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The susceptibility of *Culicoides imicola* and *Culicoides oxystoma* to oral infection with epizootic hemorrhagic disease virus serotype 7 (EHDV-7) in Israel

הרגישות של *Culicoides imicola* ושל *Culicoides oxystoma* להדבקה אוראלית ב-(EHDV-7) hemorrhagic disease virus serotype 7 בישראל

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Abstract

The 2006 outbreak of Epizootic hemorrhagic disease virus serotype 7 (EHDV-7) that occurred in Israel resulted in a significant economic loss to the Israeli dairy industry. One of the most important aspects regarding the epidemiology of this newly emerging virus in the Middle-East is the identity of its vectors in Israel, which are still unknown. Several findings point towards biting midges of the species *Culicoides imicola* and *Culicoides oxystoma* as the main suspects as EHDV-7 vectors in Israel.

The present study aimed to determine if *C. imicola* and *C. oxystoma* females are susceptible to oral infection by EHDV-7. We hypnotized that these midges will acquire the virus through a blood-virus meal, undergo an infection and carry it for an assumed period which allows it to be transmitted in the following blood meal (i.e. extrinsic incubation period).

Field-collected *C. imicola* and *C. oxystoma* females were fed with cattle blood spiked with the virus using the dental cotton rolls feeding technique. Engorged females were processed individually for virus isolation immediately after feeding, and after 3-4, 11-12 or 15-16 days post feeding, with the last two testing times assumed to be equal of or higher than the usual extrinsic incubation period. Up until their processing, midges were held at 25ºC-26ºC and 50%-55% relative humidity. For virus isolation, individual midges' extract were inoculated onto Cow pulmonary artery endothelia (CPAE) cells and samples that caused cytopathic effect within 5 days were considered as a positive isolation of the virus.

Virus recovery rates were compered between the different time groups and the dynamics displayed was different than the expected one. Possible reasons for that are discussed. Further discussed are the feeding and survival rates of the midges as they were used as measures for evaluating the efficiency of the feeding technique and the incubation conditions, respectively.

Positive virus isolations were recorded from 47% (34/73) *C. oxystoma* and 80% (8/10) *C. imicola* after 3-16 incubation days. Amongst the midges that survived an assumed extrinsic incubation period of 11-12 or 15-16 days, 49% (22/45) and 71% (5/7) positive isolations were made from *C. oxystoma* and *C.*

imicola, respectively. These results indicate that the midges not only took up virus via the blood meal, but also became infected with it.

The results presented in this study indicate that *C. imicola* and *C. oxystoma* should be considered as potential vectors of EHDV-7 in Israel and warranted further investigation as to their vectorial competence and capacity.

תקציר

ההתפרצות של מחלה דימומית אפיזואוטית אשר ארעה בישראל בשנת 2006 ונגרמה ע"י סרוטיפ 7 של הנגיף Epizootic hemorrhagic disease virus (EHDV-7) היוותה מחלה מגיחה במזרח התיכון וגרמה נזקים כלכליים ניכרים לתעשיית הבקר לחלב בישראל. אחד ההיבטים החשובים ביותר בהבנת האפידמיולוגיה של הנגיף הינו זהות המעבירים הביולוגיים שלו בישראל, אשר טרם זוהו. מספר עדויות העלו את החשד כי נקבות יבחושים מן המינים *Culicoides imicola* ו-*Culicoides oxystoma* הינם המעבירים של EHDV-7 בישראל.

מטרת המחקר הייתה בחינת הרגישות של נקבות *C. imicola* ו-*C. oxystoma* להדבקה אוראלית עם EHDV-7. השערתנו היא כי היבחושות ירכשו את הנגיף דרך ארוחת דם המכילה אותו, יעברו הדבקה ויישאו את הנגיף למשך תקופת אינקובציה אקסטרינזית משוערת.

נקבות *C. imicola* ו-*C. oxystoma* אשר נלכדו בשדה הואכלו בתערובת דם בקר-נגיף תוך שימוש בשיטת הפד הדנטלי. יבחושות רוויות דם עובדו פרטנית לצורך בידוד הנגיף מיד בתום ההאכלה ולאחר 3-4, 11-12 או 15-16 ימים, כאשר שתי קבוצות הזמן האחרונות נחשבו בתור תקופות אינקובציה אקסטרינזית משוערות. עד למועד עיבודן, היבחושות גודלו תחת תנאי הדגרה של 25ºC-26ºC ולחות יחסית של 50% עד 55%. בידוד הנגיף התבצע על-ידי הדבקת תרביות תאי Cow pulmonary artery endothelia (CPAE) בתרחיף יבחושות, כאשר דגימות אשר גרמו לאפקט ציטופאתי תוך חמישה ימים נחשבו כבידוד חיובי של הנגיף.

שיעורי בידוד הנגיף הושוו בין קבוצות הזמן השונות והדינמיקה שהודגמה הייתה שונה מהמצופה. גורמים אפשריים לכך נידונים בעבודה. בנוסף, העבודה דנה בשיעורי האכילה וההישרדות של היבחושות, כמדדים המשמשים לצורך הערכת יעילות שיטת ההאכלה ותנאי ההדגרה, בהתאמה.

הנגיף בודד מ-47% (34/73) *C. oxystoma* ו-80% (8/10) *C. imicola* לאחר הדגרה של 3-16 ימים, כאשר 49% (22/45) ו-71% (5/7) בידודים בוצעו מקרב *C. oxystoma* ו-*C. imicola*, בהתאמה, אשר שרדו תקופת אינקובציה אקסטרינזית משוערת של 11-12 או 15-16 ימים. תוצאות אלו מעידות כי היבחושות לא רק רכשו את הנגיף דרך ארוחת הדם, אלא גם עברו הדבקה בו.

התוצאות המוצגות בעבודה מדגימות כי אכן יש לשקול את המינים *C. imicola* ו-*C. oxystoma* כמעבירים פוטנציאליים של EHDV-7 בישראל וכי יש להמשיך ולבחון את הכשירות (competence) והיכולת (capacity) שלהם כמעבירים של הנגיף.

Table of contents

Introduction

Epizootic hemorrhagic disease (EHD) virus (EHDV) is one of the important viruses that infect wild and domestic ruminants, and occurs in the Americas, Africa, Asia and Australia. It is a double-stranded RNA (dsRNA) arthropod-borne virus (Arbovirus), within the genus *Orbivirus* family *Reoviridae*, which causes a bluetongue-like (BT-like) disease in infected ruminants [1-3]. This virus is thought to be vectored by midges of the genus *Culicoides*, which feed on ruminant's blood, thus enabling the virus transmission to its host [1,3,4]. The first report of EHDV infection was in wild ruminants in New Jersey, USA, during august 1955 [5]. Since then, EHDV has been responsible for several large-scale epizootics in wild ruminants at various locations worldwide [1-3]. It is known to cause a disease, mainly in white tailed deer (*Odocoileus virginianus*), characterized by fever and extensive hemorrhage, which results in hemorrhagic shock and death [2,5,6].

EHDV has a typical *Orbivirus* morphology, which is characterized by icosahedral virus-core, ~80nm in diameter, composed of three concentric protein layers, encompassing the genome within the central core space. The genome includes 10 distinct segments of linear dsRNA, with each segment coding for 1 of 10 proteins [7-11]. Three of these proteins are non-structural and seven are structural proteins. The structural proteins form the double-shelled virus particle and includes 5 core proteins and 2 outer-coat proteins [7,8,12-14]. The outer-coat proteins VP2 and VP5 have a principal role in binding and penetrating to a host cell during the initiation of infection, and are encoded by genome segments 2 and 6, respectively [7,8,12,15]. These two are highly variable proteins and are known to be used for the distinguishing between the serotypes of each *Orbivirus* species [9,12,15-21].

Eight to 10 serotypes of EHDV are generally recognized in the literature based on neutralization assays and genome segments sequencing of viruses isolated from infected ruminants and/or *Culicoides* species: EHDV serotypes 1 to 8 (EHDV-1 to EHDV-8, respectively), serotype 318 (EHDV-318) and

Ibaraki virus (IBAV) [9]. EHDV-1 and EHDV-2 are known to be endemic in North America, where they were first described in 1955 and 1962, respectively [5,22,23]. Additionally, strains of EHDV-1 and EHDV-2 were later isolated in Australia [24,25]. EHDV-3 and EHDV-4 were initially isolated in Nigeria in the late 1960's [26]. EHDV-318 was first isolated in Bahrain in 1983 and subsequently recovered from sentinel calves in Sudan [27,28]. EHDV-5 to EHDV-8 were originally identified in Australia during 1977–1982 [29]. Since 2006, EHDV-6 has been proposed as an additional potentially endemic serotype in North America, and EHDV-7 was isolated in Israel [22,30]. IBAV was isolated from cattle herds in Japan [31]. Originally IBAV was considered as a distinct serotype but was later on identified as a strain of EHDV-2 [32,33].

