Introduction and Establishment of the Exotic Mosquito Species *Aedes japonicus japonicus* (Diptera: Culicidae) in Belgium

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ABSTRACT The establishment of the potential vector species *Aedes* (*Finlaya*) *japonicus japonicus* (Theobald) (Diptera: Culicidae) in southern Belgium is reported. The species was most likely introduced through the international trade in used tires. It was first collected in 2002 on the premises of a second-hand tire company and was sampled using different sampling methods in the two consecutive years (2003–2004). It was only in 2007 and 2008, during a national mosquito survey (MODIRISK), that its presence as adults and larvae at the above-mentioned site and at another tire company in the area was confirmed based on morphological and molecular identification. This discovery is the first record for Belgium of an exotic mosquito species that established successfully and raises the question on the need for monitoring and control. Considering the accompanying species found during the surveys, we also report here the first observation of *Culex* (*Maillotia*) *hortensis hortensis* (Ficalbi) in Belgium.

KEY WORDS *Aedes j. japonicus*, *Culex h. hortensis*, establishment, exotic species, Belgium

Aedes (*Finlaya*) *japonicus japonicus* (Theobald 1901) (=*Ochlerotatus japonicus japonicus* sensu Reinert 2000), is one of four morphologically similar subspecies originating from Japan, Korea, Taiwan, eastern China, and Russia (Tanaka et al. 1979). So far, it has been reported in four countries out of its native area. The first establishment outside its endemic zone was observed in the United States where it was collected in 1998 from the eastern part of the country (Peyton et al. 1999). Since then, the species has spread rapidly and is known from >22 states including Hawaii. It has been reported in parts of Canada (Williges et al. 2008), was intercepted in New Zealand in 1993 (Laird et al. 1994), in 1998 and 1999 (Sandlant 2003), and recorded in France in 2000 (Schaffner et al. 2003).

In its native range, *Ae. j. japonicus* prefers to breed in rock holes (LaCasse and Yamaguti 1948). However, larval collections in most countries where the species has been introduced, revealed its preference for artificial sites, mostly tires filled with water and debris (Andreadis et al. 2001, Morris et al. 2007). Females are known to feed on mammals, including humans, in the field (Apperson et al. 2004) and on avian hosts under laboratory conditions (Sardelis et al. 2003) and could therefore act as a zoonotic bridge vector species. In laboratory conditions, this mosquito has been shown to be a competent vector of Eastern encephalitis virus, LA Crosse virus, St. Louis encephalitis virus, and a highly competent vector for West Nile virus (Sardelis et al. 2001, 2002a, 2002b, 2003). However, its role as a disease vector species in natural conditions in the United States, where the species has been established for almost a decade, remains unclear.

Because of health risks, a tire trade surveillance program for *Aedes* (*Stegomyia*) *albopictus* (Skuse) has been implemented and conducted in France since 1998 focused on storage centers importing used tires from the United States, Japan, or Italy (Schaffner et al. 2004). The discovery of *Ae. albopictus* in metropolitan France in 1999 (Schaffner and Karch 2000) and subsequently of *Ae. j. japonicus* in 2000 (Schaffner et al. 2003) resulted in an extension of this program; three Belgian companies exporting to France were identified as importing second-hand tires from diverse countries, including those colonized by *Ae. albopictus*. Therefore, the companies were visited once or twice from 2000 to 2003. In 2007, a national mosquito survey (MODIRISK, www.modirisk.be) started, aiming to study the taxonomic and functional biodiversity of both endemic and invasive mosquito species in Belgium as well as the factors driving change. This paper reports the introduction and establishment of *Ae. j. japonicus* in Belgium as revealed by these surveys.

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Table 1. Overview of the adult collections

Site	Collection place	Collection date	Collection method	*An. plumbeus*	*Cx. pipiens* s.l.	*Cx. torrentium*	*Ae. geniculatus*	*Ae. j. japonicus*
Natoye1	Forest	16 July 2004	Human landing	47 F	0	0	214 F	72 F
	Forest edge	17 July 2004	Gravid trap	0	1 F	0	0	1 F
	Forest edge	17 July 2004	Dry ice trap	3 F	1 F	0	1 F	1 M
	Forest 100 m	17 July 2004	Dry ice trap	1 F	0	0	13 F, 5 M	0
	Tires edge tire storage	2 Oct. 2007	CO_2 trap	1 F	138 F, 7 M	2 F	0	1 F, 2 M
	Forest edge	2 Oct. 2007	CO_2 trap	2 F	124 F, 8 M	0	0	0
Natoye2	Tires, edge tire storage	9 Oct. 2008	CO_2 trap	4 F	25 F, 1 M	0	0	0

F, female; M, male.

