


STtech: sampling and transport techniques for *Aedes* eggs during a sampling campaign in a low-resource setting

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Abstract

Container-breeding *Aedes* spp. (Diptera: Culicidae) mosquitoes can be surveilled at low cost using ovitraps. Hence, this method is a preferred monitoring approach of dengue vectors in low-resource settings. The ovitraps consist of a cup filled with water and an oviposition substrate for female mosquitoes. The attractiveness of the substrates for female mosquitoes can greatly differ due to differences in texture, color, and smell of the materials used. We compare four oviposition substrates, which are all low priced, easy to transport, and easy to purchase, to maximize the success of *Aedes* egg sampling. Sampled egg material is often reared to adulthood for further taxonomic identification and transported to (international) laboratories for specialized vector research. Here we introduce a transport technique for sampled eggs. In addition, we explored the impact of international transport by means of a bilateral hatching experiment in Nepal, the country of origin, and in Germany, in a laboratory specialized in ecophysiological research. The best low-cost oviposition substrate for the dengue vectors *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.) was found to be a white cotton sheet. The introduced transport technique of sampled eggs is easy to build from laboratory and household materials and ensures good transport conditions (i.e., temperature and relative humidity). Even under good temperature (17.4–31.0 °C) and humidity conditions (58.9–94.2%), hatching success of eggs was found to be reduced after international transport to Germany when compared to the hatching success of eggs in Nepal. We postulate that air pressure during international transport may have reduced the hatching success and strongly recommend pressure-regulated transport boxes for egg transport via airplane. As the proposed operation procedure is useful in assisting the monitoring of *Ae. albopictus* and *Ae. aegypti* in low-resource settings, *Aedes* researchers are encouraged to follow it for the sampling and transport of *Aedes* eggs.

Introduction

Aedes aegypti (L.) and *Aedes albopictus* (Skuse) (Diptera: Culicidae) transmit vector-borne diseases such as dengue, chikungunya, and zika virus. *Aedes aegypti* is common in

subtropical regions whereas *Ae. albopictus* is common in subtropical to temperate regions (Kraemer et al., 2019). Species-specific surveillance strategies identifying the presence and distribution of the vectors are important elements of successful integrated vector management. In this regard, various monitoring tools are used to sample *Aedes* eggs, larvae/pupae, and adults (ECDC, 2012). Compared to larval surveys, sampling eggs via oviposition traps shows a high capacity to detect the occurrence of vectors (Anália et al., 2010).

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Sampling methods of *Aedes* eggs display a high variance. Black-plastic cups show the best egg sampling results (Yap et al., 1995; Bellini et al., 1996; Hoel et al., 2011). In general, various oviposition substrates are used for sampling of *Aedes* eggs. For *Ae. albopictus* egg sampling, seed germination paper #76 (Anchor Paper Company, St. Paul, MN, USA) is common (Hoel et al., 2011; Velo et al., 2016; Reed et al., 2018), whereas for *Ae. aegypti*, egg sampling on a brown hard board strip (Chadee et al., 1995; Arunachalam et al., 1999), a plywood sheet (Anália et al., 2010), or a panel of brown blotting paper attached to a wooden tongue depressor was used (Fay & Eliason, 1966). By examining the color preferences of *Ae. albopictus* and *Ae. aegypti* for oviposition substrates, most eggs were laid onto a black substrate whereas the fewest eggs were laid onto a white substrate (Panigrahi et al., 2014). However, the authors only tested velvet papers as egg-laying substrate and did not check the substrate preferences of particular species. Fay & Perry (1965) tested the oviposition preference of *Ae. aegypti* laboratory populations for various colored substrates as well as various substrates themselves. Most eggs were laid onto brown blotter and the second most eggs were laid on white toweling. Thus, the material of the egg-laying substrate itself seems to be as important as color. Consequently, light cotton fabric (as well as velour paper) was found to be the favourable substrate for oviposition in *Ae. aegypti* contrary to a wooden paddle and blotting paper (Chanampa et al., 2018).

The aim of this study was to develop an operation procedure for sampling, documentation, and domestic as well as international transport of *Aedes* eggs in low-resource settings. Based on the earlier studies, we decided to work with black 100-ml plastic cups as ovitraps and test four oviposition substrates, all low priced, easy to transport, and easy to obtain also in low-resource settings. In addition, we tested the effect of international transport on egg quality and we identified the species of emerged adults.

Materials and methods

At first, four substrates for oviposition were tested in a simple choice experiment. Based on the results a guideline for the sampling of *Aedes* eggs in low-resource settings was developed. Second, the effect of international transport on *Aedes* eggs to specialized laboratories was assessed in a comparative hatching experiment in Nepal and Germany (Table 1). This sampling survey was approved by the Ethical Review Board of the Nepal Health Research Council (#1058) and all transported material complies to the agreement between the Nepal Health Research Council and the Goethe University, Frankfurt am Main, Germany (#381/2017).