As opposed to the above described taxonomy, recent work demonstrated fewer serotypes of the virus [12]. VP2 is the major component of the outer capsid, and due to its location and role, it is the virus's most exposed protein to the host's immune system. Thus, the gene coding for it is placed under selective pressure to adapt in order to evade neutralizing antibodies. As expected, VP2 is the most variable of the EHDV proteins, with sequence differences that correlate with both the serotype and geographic origin (topotype) of the virus lineage [9,12,15,16,18,19,22]. Based on molecular and serological analysis, mainly of the genome segment 2 of EHDV and protein VP2 for which it codes, the current classification of the EHDV serogroup has been proposed to be condensed into seven serotypes, with the inclusion of EHDV-3 into EHDV-1, and EHDV-318 into EHDV-6 [12].

In 1959 a BT-like disease, characterized by fever and stomatitis, was reported from cattle herds in Japan [31]. The disease was named 'Ibaraki disease' and the causative agent was Ibaraki virus, which was later recognized as a strain of EHDV-2 that was isolated in Australia [33]. Since its initial report, IBRV has caused several outbreaks in Japan and Korea, in which thousands of cattle heads were affected [34].

There is a widespread evidence of EHDV infection in cattle [3,27,35-37]. Viremia and seroconversion were demonstrated in calves that were experimentally inoculated with several serotypes of EHDV, but calves did not develop detectable clinical disease during the course of these experiments [38-42]. Thus, although all ruminants appear to be susceptible to EHDV infection ,with the exception of the Japanese and Korean outbreaks, it was a common belief that EHDV infection in cattle is usually subclinical, but recent reports of EHD outbreaks in dairy cattle has shown otherwise [1-3,30,43-51].

Since 2003 reports of clinical disease in cattle associated with EHDV have increased in some parts of the world. Recent outbreaks of EHD in dairy cattle were reported from the French island of Réunion in 2003 and 2009 [43,44]. Outbreaks appeared in Morocco and Algeria in 2006 [45,46], and in Turkey in 2007 [47]. In all three cases the causative agent was EHDV-6 [18,48]. A strain of EHDV-6 was recently isolated in USA [18,22]. This was parallel to an EHDV associated cattle morbidity that was reported from Kentucky and Ohio on 2007 [49-51].

In the summer and autumn of 2006 the dairy industry in Israel suffered a significant economic loss due to an outbreak of a BT-like disease that affected numerous cattle herds [30,52]. The causative agent was identified as EHDV serotype 7 that prior to the Israeli outbreak was reported only in Australia [29,30]. The southern Jordan River Valley in Israel (part of the Rift Valley) was the primary outbreak region, and the first affected herd was reported on August 28, 2006. By November 2006 the disease outbreak had spread to the northern, southern, and western parts of Israel. The duration of the outbreak was about 2.5 months, with a total of 83 dairy and 22 beef infected cattle herds [30,52,53].

A variety of clinical signs was observed, some of which were similar to those noted in several other hemorrhagic syndromes of ruminants. This included anorexia, reduced rumination, a short-term low fever, weakness and stiff gait, conjunctivitis, congested mucous membranes and many more clinical signs of EHD, with some or all of them reported from each of the infected herds. The most consistent clinical sign observed was reduced milk production, which together with a low mortality rate caused an

estimated loss of 2.5 million USD to the dairy industry [30,52]. This was the first time that EHD was seen in Israel or elsewhere in the Middle East, and an outbreak of the disease did not reoccur in Israel since [30,52]. To date, the vector of EHDV in Israel is still unknown [30].

Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are haematophagous insects that occurs throughout most of the inhabited world, with the exception of Antarctica and New Zealand [1,3]. They are among the world's smallest haematophagous flies measuring up to 3 mm in body length and are generally short-lived, with most of the adult *Culicoides* surviving for fewer than 20 days [3,54]. These midges are considered as abundant vectors for various vertebrate pathogens, but their primary veterinary and economic importance is as vectors of Arboviruses that infect domestic livestock [3,54,55]. More than 1400 *Culicoides* species have been identified globally, of which about 96% are obligate blood suckers, attacking mammals and birds. Only a few are potential vectors, from which more than 50 Arboviruses have been isolated, including 19 viruses of the family *Reoviridae* [3]. Only adult females are blood suckers that feed on vertebrates. Thus, viruses are transmitted from the midges to their hosts only by infected adult females of these vectors species [1,3,55].

The vectorial capacity of a vector species is the relative measure of its population to transmit a pathogen to a host population during a defined time period. This depends on the vector's abundance and seasonal occurrence at location within its geographical distribution, a short generation time, vector survivorship, biting rate, vector competence, and the duration of the extrinsic incubation period (EIP) of the virus [3,55-57]. Climatic variables and the annual cycle of meteorological conditions have a profound impact on some if not all of these criteria [1,3,27,35,55,56,58,59].

Culicoides activity and abundance is often positively correlated with temperature. Low temperature suppresses their biting rate and other activities, although if the temperature exceeds an upper limit, activity will be suppressed as well [3,55,59]. Laboratory studies have shown that higher temperatures

support a faster rate of virogenesis, which corresponds to a shorter EIP, but the survival rate of adult *Culicoides* was diminished [3,55,60]. Humidity is a key factor influencing the midges, and like temperature, there is an optimal humidity range that deviation from it can reduce the activity levels of *Culicoides* and diminish their survival capacity. The exact range of optimal temperature and humidity can vary among species and locations [3,55]. Rainfall is another impacting factor, with both positive and negative influences on *Culicoides* populations. The life cycle of *Culicoides* includes egg, four larval stages, pupa, and imago (the adult form). The pupae of some species do not float on water and will drown if breeding sites are flooded due to rainfall, and the adults' activity of most species is inhibited during the rainy season. On the other hand, the development of the young stages of almost all *Culicoides* requires moisture-rich habitats and the availability of such breeding sites is a key determinant limiting distribution, abundance and seasonal occurrence. It is likely that rain provides more suitable breeding sites, and it has been shown that *Culicoides* abundance and biting rate increases after a rainy season, in particular when rainfall follows a drought [3,27,35,58,61]. Most adult *Culicoides* are crepuscular or nocturnal, therefore activity levels peaks around sunset and sunrise, and may be suppressed due to complete darkness [3,58]. Negative correlations between midges' activity levels and wind speed have been reported and a significant positive correlation is also found between wind speed and midges' mortality rates [3,58].

The above mentioned variables have been shown to have different strengths of effect on different species, and on a single species of *Culicoides* at different locations. For example, different factors have been shown as the major influence on the abundance of *Culicoides imicola*, a well-studied species. In region with a cold season, there is a positive correlation with temperature, whereas in areas where temperatures are more suitable throughout the year, abundances tend to be more strongly related to rainfall [3].

The role of *Culicoides* as biological vectors of bluetongue virus (BTV), the prototype species of the genus *Orbivirus*, has been long documented [2,9]. Du Toit was the first to demonstrate *C. imicola* as the vector of BTV [62]. BTV was later isolated from pools of *C. imicola* in Israel [63,64], several areas in Africa [27,35,58,65,66] and Southern Europe [67-69]. In some of these regions where *C. imicola* is known as the major vector of BTV, the virus was isolated from additional species such as members of the *Culicoides schultzei* group, *Culicoides bolitinos*, *Culicoides milnei* [3,27,35,58,63,70,71], and members of the *Culicoides obsoletus* and *Culicoides pulicaris* complexes that were also found to be the potential vectors in parts of Europe were *C. imicola* is not abundant [69,72,73]. *Culicoides sonorensis* is a known vector in North America, while serotypes in Central and South America are transmitted by *Culicoides insignis* [36,71,74]. *Culicoides wadai* and *Culicoides brevitarsis* were found to be the vectors in Australia [71]. These findings demonstrate the possibility of one virus being vectored by several species at a given location, and that the major vector species can differ between geographic regions according to the abundant vector species at each region.

Several findings suggest that EHDV is also transmitted by *Culicoides*. In North America *C. sonorensis* is a confirmed vector of EHDV-1 and EHDV -2 [36,74-76], and was recently demonstrated as a potential competent vector for EHDV-7 [77]. *Culicoides lahillei* was suggested as a potential vector in locations where *C. sonorensis* is scarce or absent, due to a successful isolations of the virus from orally infected midges of this species [78]. EHDV was isolated in South Africa from *Culicoides cornutus* and *Culicoides nevilli* collected at cattle farms where cases of the disease had been detected [35]. In Australia isolations have been made from *C. brevitarsis* [29,37], in Africa from species within the *C. schultzei* group [27,66,79], and in Japan from *Culicoides oxystoma*, a member of the *C. schultzei* group [4,34,54].

There is a high probability that *Culicoides* biting midges are the vectors for EHDV in Israel. In addition to the above mentioned reports of the association between the virus and a few of these vectors species, characteristics of the EHD outbreak in Israel further supports this assumption [30,53,80].

As already discussed, climatic variables and their annual cycle can impact the seasonal abundance and activity rate of *Culicoides* at a given location. Consequently, the epidemiology of *Culicoides*-borne viral diseases is strongly linked to climate and weather, and transmission is limited to those times of the year when adult insects are active [1,3,58,59,69]. In Israel the annual peak in *Culicoides* abundance and activity occurs at the end of the summer, which corresponds to the outbreak period in 2006 [3,30,53,61].