Materials and Methods

Study Sites. One of the Belgian sites implemented in the French study, revealed infestation by *Ae. j. japonicus* in 2003 and was therefore visited at several occasions. The company, located in the village of Natoye (Namur, 50.3389587° N, 5.0448799° E; subsequently named Natoye1), imports mainly tires for trucks and heavy vehicles originating from various countries. Tires are stacked outside and many of these are exposed to rainfall and contain water and organic material; other nearby potential breeding sites are present. In October 2008, a similar second-hand tire company (subsequently named Natoye2) in the same area (50.33588° N, 5.0714698° E) was surveyed.

Adult Survey. Adult species were collected in 2004, 2007, and 2008 by using various methods. On 16 July 2004, mosquitoes were collected from the tire storage and the neighboring forest by a CO_2 (dry ice)-baited trap (similar to Bioquip model, EID Méditerranée, Montpellier, France), a CDC Gravid trap (model 1712, John W. Hock Co., Gainesville, FL) (from 1830 hours to 1030 hours, 17 July 2004) and on human landing catches from 1900 hours to 2110 hours. Traps were placed at the rear side of the company, on the forest edge. After 2004, the site was not revisited until the beginning of the national mosquito survey (MODIRISK, www.modirisk.be) in 2007. The site was resampled during 1 wk in September–October 2007, with two Mosquito Magnet Liberty Plus traps (Woodstream Corporation, Lititz, PA). One of the two traps was placed near tires no longer used for commercial sale; the second trap was placed near a small forest fragment, ≈100 m away from the tires. The same trap type was used in Natoye2. All adult collection dates are listed in Table 1.

Larval Collections. Sampling for immature mosquitoes was conducted in 2002–2004, 2007, and 2008. In October 2002, February 2003, and July–September 2004 larvae were sampled at Natoye1 and the surrounding area by using a net or a sieve and small dippers. Tires at Natoye1 were checked for larvae, by using small sieves and pipettes on four occasions between September and November 2007. Larvae were identified or reared to adult in a secured lab for identification. In 2008, larvae were collected at the end of March in Natoye1 and at the beginning of October in Natoye2.

Morphological Identification. Mosquito larvae and adults were identified using identification keys of Tanaka et al. (1979), Schaffner et al. (2001), Schaffner (2003), and Becker et al. (2003).

Molecular Identification. Molecular identifications were performed on adult specimens caught in 2007 to confirm morphological identification. The entire ribosomal internal transcribed spacer (ITS) 2 region and a part of the mitochondrial cytochrome oxidase I (COI) gene for four individuals were amplified and sequenced. DNA extraction was performed using the protocol described by Collins et al. (1987). The rDNA ITS2 was amplified using the primers described in Proft et al. (1999), whereas the mitochondrial DNA COI was amplified using the barcode primers of Folmer et al. (1994); amplification reaction was done following Van Bortel et al. (2000). Positive polymerase chain reaction) products were sequenced by Genoscreen (Lille, France). Sequence data were aligned (BioEdit) and compared with data available in CBOL (www.barcodinglife.org).

Results and Discussion

In 2004, 360 adult mosquitoes belonging to four species were collected from Natoye1. The majority of *Ae. j. japonicus* individuals (72 females; 21.6% of the mosquitoes) were manually collected on human landing during 2 h at sunset; trap catches yielded only one male and one female. Other species collected were *Aedes* (*Finlaya*) *geniculatus* (Olivier), *Anopheles* (*Anopheles*) *plumbeus* (Stephens), and *Culex* (*Culex*) *pipiens* L. s.l. (Table 1). Of the 285 mosquitoes collected with the MMLP in 2007, only three were *Ae. j. japonicus* (one female and two males; 1.05% of the mosquitoes). The most abundant species was *Cx. pipiens* s.l.; other species collected were *Culex* (*Culex*) *torrentium* (Martini) and *An. plumbeus* (Table 1). The MMLP used in 2008 at Natoye2 yielded no adults of *Ae. j. japonicus,* only *Cx. pipiens* s.l. and *An. plumbeus* (Table 1).