Choice experiment for oviposition substrate

Four substrates were offered in a choice experiment in spatiotemporal replication in (urban) Chitwan District, Nepal, in June 2018. During the 1st sampling week, heavy rain occurred on 5 and 6 June, prior to the official onset of monsoon, which was 8 June in 2018 according to the Nepalese government (Department of Hydrology and Meteorology). During the rest of the sampling campaign, rainfall per week was similar between weeks, as were also the minimum and maximum temperatures (Figure 1). Increased mean relative humidity in the evening over the sampling weeks ranged from ca. 20 to ca. 90%. Mean rainfall and minimum and maximum temperature and relative humidity during the sampling weeks were assessed by a weather station in Bharatpur, Chitwan (Figure 1) which is operated by the Department of Hydrology and Meteorology, Nepal. The station was in a 23-km radius of all sampling sites. In contrast, mean relative humidity in the morning was ca. 70–90% over all sampling weeks.

The following oviposition substrates were tested: (1) white filter paper (WHI; attached with a wooden clip), (2) wooden stick (WOD; wooden tongue depressor), (3) white cotton sheet (COT), and (4) blue synthetic sheet (BLU; Figure 1). Each oviposition substrate was tested in 30 ovitraps (180-ml black cups) and replicated 5× in space and 5× in time using a block design: five time points × five spatial clusters × 30 ovitraps per oviposition substrate type. A choice for oviposition was offered by placing four cups with different substrate randomly next to each other. Ovitrap were filled two-thirds with rain or tap water, depending on availability, and placed at more or less sheltered sites, as well sites protected against rain and direct sunlight as possible given the local circumstances. Per cluster, sketches were drawn and photographs were taken for the successful recollection of the cups later. It has to be noted that due to heavy rain over the whole study period, most of the ovitraps from two clusters of the 1st week were swept away and egg sampling started on these spots during the 2nd week.

Operation time of the ovitraps was 5 days, but they were checked every 2 days and refilled with water if found dry. After 5 days, the oviposition substrates with eggs were moved into numbered, 1/5 opened plastic Petri dishes and left to dry for 24 h. The parts of the WOD that did not contain eggs were cut off to make the stick fit in the Petri dishes. By applying a rubber band around the Petri dishes, the sampling substrates were easily transported. After the substrates were dried, the eggs were counted visually.

The numbers of eggs sampled per substrate were analyzed using a one-way ANOVA followed by Tukey's multiple comparison test. In addition, the preference of females

Table 1 *Aedes* egg sampling in Nepal in 2018 and usage of sampled egg substrate in the experiments described here. The installation period of oviposition cups, the number of oviposition cups, and the location of sampling are given for eggs sampled in June (trial 1) and eggs sampled in September and October (trial 2). In trial 1, the sampling of eggs on different oviposition substrates was tested in a choice experiment, whereas in trial 2, the most successful substrate of trial 1 was used

Trial	Installation of oviposition cups		No. cups	Experiments			
	Location of egg collection	Installation period		Choice experiment (oviposition substrate)	International transport	Bilateral hatching experiment	Species identification
1	Chitwan	4 June–8 June	120	X	X	X	
		8 June–12 June	120	X	X	X	
		12 June–16 June	120	X	X	X	
		16 June–20 June	120	X	X	X	
		20 June–25 June	120	X	X	X	
2	Chitwan	4 September–10 September	150		X		X
	Kathmandu	13 September–19 September	150		X		X
	Dhading	19 September–25 September	125		X		X
	Kathmandu	27 September–1 October	80		X		X
	Dharke	26 September–5 October	100		X		X
	Naubise	26 September–5 October	80		X		X
	Kakani	3 October–7 October	55		X		X

for ovitraps was analyzed, because heavy rain during the sampling campaign may have disturbed oviposition behavior. Choices of females for oviposition substrates over the 5-week period (positive choice: eggs deposited = 1; negative choice: no eggs = 0) were calculated and analyzed via one-way ANOVA followed by Tukey's multiple comparison test. In order to analyze preference for the various substrates along with the sampling week and (spatial) replicate, a general linear mixed-effects model (GLMM) with binomial distribution was used. All statistical and graphical analyses were conducted using Prism v.7 (GraphPad Software, San Diego, CA, USA), except the GLMM model which was analyzed using RStudio v.1.1.423.

Domestic and international egg transport

In total, three long-distance international transports of eggs were conducted using specifically designed transport boxes (Figure 2). The first transport took place from 7 to 8 August 2018 (transport A), the second from 28 to 29 September 2018 (transport B), and the third from 28 to 29 October 2018 (transport C). Transport A was used for the transportation of eggs collected using 120 oviposition cups in Chitwan during the choice experiment. Similarly, in transports B and C, eggs were sampled along an altitudinal gradient with decreasing population size from Chitwan (ca. 200 m), Dhading (ca. 600 m), Dharke (ca. 800 m), Naubise (ca. 930 m), Kathmandu (ca. 1 300 m), and Kakani (ca. 2 000 m) using oviposition cups (55–150) with COTs as the only oviposition substrate in September

and October 2018 (Table 1). Eggs were transferred onto white laboratory filters for further transportation, after drying for 24 h in Petri dishes.