The flight range of *Culicoides* is short, and the midges' active dispersal is usually only a few hundred meters to 3 km from their breeding sites [3]. However, *Culicoides* are capable of being dispersed passively on prevailing winds over much greater distances and several studies have suggested a relationship between the spread of *Culicoides*-borne viral diseases and wind movements [3,27,81-85]. An epidemiological investigation showed that air movement rather than animal movement is probably the most important factor in the spread of EHDV during the Israeli outbreak [80].

BTV and EHDV are both members of the genus *Orbivirus* and are closely related viruses in their genetic and morphological features, hosts and clinical signs which they cause [1,3,9]. Thus, it is reasonable to assume that they also share the same vectors species. In a serological survey that was conducted in Israel, there was a correlation in the seroprevalence of both viruses in cows, a finding that implies that the transmitting vectors' abundance is influenced by similar environmental conditions or even that both are transmitted by the same vectors [53].

Fifty-eight *Culicoides* species were identified in Israel, the Golan Heights, and Sinai [4]. There are several suspects as EHDV vectors in Israel, in particular *C. imicola,* a major vector species of BTV in Israel [64], and *C. oxystoma*, a suspected vector of BTV [4,63]. These two *Culicoides* species are blood-

sucking midges that feed on mammals, and both are abundant in Israel, particularly in livestock farms [4,57].

C. imicola is a widely distributed Afrotropical species that extends throughout much of Africa, East, South and Southeast Asia, and many of the countries in the Middle East. It is also found in Western, Eastern and Southern Europe [3,4,54]. In addition to the above mentioned role as a vector of BTV, *C. imicola* is a proven and most important vector of African horse sickness virus (AHSV) in Africa and the Middle East [3,4,54,62,65,86]. It is also a suspected vector of a few other Arboviruses, due to their isolations from these insects [3,4]. This includes the Akabane virus (AKAV) [65,87,88], bovine ephemeral fever virus (BEFV) [65] and equine encephalosis virus (EEV) [89,90].

It has been demonstrated that *C. imicola* may be a competent vector of EHDV. In a study conducted in South Africa, EHDV was recovered from *C. imicola*, *C. bolitinos* and other *Culicoides* species after an oral infection with eight serotypes of the virus [91]. BTV was isolated from field-collected *C. imicola* and *C. bolitinos* in South Africa [35], and midges of these two species were also found to be susceptible to oral infection with several serotypes of BTV [60,92-94]. These findings indicate that EHDV and BTV can share common vectors at the same region, which is further supported by the confirmed common vector of both viruses in North America (i.e. *C. sonorensis*) [36,71,74-76]. Therefore, this may also be true for *C. imicola* in Israel.

C. oxystoma is a member of the *C. schultzei* group, and it is a common species in the Mediterranean area [4]. Its known distribution includes East and Southeast Asia, Australia, the Middle East, Greece and Russia [34,54,95]. The medical and veterinary importance of this species is not well defined, but it is assumed to be an important vector of Arboviruses, especially in Japan [4,34,54]. Viruses that were isolated from midges of this species include BTV, AKAV, Aino virus (AINOV), IBAV and members of the Palyam serogroup (Kasba and D'Aguilar viruses) [4,34,96-99].

Although the role of *C. oxystoma* as a vector species in Israel is not yet determined, as already mentioned, it is a suspected field vector of BTV [4,63], and laboratory infection trial demonstrated that it is indeed a potential vector of this virus [100]. As for EHDV, the involvement of *Culicoides* species within the *C. schultzei* group in its transmission has been implied. In Sudan, a member of the EHD serogroup was isolated from a pool of *Culicoides kingi*, another member of the *C. schultzei* group, and in a later study EHDV-4 was isolated from midges within this group [27,66]. EHDV was also isolated from members of the *C. schultzei* group in Nigeria [79]. Ibaraki virus was isolated from *C. oxystoma* in southern Japan, and the isolation showed correlation with serological data and reported cases of disease in cattle, suggesting that *C. oxystoma* is a potential vector in Japan [34]. In a study conducted in Israel, molecularly identified *C. oxystoma* was found to be closely related to *C. oxystoma* from Japan [57].

Taking all the above under consideration, we believe that *C. imicola* and *C. oxystoma* could be the biological vectors for EHDV in Israel. Therefore, the present study was conducted in order to evaluate the susceptibility of field-collected midges of these two species to an oral infection with EHDV-7, which was isolated during the Israeli outbreak in 2006.

Vector competence is the ability of a vector to acquire a pathogen, support its replication and successfully transmit it to a susceptible host [59]. Therefore, in order to function as field biological vector of an *Orbivirus*, the midge must take a viremic blood meal from a host, and the virus must go through an extrinsic incubation period (EIP), during which it infects and replicates in the midge's mid-gut cells, disseminates through the haemocoel and subsequently infects the salivary gland cells. There, the virus replicates and released in the saliva. Only then the EIP is completed and the virus can be transferred to a new naïve host, during the infected midge's next blood meal [3,55]. Thus, the demonstration that field-collected *C. imicola* and *C. oxystoma* are susceptible to oral infection with EHDV is an essential step in the determination of EHDV's vectors in Israel, and in the understanding of

the transmission and epidemiology of an emerging virus with primary economically significant to the Israeli dairy industry.

Basic hypotheses

Biting midges of the species *C. imicola* and *C. oxystoma* that are fed with cattle blood-EHDV-7 mixture will be positive for the virus after 11-16 days of post-infection incubation.

The research goal

Primary goal:

Demonstrating the ability of *C. imicola* and *C. oxystoma* females to acquire EHDV-7 through a blood-virus meal, undergo an infection with the virus and carry it for an extrinsic incubation period of 11-16 days (a period during which the virus is assumed to arrive the salivary glands) [3,55,77,91].

Secondary goal:

Establishing an efficient experimental protocol that will enable field-collected midges to feed on a blood-virus meal and survive incubation under laboratory conditions, and will enable the isolation of the virus from infected midges.

Materials and methods

Research plan

Laboratory experiment.

Research population

Field collected females of *C. imicola* and *C. oxystoma* during the summer and autumn of 2011-2012.

Research variables

Outcome variable: Prevalence of successful infection.

Independent variable: *Culicoides* species.

Tissue culture

Two tissue culture cell lines were employed in this study: Baby Hamster Kidney (BHK-21) cells and Cow pulmonary artery endothelia (CPAE) cells [courtesy of Elizabeth Howerth, University of Georgia, USA]. CPAE cell lines were used for virus proliferation, virus stock quantification and virus isolation *in vitro*. BHK-21 cell lines were used for virus isolation *in vitro*.

CPAE cells were grown in Minimal Eagle Medium (MEM) medium [Bioind, Kibbutz Beit Haemek Israel. cat.no. 01-025-1A] supplemented with 10% Fetal Calf Serum (FCS) [Bioind, Kibbutz Beit Haemek Israel. cat.no. 04-121-1A], 1% L-Glutamine [Bioind, Kibbutz Beit Haemek Israel. cat.no. 03-020-1B] and 1% Penicillin-Streptomycin-Amphotericin [Bioind, Kibbutz Beit Haemek Israel. cat.no. 03-033-1B].

BHK cells were grown in MEM medium [Bioind, Kibbutz Beit Haemek Israel. cat.no. 01-025-1A] supplemented with 10% Triptose phosphate broth [Sigma, St. Louis MO USA. cat.no. T9157], 3% FCS [Bioind, Kibbutz Beit Haemek Israel. cat.no. 04-121-1A], 1% L-Glutamine [Bioind, Kibbutz Beit

Haemek Israel. cat.no. 03-020-1B], and 1% Penicillin-Streptomycin-Amphotericin [Bioind, Kibbutz Beit Haemek Israel. cat.no. 03-033-1B].

Both cells lines were grown in a standard tissue culture incubator at 37°C and 5% CO_2.

Virus proliferation

For virus proliferation the strain of EHDV-7 that was isolated during the 2006 outbreak in Israel was used [courtesy of Hagai Yadin, Israel veterinary institute]. Stocks of the virus for oral infection of the midges were grown in CPAE cells. CPAE cells were inoculated with EHDV-7 at 50-70% confluence, and monitored daily for cytopathic effect (CPE). Once CPE was noticed, the cell culture media containing the virus stock was stored at 4ºC.

Virus stocks quantification

Virus stocks were quantified using plaque assay according to Shai et al. [101]. Serial dilutions, in 1180 µl of Dulbecco's Modified Eagle Medium (DMEM) medium [Bioind, Kibbutz Beit Haemek Israel. cat.no. 01-050-1A] supplemented with 1% L-Glutamine [Bioind, Kibbutz Beit Haemek Israel. cat.no. 03-020-1B] and 1% Penicillin-Streptomycin-Amphotericin [Bioind, Kibbutz Beit Haemek Israel. cat.no. 03-033-1B] were used. CPAE cells were seeded in a 6 well-plate format [NUNC, Roskilde, Denmark. cat.no. 140685] and were inoculated with the virus dilutions at ~ 60-70% confluency. 10^{-3} - 10^{-8} dilutions of the virus stocks were used, 2 wells per dilution. Two wells were used as control, and were supplement only with media. The cells were incubated at 37ºC and 5% CO_2 for 45 min, then washed and overlayed with 0.8% tragacanth [Sigma-Aldrich, St. Louis, Mo, USA cat.no. G1128] in MEM. The cells were incubated at the same conditions and monitored daily for plaques formation. Virus titer (PFU/ml) was calculated according to number of plaques/dilution factor.

