimens available or no photographs taken during dissection process

### Table 11: Sizes of developmental stages of *Dirofilaria immitis* reared under laboratory conditions at 27 °C

| Day post-infection | Measurement (μm) | *Ae. japonicus* PEN | *Ae. japonicus* CH | *Ae. geniculatus* IPZ | *Ae. aegypti* IPNC |
|---------------------|------------------|---------------------|-------------------|---------------------|-------------------|
| 1–2                 | Mean length (range) | – | – | – | 283 (256–290) |
|                     | Mean width (range) | – | – | – | 8 (6–10) |
| 4–6                 | Mean length (range) | 166 (122–215) | 218 (171–250) | – | 260 (244–270) |
|                     | Mean width (range) | 22 (14–35) | 25 (23–28) | – | 5.9 (5.5–6.2) |
| 7–9                 | Mean length (range) | – | 284 (170–353) | 359 (283–418) | – |
|                     | Mean width (range) | – | 28 (25–33) | 32 (28–35) | – |
| 10–14               | Mean length (range) | – | 859 (477–922) | 811 (552–1050) | – |
|                     | Mean width (range) | – | 33 (27–36) | 29 (23–37) | – |
| More than 14        | Mean length (range) | – | 1022 (906–1130) | – | – |
|                     | Mean width (range) | – | 29 (25–32) | – | – |

Abbreviations: na not applicable (only single specimens available); –, no specimens available or no photographs taken during dissection process
Ae. japonicus, whereas they had only marginally shortened in the refractory Ae. aegypti IPNC, indicating that development did hardly take place in this mosquito species, and microfilariae most probably died in these first days post-inoculation. Ae. aegypti has been considered a rather unsuitable natural host for D. immitis, but a large variability of its susceptibility has been reported on many occasions [40, 43, 44, 61]. Both D. immitis and D. repens larvae started to elongate to L2 stage after 6 days at sizes comparable to what has been described earlier [40]. After 14 days and later, the infectious D. immitis L3 larvae reached their full length [40], with a lower size variation than described previously [22]. Interestingly, several larval stages of D. repens observed also in thorax and proboscis were shorter than previously described for L3 stages, and many of them were under 1000 μm. Previously, it was reported that the infectious stages reached lengths above 1000 μm [39].
Microscopy is a good and sensitive tool to observe the developmental stages of *Dirofilaria* spp. in mosquito samples. However, especially in the early stage of infection, additional positive specimens can be detected by PCR when low numbers of microfilariae originating from the infectious blood meals may be overlooked. In addition, PCRs remained positive until the end of the trials although development ceased at 5 dpi for *D. immitis* in *Ae. japonicus* PEN, and in *Ae. aegypti* IPNC for both filarial species. Thus, PCR positivity gives no clue whatsoever about infection rates and vector efficiency of any mosquito species, but can merely be of use in epidemiological studies to make a general assessment of occurrence of a filarial species in a certain area. This means that DNA reports from field-collected mosquitoes need to be very critically assessed.

We observed a large variation of microfilaria in the blood meals at 1 dpi (Tables 2, 3, 4, 5, 6, 7, 8 and 9). Furthermore, altogether only 75–94% of the engorged mosquitoes harboured microfilariae at all. This has to be taken into account additionally when making risk assessments. The calculations of VEI are based on the observed number of microfilariae at the beginning of the trial. Due to the low numbers of mosquitoes involved in some experiments, only between 1 and 3 mosquitoes could be used to determine the number of ingested microfilariae. Thus, the obtained VEI values have therefore to be treated with some caution. However, several mosquito species had L3 larvae in the proboscis and those can as such be seen as suitable vectors for the respective filarial species.

A crucial factor for vector competence and vector capacity is that mosquitoes need to survive the extrinsic incubation period in order to be able to transmit the pathogen to the next host. The results indicate that the parasite does not cause an overall higher mortality in the mosquitoes at the infection dose of 3000 mf/ml, which was chosen because it was shown to be suitable for such investigations in previous studies [31]. Higher mortalities
with inocula containing higher microfilaraemiae were observed in various studies [31, 43, 48]. In endemic foci, very high microfilaraemiae can be observed (up to 70,000/ml) [31], and it was speculated that dogs with low microfilaraemiae might be the relevant reservoirs for Dirofilaria transmission [48].

Conclusions

To our knowledge, field-collected Ae. japonicus were for the first time shown to be an efficient vector for both D. immitis and D. repens, indicating that this invasive and locally highly abundant species may contribute to a local transmission of filarial worms, as has been described for Ae. albopictus and D. immitis in Italy. Additionally, also the indigenous Ae. geniculatus, sharing the same larval breeding sites with Ae. japonicus, is a suitable vector for Dirofilaria spp. Aedes japonicus from a laboratory colony were refractory to D. immitis, confirming the necessity to perform vector competence studies and risk assessments based on such studies with local mosquito populations. Our results further demonstrate that by DNA detection alone no reliable conclusions can be drawn with regard to the vector competence of a mosquito species.

Abbreviations

DDU: Dirofilaria development unit; DIC: differential interference contrast; dpi: day post-infection; HDU: heartworm development unit; IR: infection rate; L1: first-stage larva; L2: second-stage larva; L3: third-stage larva; mf: microfilaria; PBS: phosphate buffered saline; VEI: vector efficiency index

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Availability of data and materials

The data supporting the conclusions of this article are included within the article. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

CS and AM had the idea for the study. CS set up the experimental design, performed the experimental infection, laboratory analysis and analysed the data. RB provided study material. GC and FM helped with the experimental design and gave valuable scientific input. CS wrote the manuscript. All authors critically revised the manuscript, read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

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Ethics approval

Not applicable.

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