The morphological identification was confirmed by COI sequence showing 99% similarity with sequences of the COI gene of *Ae. j. japonicus* species from Canada (www.barcodinglife.org; Mosquitoes of Canada). The nucleotide sequences are deposited in GenBank un-

Table 2. Occurrence of sampled container and groundwater habitats in Natoye for immature *Ae. j. japonicus*

Habitat	*An. plumbeus*	*An. maculipennis* s.l.	*Cu. annulata*	*Cu. morsitans*	*Culex* × *h. hortensis*	*Cx. pipiens* s.l.	*Cx. torrentium*	*Aedes j. japonicus*	*Ae. rusticus*
Used tire castings (onsite)	x		x		x	x		x	
Used tire castings (1.75 km away)						x	x		
Treehole (onsite)								x	
Road hole (250 m away)						x			
Temporary surface water pools (1 km away)		x							
Permanent surface water pools (1 km away)				x					x

der accession numbers FJ641869 (COI sequence) and FJ641870 (ITS2 sequence).

Larvae were collected in tires at Natoye1 on different visits in 2002, 2003, and 2004. On 30 October 2002, larvae were observed in tires at low density, including *Ae. j. japonicus* and four other species: *An. plumbeus, Culiseta* (*Culiseta*) *annulata* (Schrank), *Culex* (*Maillotia*) *hortensis hortensis* (Ficalbi), and *Cx. pipiens* s.l. During winter 2003, no larvae were found under the ice cover. In July 2004, *Ae. j. japonicus* larvae were observed at high density in the tires and in a tree hole next to the tire company. Associated species were *An. plumbeus, Cx. h. hortensis, Cx. pipiens* s.l., and *Ae. geniculatus.* In 2003 and 2004, in a range of 0.24–1.75 km away from the company, artificial and natural niches were screened for *Ae. j. japonicus* larvae; however, none were found (Table 2). Larvae collected from a single tire in September and October 2007 and reared in the lab yielded 11 adults of *Ae. j. japonicus* ($n = 8$ females, $n = 3$ males). Other species emerging were *Cs. annulata* ($n = 4$) and *Cx. torrentium* ($n = 2$). In March 2008, 10 *Ae. j. japonicus* emerged from larvae collected ($n = 6$ females, $n = 4$ males) at Natoye1 from a single tire; accompanying species were *Cs. annulata* ($n = 4$), *Cx. pipiens* s.l. ($n = 15$), and *An. plumbeus* ($n = 1$). From larvae collected at Natoye2 on 2 and 9 October 2008, two *Ae. j. japonicus* (one male, one female) emerged in the lab; other emerging larvae belonged to *Cx. pipiens* s.l. ($n = 22$) and *Anopheles* (*Anopheles*) *claviger* Meigen ($n = 5$). These larval collections done early spring of 2008 indicate the ability of *Ae. j. japonicus* to overwinter in Belgium as diapausing eggs, the known overwintering stage of the species (Andreadis et al. 2001). Moreover, during the larval survey in winter no larvae were found. Furthermore, we found that larvae were particularly common in algal and leaf filled tires, in both shaded and sunlit areas. Although *Ae. j. japonicus* was first discovered in these discarded tires, both larvae and adults also were collected in natural habitats. These findings are consistent with data from its native region (Tanaka et al. 1979) and the recently colonized areas (Andreadis et al. 2001, Grim et al. 2007).

Ae. j. japonicus has proven to be a successful invasive species in the United States (Morris et al. 2007) due to: its capacity to survive human induced long-distance dispersal; its ability to survive winter in temperate regions; and its broad range tolerance of organic concentrations in natural and artificial aquatic containers (Tanaka et al. 1979, Andreadis et al. 2001). To date, it seems that *Ae. j. japonicus* is well-established at least on one site, even if recurrent infestations cannot be completely ruled out yet. There are currently no indications that the species, which has been present for at least six years in Belgium, has spread from the surroundings of the two companies. The most likely route of importation into Belgium seems the international used tire trade. First, the primary infested site belongs to a company importing used tires from various countries, including countries colonized by the species (Japan, United States). Second, the site is isolated from motorways and airports. And third, the species has so far not been observed in other surrounding sites. In general, the international movement of tires is known to be the primary pathway of introduction of container breeding species (Sardelis and Turell 2001, Schaffner et al. 2003). No control measures have been implemented so far, despite repeated information and alerts to regional and national health authorities. Because the anthropophily of *Ae. j. japonicus* is confirmed by our field observations, the establishment of this invasive species implies the need for monitoring and control measures at least to keep down its population and to limit its spread. It would be appropriate to aim the elimination of the species from the area where it was found before its spread to other parts of the country. At least the used tire storage should be free of the species, to avoid the export of eggs in tires that are exported to other regions and countries.

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