Insect collections

Insects were collected during the summer and autumn 2011-2012 using UV-light traps with a suction fan [John W. Hock Company, Gainesville, FL, USA, model 1212]. The traps were situated at the Netzer Sereni's horse farm (31˚55'20"N, 34˚49'21"E, at an altitude of 77 m a.s.l) and placed 1.5-2 m above the ground. The traps were turned on before sunset and insects were collected overnight into organic cups covered with nylon stocking (figure 1). The insects were transferred to the laboratory the next morning and the organic cups were used as incubation cages of the midges for the entire duration of the experiment.

Figure 1. UV-light and suction fan trap system. Traps were used for the collection of insects into organic cups covered with nylon stocking. The traps were turned on before sunset and insects were collected overnight.

Feeding technique

Upon arrival to the laboratory the insects were starved for 24 h (with an ample water supply) and kept at the laboratory standard incubation conditions of darkness, 25ºC-26°C and relative humidity of 50-55%, followed by blood feeding.

Cattle blood was collected in heparin tubes [BD, UK cat.no. LH170 I.U]. To avoid a specific EHDV antibody reaction the blood plasma was removed, red blood cells (RBCs) were washed three times with Phosphate buffered saline (PBS) [Bioind, Kibbutz Beit Haemek Israel. cat.no. 02-023-1A] and supplemented with Fetal Calf Serum (FCS) [Bioind, Kibbutz Beit Haemek Israel. cat.no. 04-121-1A].

To do so, the blood was centrifuged at 3000g for 8 min at room temperature and the plasma volume (PV) was measured. The plasma was removed. The red blood cells were washed three times using a PV equivalent of sterile PBS. During each wash the RBCs were mixed with PBS and centrifuged at 3000g for 5 min at room temperature. Then, the RBCs were supplemented with a PV equivalent of FCS, vortexed and immediately used or, at most, during the next 24 h, in which case they were stored at 4ºC.

Midges were fed with one of two possible blood based feeding media: the first containing a 1:5 virus stock to blood ratio (respectively) supplemented with 0.2 gr/ml sucrose powder [Biolab, Jerusalem, Israel. cat.no. 19220591] and the other containing a 1:2 virus stock to blood ratio (respectively) supplemented with 0.4 gr/ml sucrose powder (dissolved). This yield one of two possible mean virus concentrations in the feeding media: $10^{4.6}$ or $10^{5.0}$ PFU/ml, respectively. Each cup constraining the trapped midges (as well as other insects) was fed by soaking up dental cotton rolls, [Dental Depot WOHL, Nesher, Israel. cat.no. 004943] as previously described [102], with a total of 9 ml of warmed (at 40-41ºC) and vortexed blood based feeding media, and placing them on top of the nylon stockings (figure 2). The midges were allowed to feed for 2 h under the laboratory

Figure 2. Dental cotton rolls feeding technique
The midges are constrained within organic cups covered with nylon stocking. Dental cotton rolls are soaked with a total of 9 ml per cup of cattle's blood media and placed on top of the nylon stockings.

incubation conditions. During blood-feeding the insect containing cup was coated with aluminum foil.

In order to verify the efficiency of the feeding technique, prior to the feeding trials described above, feeding trials were conducted without adding the virus. Midges were fed with blood supplemented with 0.2 gr/ml sucrose. For the first 3 trials, midges were able to feed for 45-60 min, and for the rest of the trials the midges could feed for 2 h.

Insects sorting

Post feeding the insects were anesthetized with CO_2 and sorted out under binocular microscope according to the following:

1. Separating midges from other insects.
2. Separating the male and female midges according to their antennae and genitalia [54].
3. Separating *C. imicola* females from *C. oxystoma* females according to wing pattern [54].
4. Separating blood engorged midges for future experimentation according to the red color of undigested blood in the abdomen of fed insects (figure 3).

Blood fed midges were analyzed for presence of the virus. Midges were taken at 0, 3-4, 11-12 and 15-16 days post feeding (DPF) [55,77]. For the duration of the experiment, midges were incubated under standard laboratory conditions and were provided with 10% sucrose solution via soaked cotton wool that was placed on top of the nylon stockings. The insects were monitored daily and dead insects were removed. Only live insects were taken for analysis.

The females were counted according to species, and for each species according to blood fed, not blood fed and number of days they survived in incubation post-feeding.

Virus isolation

Midges taken for viral isolation were grinded individually at the predetermined DPF in transport media, as described by Ruder et al. [77]. Transport media was prepared by increasing the CPAE growth medium antibiotics concentration to 5%.

Each insect was placed into an individual 2ml sterile eppendorf tube containing 500ul of virus transport media. The tube was prefilled with 1ml of 3 and 4mm glass beads [Hatzvi, Rosh-Haayin Israel. cat.no. 370-35-004]. The tube was vortexed on a specially designed vortex wrack, resulting in an efficient grinding of the insect. The ground insect extract was briefly centrifuged at 1,500g for 12 min at 4°C and the clear supernatant was transferred into a fresh tube and stored at 4ºC.

BHK-21 cells were grown in a 24 well-plate format [NUNC, Roskilde, Denmark. cat.no. 142475] to 50-70% confluence before inoculation with the midge extract. Inoculation was performed by adding 200µl of the clear midge extract and 1ml fresh MEM medium containing 5-7% antibiotics. On day 5 post inoculation the cells from each well were transferred to a well in a 6 well-plate [NUNC, Roskilde, Denmark. cat.no. 140685]. On day 10 cell culture media supernatant was transferred to a CPAE cell culture plate. The CPAE cells were previously grown in a 24 well-plate format to 50-70% confluence before inoculation and inoculation was performed using 200µl midge inoculated BHK medium and 1ml fresh medium. The CPAE cells were monitored daily for CPE.

As a control, midges that were fed with feeding media that did not contain the virus were incubated and analyzed at 0, 3-4, 11-12 and 15-16 DPF as described for the infected midges.

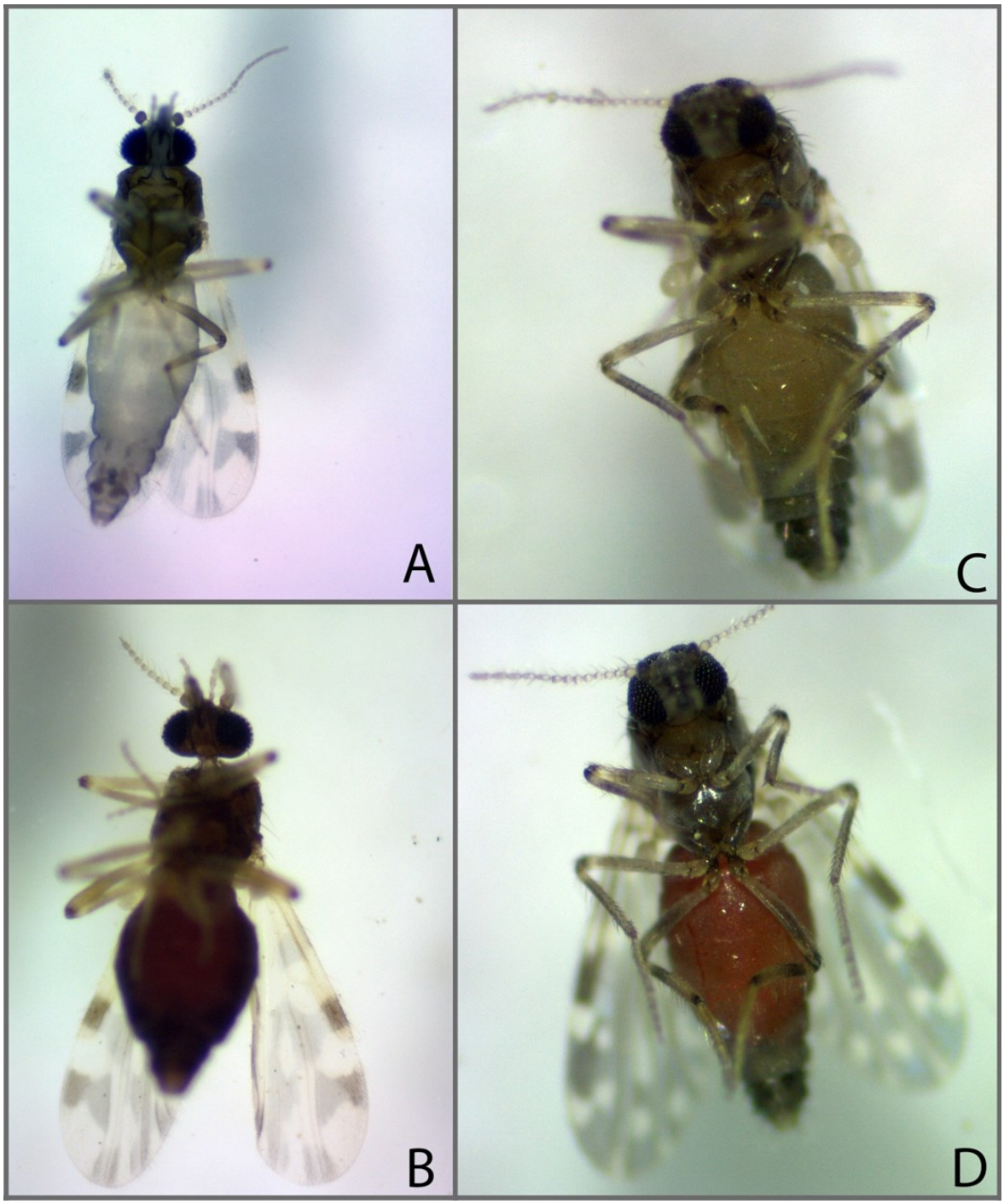

Figure 3. Engorged and non-engorged *Culicoides*.

Blood engorged midges are distinguish from non-engorged midges by the red blood color in their abdomen. The two species are recognized by their wing pattern. **A**: non-engorged *C. imicola*; **B**: engorged *C. imicola*; **C**: non-engorged *C. oxystoma*; **D**: engorged *C. oxystoma*.

Statistical analysis

Feeding rates as well as survival rates at each time point and infection rates were calculated for both *C. imicola* and *C. oxystoma* and compared using the Chi square test (χ^2) for each trial. Results of all the trials were combined by using the Mantel Heanszal Chi square test. P-value smaller than 0.05 was considered statistically significant. All analyses were performed using WinPepi statistical software [103].

Results

Virus proliferation

Preliminary trials were performed with CPAE cells, Ovine Kidney cells (OKC) and Vero cells lines. Each cell line was grown in a 24 well-plate format to 50-70% confluence before inoculation with EHDV-7, and then monitored daily for CPE. Four to five days post inoculation CPE was noticed for CPAE, while OKC and Vero cells have grown to 100% confluence, and the transfer to a 6 well-plate was necessary before CPE was noticed. CPAE cells were more suitable for our purposes due to slower growth rate and higher sensitivity to the virus, and therefore were the cells that were employed in this study.

Virus stocks quantification

Gel plaques were apparent 4-5 days post infection in the wells that contained cells that were inoculated with 10^{-3}-10^{-5} dilutions and were absent in all other wells. Virus titer was calculated according to the 10^{-4} dilution. The average for these two wells was 18 plaques; therefore the virus concentration of our stock was $2.0\times10^{5.0}$ PFU/ml.

Insect collection

Our study required live midges and delivery of the midges to the incubation conditions at our lab in a short time period was essential for their survival. Therefore, we chose a collection site that is in close proximity to our lab that is located at the Koret School of Veterinary Medicine, The Robert H. Smith Faculty of Agriculture, Food and Environment, Rehovot, Israel. A few possible locations were considered and we chose the Netzer Sereni's horse farm because the number of midges per trap was the highest. Traps were initially located at 5 additional livestock farms, and most trappings yield les then 20 midges per trap. All of these farms are highly active at most hours of the day; hence lighting systems were turned on from around sunset and throughout most of the night. This was not the situation at the Netzer Sereni's horse farm. At this location an average of 80 midges per trap were viable at the time of their post-feeding sorting.

After each feeding trial the midges were sorted and the females were counted as described.

A total of 326 *C. imicola* and 1838 *C. oxystoma* were counted post-feeding. This excludes dead midges at the time of their sorting, but the relative number of midges that were dead at this time was higher for *C. oxystoma*. Therefore, even with including dead midges, *C. oxystoma* were about 5-fold more abundant.

Insect feeding and sorting

We first conducted feeding trial without adding the virus. At a feeding duration of 45-60 minutes less than 5% of the midges were engorged (data not shown). Therefore, we conducted additional trials in which the midges could feed for 2 h. At this duration the feeding rate ranged between 29% to 67% for *C. oxystoma* and 25% to 60% for *C. imicola* with an overall average of 51% and 41% engorged *C. oxystoma* and *C. imicola,* respectively (table 1). No significant difference was found between the two species' feeding rate ($\chi^2 = 2.679$, d.f. = 1, P = 0.102).

Once a valid feeding method was established, we conducted trials in which the midges were fed on blood-virus mixture, and were able to feed for 2 h.

We first fed the midges with a virus:blood mixture at a ratio of 1:5. We also conducted feeding sessions with virus:blood mixture at a ratio of 1:2, in order to examine whether it will improve our results regarding CPE positive wells. The virus concentration in the stock used was $2.0\times10^{5.0}$ PFU/ml, thus the mean virus concentration in the blood meal was either $10^{4.6}$ or $10^{5.0}$ PFU/ml, respectively.

When fed upon feeding media with a mean virus concentration of $10^{4.6}$ PFU/ml, an average of 42% *C. oxystoma* and 43% *C. imicola* were engorged, with feeding rates ranging between 32% to 59% and 20% to 100%, respectively. When fed upon feeding media with a mean virus concentration of $10^{5.0}$ PFU/ml, an average of 28% *C. oxystoma* and 36% *C. imicola* were engorged, with feeding rates ranging between 21% to 32% and 13% to 80%, respectively. No significant difference was found between the two species' feeding rate for both of the feeding media (table 2) (χ^2 = 0.977, d.f. = 1, P = 0.323; χ^2 = 2.919, d.f. = 1, P = 0.088, respectively).

For *C. oxystoma*, the average of engorged females was the highest when fed upon feeding media that was not mixed with virus suspension, lower when fed on virus:blood mixture at a ratio of 1:5, and the lowest for virus:blood mixture at a ratio of 1:2 (51%, 42% and 28%, respectively). There was a statistically significant difference in the rate of engorged females when comparing between feeding trials with virus free to 1:5 virus: blood feeding media (P = 0.002), and 1:2 virus: blood feeding media (P < 0.001), and between 1:5 and 1:2 virus: blood feeding media (P < 0.001).

For *C. imicola*, the average of engorged females did not differ in the same manner when fed upon feeding media that was not mixed with virus suspension, on virus:blood mixture at a ratio of 1:5, and virus:blood mixture at a ratio of 1:2 (41%, 43% and 36%, respectively). There was no statistically significant difference in the rate of engorged females when comparing between feeding trials with virus

free to 1:5 virus:blood feeding media (P = 0.889), and 1:2 virus:blood feeding media (P = 0.493), and between 1:5 and 1:2 virus:blood feeding media (P = 0.329).

Midges were monitored for survival rate up to the day they were processed. For all of the incubation trials over 80% of the mortality occurred at the first 24 h post sorting. The overall survival rate of the midges following the extrinsic incubation period of 11-16 days was comparable to survival rates after 3-4 incubation days and ranged from 25% to 73% and an average of 51% for *C. oxystoma*, and 29% to 100% and an average of 53% for *C. imicola*. For each of the trials, there was no statistically significant difference in the survival rate between *C. imicola* and *C. oxystoma* (table 3).

Table 1. Feeding rates of *C. oxystoma* and *C. imicola* fed with cattle blood, not containing the virus.

Trial no.	% blood fed (no. fed/n)		P-value	χ^2 (d.f.=1)[a]	Estimated Odds Ratio	CI (95%)[b]
	C. oxystoma	*C. imicola*				
1	54 (7/13)	32 (10/31)	0.309			
2	40 (48/120)	25 (1/4)	1.000			
3	29 (21/73)	33 (3/9)	0.717			
4	67 (48/72)	60 (6/10)	0.729			
5	56 (60/108)	33 (2/6)	0.409			
6	62 (31/50)	50 (2/4)	0.638			
7	63 (19/30)	50 (2/4)	0.627			
8	50 (24/48)	43 (3/7)	1.000			
9	63 (20/32)	60 (9/15)	1.000			
10	45 (9/20)	35 (7/20)	0.748			
total	51 (287/566)	41 (45/110)	0.102	2.679	1.224	0.95 - 1.58

[a] d.f. = degree of freedom; [b] CI = Confidence Limits.

Table 2. Feeding rates of *C. oxystoma* and *C. imicola* fed with cattle blood: EHDV-7 mixture with one of two possible virus concentrations.

Trial no.	Mean virus concentration in blood meal (PFU/ml)	% blood fed (no. fed/n)		P-value	χ^2 (d.f.=1)[a]	Estimated Odds Ratio	CI (95%)[b]
		C. oxystoma	*C .imicola*				
1	$10^{4.6}$	32 (16/50)	20 (5/26)	0.288			
2	$10^{4.6}$	43 (41/95)	25 (2/8)	0.463			
3	$10^{4.6}$	40 (56/139)	46(11/24)	0.657			
4	$10^{4.6}$	59 (22/37)	100 (2/2)	0.514			
5	$10^{4.6}$	43 (60/141)	67 (10/15)	0.101			
6	$10^{4.6}$	42 (25/59)	67 (4/6)	0.395			
7	$10^{4.6}$	37 (48/130)	45 (9/20)	0.621			
total	$10^{4.6}$	42 (268/641)	43 (43/101)	0.323	0.977	0.881	0.70 - 1.12
8	$10^{5.0}$	23 (3/13)	32 (10/31)	0.722			
9	$10^{5.0}$	21 (30/142)	25 (4/16)	0.750			
10	$10^{5.0}$	32 (16/50)	80 (4/5)	0.053			
11	$10^{5.0}$	27 (22/85)	13 (1/8)	0.674			
12	$10^{5.0}$	28 (9/32)	50 (6/12)	0.284			
12	$10^{5.0}$	32 (55/170)	32 (8/25)	1.000			
14	$10^{5.0}$	28 (39/139)	44 (8/18)	0.176			
total	$10^{5.0}$	28 (174/631)	36 (41/115)	0.088	2.919	0.761	0.56 - 1.03

[a] d.f. = degree of freedom; [b] CI = Confidence Limits.

Virus isolation

Midges were taken for analyzes at 0, 3-4, 11-12 and 15-16 DPF. Days were grouped together as the rate of positive virus isolations did not differ between them. Blood fed midges were analyzed for presence of the virus by inoculations of CPAE cells with midge inoculated BHK medium. The usage of

1 BHK cells was due to our finding that when CPAE cells were inoculated directly with midges extract,
2 they did not survive the first 24 h.

Table 3. Survival rates of *C. oxystoma* and *C. imicola* following a post-feeding incubation period of 3-16 days.

Mean virus concentration in blood meal (PFU/ml)	Processing day (DPF)[a]	% survival until processing day (no. survived/n)[b]		P-value
		C. oxystoma	*C. imicola*	
$10^{4.6}$	3	62 (23/37)	50 (1/2)	1.000
$10^{4.6}$	4	50 (9/18)	75 (3/4)	0.594
$10^{4.6}$	11	73 (27/37)	71 (5/7)	1.000
$10^{4.6}$	12	53 (20/38)	57 (4/7)	1.000
$10^{4.6}$	15	58 (26/45)	60 (3/5)	1.000
$10^{4.6}$	16	25 (16/65)	29 (2/7)	1.000
$10^{5.0}$	3	65 (17/26)	75 (3/4)	1.000
$10^{5.0}$	4	57 (4/7)	50 (1/2)	1.000
$10^{5.0}$	11	57 (4/7)	100 (2/2)	0.500
$10^{5.0}$	12	71 (22/31)	57 (4/7)	0.656
$10^{5.0}$	15	40 (22/55)	50 (4/8)	0.707
$10^{5.0}$	16	57 (20/35)	50 (3/6)	1.000
–[c]	4	56 (9/16)	100 (2/2)	0.497
–[c]	12	60 (12/20)	50 (3/6)	1.000
–[c]	16	56 (5/9)	43 (3/7)	1.000

[a] DPF = days post-feeding.

[b] not including midges that did not recover from the anesthesia preformed after feeding in order to sort out the midges.

[c] control groups.

3 Post inoculation, CPAE cells were monitored for CPE. The number of seeded wells that showed CPE
4 was counted according to *Culicoides* species, the 4 time groups and to the two virus concentration in the

blood meal. If CPE was not noticed within 5 days from inoculation, the well was considered negative for the virus. When comparing between the two virus concentration in the blood meal for each of the time groups, there was no significant difference in virus recovery rates form cells inoculated with *C. oxystoma* extract (P = 0.130, P = 0.223, P = 0.290, P = 0.097 for 0, 3-4, 11-12 and 15-16 DPF, respectively). Therefore, virus recovery prevalence's at each time group were combined for the two virus concentration in the blood meal (table 4).

Table 4. Prevalence of positive virus isolation.

	% CPE-positive cell-wells inoculated with midges extract (no.positive/n)	
DPF [a]	*C. oxystoma* fed with a mean virus concentration of $10^{4.6}$ or $10^{5.0}$ PFU/ml	*C. imicola* fed with a mean virus concentration of $10^{4.6}$ or $10^{5.0}$ PFU/ml
0	63 (20/32)	63 (10/16)
3-4	43 (12/28)	100 (3/3)
11-12	35 (7/20)	67 (2/3)
15-16	60 (15/25)	75 (3/4)

[a] days post-feeding.

Each midge that was used for inoculation of cells that resulted in a CPE-positive CPAE cells' well was considered as positive for EHDV. For *C. oxystoma*, There was no significant difference in virus recovery rates when comparing between 0 DPF to 3-4, 11-12 and 15-16 DPF (P = 0.195, P = 0.087, P = 1.000, respectively), between 3-4 to 11-12 and 15-16 DPF (P = 0.766, P = 0.275, respectively) and between 11-12 and 15-16 DPF (P = 0.136). Eleven to 1 and 15-16 DPF were examined as two possible EIPs. As there was no significant difference in the prevalence of positive virus isolation between these

two testing times, the results for these two time groups are combined (figure 4). Due to the low number of *C. imicola* it is difficult to discuss differences in the results associated with each feeding regime and incubation duration. For the graphic illustration results for *C. imicola* are combined in the same manner as for *C. oxystoma* (table 4 and figure 4).

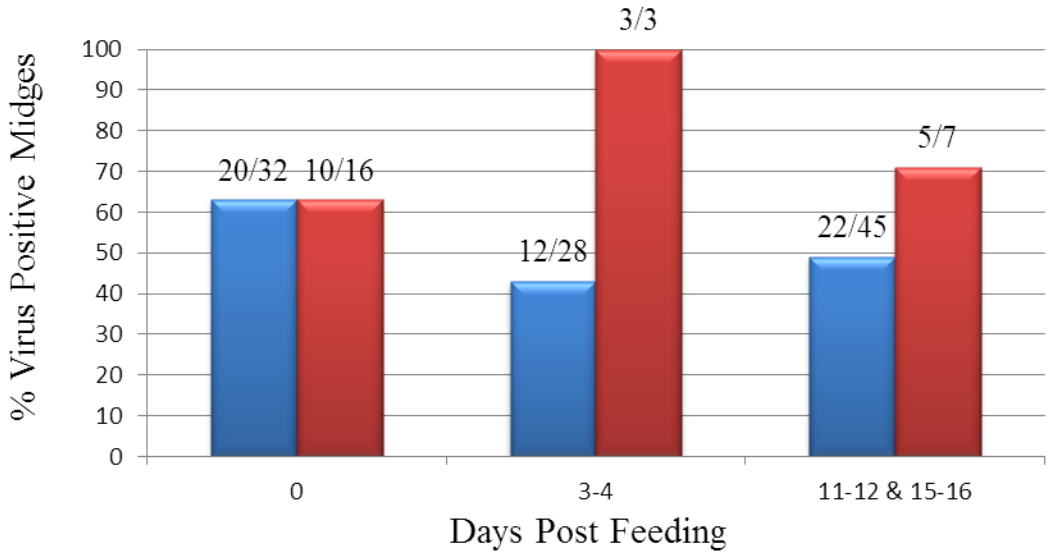

figure 4. virus positive *C. oxystoma* and *C. imicola* after an oral infection with EHDV-7.
The bars show the rate of midges that were positive for the virus (no. positive/no. tested x 100) acording to three time groups of processing day post feeding and *Culicoides* species
Numbers above bars represent the number of positive midges/number of blood fed midges processed at each category.
blue bars = *C. oxystoma* fed with mean virus concentration of $10^{4.6}$ or $10^{5.0}$ PFU/ml.
red bars = *C. imicola* fed with mean virus concentration of $10^{4.6}$ or $10^{5.0}$ PFU/ml.

The gap between the numbers of midges surviving until processing day to the number of results shown at table 4 is due to a high contamination rate with mold. A total of 59% (228/384) midge inoculated BHK medium were not used for inoculation of CPAE due to contamination.

For midges that were fed with feeding media that did not contain the virus, no virus was recovered form cells inoculated with extract of *C. oxystoma* or *C. imicola* that were analyzed at 0 DPF (0/6 and 0/3, respectively), at 3-4 DPF (0/4 and 0/2, respectively) and at 11-12 or 15-16 DPF (0/8 and 0/4, respectively). For all of the inoculation trials that were performed with midge inoculated BHK medium of midges that fed on feeding media containing virus suspension, CPE was noticed in some of the seeded wells. Prevalence of CPE-positive cell wells inoculated with extract of midges incubated for 3-16 DPF ranged from 35% to 60% for *C. oxystoma* and 67% to 100% for *C. imicola* and an average of 47% and 80%, respectively. 49% and 71% positive isolations were made from *C. oxystoma* and *C. imicola*, respectively, incubated for 11-12 and 15-16 days.

Discussion

Our findings demonstrate that *C. imicola* and *C. oxystoma* are susceptible to oral infection by EHDV-7, as evidenced by the ability of the midges to become infected following ingestion of a blood meal containing the virus. Virus was isolated from 47% *C. oxystoma* and 80% *C. imicola* analyzed after an incubation period of 3-16 days post feeding. Amongst the midges that survived an assumed extrinsic incubation period (EIP) of 11-12 or 15-16 days, the virus was isolated from 49% *C. oxystoma* and 71% *C. imicola*.

Insects were collected during the summer and autumn of 2011-2012 at the Netzer Sereni's horse farm. This was the final trapping location chosen after several other locations were excluded due to very low numbers of midges in each of the collection attempts. Light traps operate less efficiently when alternative sources of light are present [3]. Although not statistically verified, we believe that the unsuccessful collections were due to such light sources that were activated during collection attempts at these additional locations but not at Netzer Sereni's horse farm. *C. oxystoma* females were far more abundant in the traps then *C. imicola* females. High abundance of a vector species is an important component in its vectorial capacity [3,55-57]. However, this should not be interpreted as the relative abundance of these species as several studies have postulated that the midges' populations seen in traps do not necessarily represent the situation at the collection site [104-108]. Moreover, only one collection site was employed, and previous studies had demonstrated that both species are abundant at various locations in Israel [4,57].

Trapped insects were transferred to the laboratory where feeding trials were conducted. After each feeding trial the insects were sorted, and the identification of female midges of the desired *Culicoides* species was based on morphological parameters. We distinguished between females and males according to their antennae and genitalia [54]. As there is no published evidence to suggest venereal or

transovarial transmission of Arboviruses through *Culicoides,* it is believed that midges acquire viruses only by feeding on a viremic host [1,3,55]. In their natural environment only adult females are known to feed on vertebrates, thereby males do not take part in Arboviruses' transmission to susceptible hosts [1,3]. Thus, it was essential that only females will be included in our assessments. Furthermore, as opposed to the condition in the field, after the midges were fed males were found to be engorged as well. Thereby, wrongfully including them in the inoculation of cell lines would have yield higher numbers of virus recovery immediately after feeding and would have also resulted in a lower percentage of positive midges later on assuming that males do not harbor the virus. This enlightens the importance of differentiating females from males and that one should not automatically assume that engorged midges post a feeding trial are females.

The morphological identification of *C. imicola* and *C. oxystoma* was according to their wing-spot pattern [54]. The wings of *C. imicola* are pale with darker markings and it is quite easy to identify and differentiate these midges from other *Culicoides* species [54,57]. *C. schultzei* group consists of several species, one of which is *C. oxystoma* [4,54]. The wings of *C. oxystoma* and other species within the *C. schultzei* group are grayish with pale markings and differentiating between species of this group is considered as a difficult task that requires some expertise [54,57]. Therefore, the usage of morphological characteristics alone for the identification of *C. oxystoma* may be considered as questionable. With that said, a molecular identification study that was conducted in Israel supports our morphological identification. Midges were collected at different climatic regions throughout Israel using the same traps as in our study. One of those regions was the Mediterranean region, where Netzer Sereni is located. Furthermore, Netzer Sereni was one of the trapping locations used. All the midges that were collected at the Mediterranean region and were morphologically identified as members of the *C. schultzei* group were molecularly identified as *C. oxystoma*. Other members of the *C. schultzei* group that were molecularly distinguished from *C. oxystoma* were collected from settlements located outside of the

Mediterranean region of Israel. Furthermore, the identification of *C. imicola* by morphological features was also verified by molecular methods [57].

The feeding rates and the survival rates of the midges were calculated in order to evaluate the efficiency of the dental cotton rolls feeding technique and of the chosen incubation conditions, respectively. To the best of our knowledge there are no published data of *C. oxystoma*'s feeding and survival rates, but according to our results they are comparable to that of *C. imicola*, as no significant difference was found between the feeding and survival rates of the two species (tables 1-3).

The feeding rates were measured by the proportion of engorged female midges immediately after a feeding trial. When fed upon blood that was not mixed with virus suspension the feeding rates ranged between 25% to 67% with an average of 51% for *C. oxystoma* and 41% for *C. imicola* (table 1). A common method that is successfully used for oral infection of *Culicoides* with Arboviruses is the skin membrane blood-feeding technique [109]. Several studies have each reported feedings rates of 10% to 70% using this technique [86,90-93]. In all of these studies *C. imicola* was one of the *Culicoides* species that were fed. Although we used a different feeding technique we found it to be efficient as the range of the feeding rates was comparable to these reports. However, it might be less efficient as the duration of each feeding trial was 2 h, and in the above mentioned studies midges were able to feed only for 30-45 min, a duration that was found to be too short in our feeding trials. In order to infect the midges with the virus they were fed with one of two possible blood based feeding media, the first containing a mean virus concentration of $10^{4.6}$ PFU/ml and the second of $10^{5.0}$ PFU/ml. because we used the same virus stock for all feeding trials, the higher concentration was achieved by adding a larger volume of the virus suspension per ml of blood. In the field female *Culicoides* ingest a range of liquid foods including blood, sugars, water, and nectar [3]. One of our concerns was that the cell culture media containing the virus stock, when added to blood, would reduce feeding rates as it is not part of the natural food of the midges.

Indeed, although the feeding rates did not deviate below the reported ranges, a reduction was noticed (table 2). There was no reduction in the average feeding rate of *C. imicola* when fed with feeding media containing virus concentration of $10^{4.6}$ PFU/ml, and a reduction did occur when fed with the feeding media containing the larger volume of virus suspension, although not a significant one. As for *C. oxystoma*, there was a significant reduction in the average feeding rate in both of the feeding media when comparing to blood without virus suspension. Furthermore, when fed upon feeding media containing the larger volume of virus suspension, the highest feeding rate was only 32%, and the reduction in the average feeding rate was also significant when compared to feeding media containing the lower volume of virus suspension.

The overall survival rate of the midges following the assumed EIP of 11-16 days ranged from 25% to 73% with an average of 51% for *C. oxystoma*, and from 29% to 100% with an average of 53% for *C. imicola* (table 3). For all of the incubation trials over 80% of the mortality occurred within the first 24 h post feeding and we consider these midges as those who did not recover well from the anesthesia and sorting procedures. This may be expected as these midges are very small and fragile. Venter et al. [93] reported very low survival rate of *C. imicola* during feeding attempts ranging from 0.3 to 54.1% which corresponds with our results. Survival rate of the midges can depend on the incubation condition provided, in particular temperature and relative humidity, and on the incubation duration [3,55]. Several studies have reported survival rates of field-collected *Culicoides* species, including *C. imicola,* that were incubated for 10 days at 23.5ºC and 50% to 70% relative humidity, and the survival rates reported varies between these studies [86,90-93,110]. Among these studies the lowest overall survival rate was 15.1% [86] and the highest 66.3% [92], with the lowest rate for a single batch being 12.6% [86] and the highest 83.3% [90]. Here, both the 25% and 29% survival rates of *C. oxystoma* and *C. imicola*, respectively, where observed at 16 DPF. For 11 DPF, the lowest rate was for *C. oxystoma*, and it was 57%. Therefore, we consider the survival rate as relatively high when comparing with other studies. Thus, the higher

temperature of 25ºC-26ºC used in our study in order to improve virogenesis did not seem to diminish the midges' survival. Moreover, the relative humidity in which we incubated the midges did not exceed 55%, and it is possible that a range of 50%-55% relative humidity is better suited for the midges. However, the optimal incubation conditions that will yield higher survival rates can differ between *Culicoides* populations of a single species that were collected at different locations [3], and even between collection batches at a single site [90,93]. Therefore, it is possible that the incubation conditions used here would not yield high survival rates for *C. imicola* and *C. oxystoma* collected elsewhere.

For virus isolation, CPAE cell lines were inoculated with midges extract and monitored for CPE. When using cell culture lines for isolation of viruses, depending on the dilutions used there is a minimum detection level of the assay and inoculation of the cells with virus concentration lower than this threshold will not yield CPE, even if the midges are positive for the virus [77,78,92]. Thus, recovery rates depend not only on the presence of the virus but also on its concentration within each midge.

Recovery rates pre-incubation (0 DPF) were used as a measure for evaluating the midges' intake of the virus via the blood meal. The amount of virus that the midges ingest depends on the meal size that they take and the virus concentration in the blood meal [55,60,77,78,86,90-92,94]. When midges are provided with a blood meal, individuals of a particular species fed via a certain technique take an average meal size [55,60,86,90,92,94]. Thus, day 0 results indicate if the infective blood meal contained sufficient virus concentrations to enable a large proportion of midges to ingest a virus amount that will be detected immediately after feeding. Depending on the concentration used and the minimum detection level of the method, reported recovery rates on 0 DPF vary between 0 to 100% [60,77,78,86,90-92], and several studies reported a linear correlation between the virus concentration in the blood meal and the proportion of virus recovery immediately after feeding [60,77,78,86,91,92]. Here, recovery rates on 0 DPF for both *C. oxystoma* and *C. imicola* were 63% (table 4). Although these results are in the higher

range of reported recovery rates, they indicate that the virus titres in the infecting blood-meal was too low to allow all engorging midges to take up enough of the virus, as recovery rates of over 90% have been reported [60,77,78,91]. Venter et al. [94] compered the membrane to the cotton wool pledgets feeding techniques and found that the meal size taken by *C. imicola* and *C. bolitinos* was about 30% smaller in the cotton wool pledgets technique. Thus, it is also possible that when using the dental cotton roll feeding technique, due to a smaller meal size, midges take up less of the virus and concentrations required for achieving a high recovery rate are higher than in the membrane technique.

In the present study the midges were fed upon feeding media containing one of two possible virus concentrations: $10^{4.6}$ or $10^{5.0}$ PFU/ml. When the virus concentration in the blood meal was increased to a mean of $10^{5.0}$ PFU/ml we expected the recovery rate on 0 DPF to increase as well. We also expected a positive correlation between the virus concentration in the blood meal and the recovery rates at each of the incubation periods, as infection prevalence in a single *Culicoides* species-virus serotype system can depend on the viral concentration in the blood meal [77,78,86,90,92-94]. However, this did not occur. As mentioned earlier, the low number of *C. imicola* samples did not allow us to compare between the two feeding regimes for this species. For *C. oxystoma*, at 0 DPF and at each of the incubation times tested there was no significant difference in the recovery rates when comparing between the two feeding regimes. The lack of increase in virus recovery rates pre- and post-incubation once the midges were fed upon a higher virus concentration may be due to an elevation of the virus concentration in the meal that was insufficient for producing such an increase. Another possible explanation is that as we assumed that the larger volume of the media reduced feeding rates, it also caused a decrease in the blood meal size taken by the midges, in case that it is indeed repellent to them. Thus, although the virus concentration in the blood meal was increased, a smaller proportion of midges took up enough of it. In order to verify this assumption, midges should be fed with the same virus concentration via several virus suspension volumes.

The Susceptibility of the midges to oral infection with EHDV-7 was evaluated by isolation of virus after the midges were incubated for 3-4, 11-12 and 15-16 days. These time groups were chosen in order to evaluate the dynamics of the relationship between the midges and the virus. At the first ~2 days after a competent midge ingested a viremic blood meal, the virus concentrations within the midge decline due to inactivation and anal excretion of some viral particles. Recovery rates on 3-4 DPF represent midges in which the virus is present after the digestion of the blood meal, but dissemination and replication in salivary glands had not yet occurred [55]. Therefore, recovery rates were expected to be the lowest at this time, as the virus concentration in some (if not all) of the midges that were infected will be too low for detection. Later on, replication begins to exceed inactivation and the viral concentration rises. Then, if the EIP, which is the interval between ingestion of the virus and the earliest time at which virus is released in the saliva, was completed, recovery rates should increase due to the rise in virus concentrations per midge [55]. Thus, if the midges are capable of virus transmission, recovery rates after 11-12 and/or 15-16 DPF were expected to be higher than after 3-4 DPF. Virus isolation immediately after feeding is considered as a reflection of the virus in the ingested blood meal [55]. Barriers to the infection and dissemination of Arboviruses have been identified in the BTV/AHSV- *C. sonorensis* system and it is hypothesized that they exist in other *Culicoides* species. These barriers are believed to be hereditary traits, and as a vector species population is genetically diverse, only a proportion of individuals is likely to be susceptible to oral infection with a particular Arbovirus [3,55,111,112]. Thus, we expected the virus recovery rates to be higher at 0 DPF than in all other time groups. Contrary to our expectations, the above described dynamic was not observed (table 4 and figure 4).

As for *C. imicola*, it is impossible to evaluate the observed dynamic as none of the groups that were incubated contained enough midges for statistical analysis. For *C. oxystoma* there was no significant difference in the recovery rates when comparing between the four different testing times. As mentioned, midges populations can be genetically diverse, and it has been postulated that population infection

prevalence's may change rapidly, independent of the viremia of the blood meal, within a few days [93]. As midges were collected over a period of 3 months at two consecutive years, it is possible that such changes were responsible for the un-expected dynamics. The above mentioned barriers to the infection and dissemination of Arboviruses do not necessarily co-exist within each individual midge, as was evident by *C. sonorensis* individuals that were successfully infected with BTV, but the virus failed to disseminate, thus the infection of and replication within the salivary glands did not occur [55,111,112]. The existence of barriers to dissemination but not to infection in a large proportion of the midges tested may be another possible reason for the lack of difference in the recovery rates between 3-4 and 11-12/15-16 DPF.

The oral susceptibility of *Culicoides* is species-dependent and viral serotype-dependent. This was demonstrated for *C. imicola* as well as other species for BTV [92-94], AHSV [86] and EHDV [91]. At this time, there is no available data for *C. oxystoma* and only one study for the *C. imicola*-EHDV-7 system [91]. In a study conducted in South Africa, the oral susceptibility of *C. imicola* and 16 other field-collected *Culicoides* species to 8 serotypes of EHDV was examined, after they were incubated for 10 days at 23.5ºC. Five of these 17 species were susceptible to infection by EHDV-7. Six EHDV serotypes were recovered from *C. imicola,* including EHDV-7. The recovery rate of EHDV-7 from *C. imicola* was 14.4%, as opposed to 0.4% - 3.4% for other serotypes. This was also the highest recovery rate in the study all together. Using the tissue culture infectious dose$_{50}$ ($TCID_{50}$) method, several studies regarded the transmission potential of midges as positive only if the titre/midge after the EIP was higher than a threshold of >2.5, $\geq$ 2.7 or $\geq$ 3 log10 $TCID_{50}$/midge, and a significant increase in the virus titre/midge after incubation when comparing to 0 DPF as an indication that replication occurred [60,86,90,92,93]. This was not the case for *C. imicola*-EHDV system, and therefore the vectorial potential was considered as marginal. However, such threshold was demonstrated for *C. sonorensis* and BTV [111,112], and it is possible that the transmission threshold value is different in other species.

Another issue is the incubation duration and temperature. The exact length of the EIP may differ between vector species, viruses species and serotypes, and incubation conditions, in particular temperature [3,55]. Paweska et al. [60] compared the replication dynamics of BTV-1 in field-collected and orally infected *C. imicola* and *C. bolitinos* over a range of different incubation periods and temperatures. In both species the highest infection rate was recorded when midges were incubated at 25°C and the highest proportion of infected females with transmission potential (according to virus titre/midge after the EIP) was recorded when midges were incubated at 30°C. Moreover, at both 23.5ºC and 25ºC infection rates and proportion of infected females with transmission potential were higher on day 15 than on day 10 post feeding. It is possible that a 10 day incubation period and/or 23.5ºC does not represent the fool magnitude of the vectorial potential of the midges in regard to EHDV-7. Therefore, in our study the incubation temperature was 25ºC-26°C, and the midges were evaluated at two longer assumed EIP: 11-12 and 15-16 DPF. Indeed, it seems that the higher temperature used and/or the longer EIP yield higher recovery rates (table 4) when comparing to the study in South Africa. However, for *C. imicola* the correct evaluation of the reason for the high infection prevalence is problematic due to the very low numbers of samples. As for *C. oxystoma*, recovery rates were relatively high at both 11-12 and 15-16 DPF. As the recovery rates did not differ significantly between these two testing times, it seems that the longer EIP of 15-16 days was not needed. Higher temperatures support a faster rate of virogenesis, which corresponds to a shorter EIP [3,55,60]. Therefore, it is possible that at a lower incubation temperature, such as 23.5ºC, longer incubation durations will yield a significant increase in virus recovery rates. Because we could not compare between the two species, at this point we cannot conclude if the high infection prevalence of *C. oxystoma* is due to the incubation conditions or to its susceptibility to EHDV-7 being very high.

One shortcoming of using field-collected *Culicoides* in an oral infection study is the possibility that they could be naturally infected with Arboviruses. In order to insure that the virus isolated is indeed the

virus used for infection, it is possible to include a field control, in which parous females (second incubation females looking for another blood meal) are assessed for the presence of the virus and virus identification methods can be implode after the isolation from experimentally infected midges. A field control for EHDV-7 was not performed because Israel is not endemic for EHDV-7, and the last and only outbreak occurred 5 years before the study began. Therefore it is highly unlikely that midges were infected in the field. A virus identification method was not performed at this time. However, we believe that this does not diminish our final conclusion. All of the virus isolations from midges in the control groups were negative. Several studies that have conducted oral infections trials of *Culicoides* with an Arbovirus such as BTV [93], AHSV [110] and EHDV [78,91] have all found the field control to be negative for the virus in question. All of these studies were conducted in endemic areas for the virus in question. Furthermore, prevalence of infected midges with Arboviruses in the field is usually very low even during epidemics [27,35,68,72,73,87,110]. Therefore, the infection prevalence recorded in this study was most likely the results of the laboratory infection.

In conclusion, although the final numbers of individual *Culicoides* analyzed in this study are insufficient for evaluating the relative susceptibility of field-collected *C. oxystoma* and *C. imicola* to oral infection by EHDV-7, they do correspond with previous reports as to being sufficient for claiming that these midges are indeed susceptible and should be considered as potential vectors of the virus [60,91,92,110]. The results of this study suggest that *C. oxystoma* and *C. imicola* could be important biological vectors of EHDV-7 in Israel. However, the demonstration that these species are susceptible to infection by the virus does not imply directly that they can transmit the virus to its hosts. Thus, further investigation as to other factors regarding their vectorial competence and capacity is required.

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Student role in the research

1. Participant in the protocols design.
2. Tissue cultures preparations, virus proliferation and virus stocks quantification.
3. Insect collection.
4. Collection of blood samples, preparation of blood meals and insects feeding.
5. Insects sorting and maintenance of the midges.
6. Virus isolation.
7. Statistical analysis.
8. Writing the final report.

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