Laboratory filters were closed with adhesive tape and moved into 50-ml tubes, each with the capacity to hold approximately five filter papers. The 50-ml tubes were covered with a small part of a surgical cap secured by rubber bands and grouped up to seven tubes in one transport box (1-l vessel) which contained up to 35 filter papers (Figure 2). An additional 50-ml tube filled with wet clay granules (or, if not available, potting soil or a small wetted cotton towel may be used), wetted for 24 h before use, was placed into each transport box to keep the humidity high during transport. In order to prevent the samples moving in the 1-l vessel, 20-ml tubes were added as place holders. During transport, the lids of each 1-l vessel were closed and secured with adhesive tape. The eggs from one location were transported in separate transport boxes to rule out mixing of the eggs from different sampling locations.

Temperature and relative humidity were recorded from one transport box per transport using a HOBO UX100-011 (Onset, Cape Cod, MA, USA) temp/RH data logger (Table 2, Figure 2) and illustrated using Prism v.7.

Bilateral hatching experiment

Eggs either stored in Nepal or transported to Germany were used for a bilateral hatching experiment. Transport boxes remaining in Nepal were opened daily to reduce humidity. Clay granules were wetted again, when

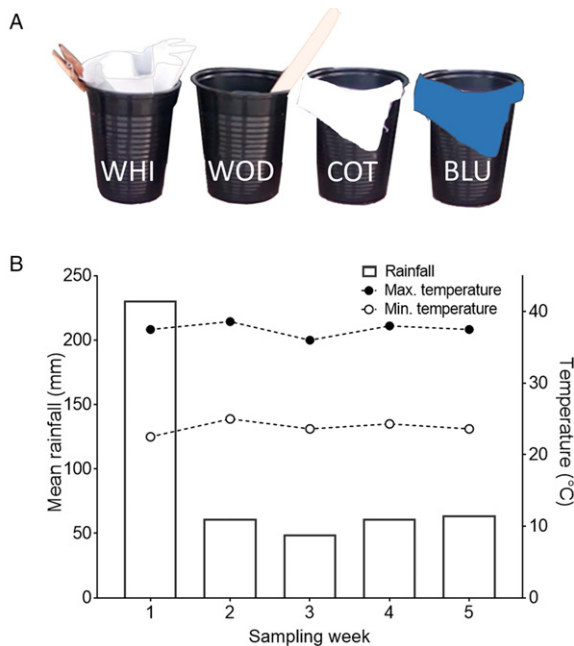


Figure 1 Oviposition substrates (ovitraps) tested in an oviposition choice experiment and climate data during sampling in Nepal. (A) Each ovitrap is composed of a black 180-ml cup, filled for two-thirds with water, and an oviposition substrate: white paper filter (WHI), wooden stick (WOD), white cotton sheet (COT), or blue synthetic fiber sheet (BLU). (B) Weekly mean rainfall (mm) and temperature range over the 5-week sampling period. [Colour figure can be viewed at wileyonlinelibrary.com]

humidity needed to be increased. For acclimation, the boxes sent to Germany were slightly opened as soon as they arrived and transferred into a climate cabinet at 26 °C, 80–90% r.h., and L13:D11 photoperiod, in line with the corresponding weather forecast in Nepal during the experimental period. Transport boxes were stored in the climate cabinet for 1 week. Then, hatching procedures were applied in parallel in Nepal and Germany on the same day and at the same time of day. To induce hatching, the protocol developed by Kreß et al. (2014) was followed, but dried yeast (that was transported from Germany to Nepal prior to the start of the experiment) was used to ensure equal yeast quality for experimental use in both countries. Moreover, non-carbonated mineral water was used to produce the hatching media. In brief, 100 eggs were placed in a 1-l vessel and 500 ml of hatching media was added ($n = 4$). A desk lamp was installed 30 cm above the vessels and was switched on for 48 h. In Nepal, room temperature was measured twice during the 48-h period and was 29.1 °C. In Germany, 1-l vessels with eggs were placed in a climate cabinet at 26 °C. The

larvae that hatched and were alive after the 48 h were counted. The effect of international transportation on the hatching success was analyzed by an unpaired t-test (Prism v.7).

Control of species emergence and species identification

Eggs collected in Chitwan, Dhading, Dharke, Naubise, Kathmandu, and Kakani, Nepal (international transports B and C) were reared up to adulthood per sampling site, the percentage of adult emergence was determined, and their species were identified. In short, the eggs were pooled per sampling site and submerged in hatching media. After 48 h, larvae were moved per population to 1-l vessels filled with water and fed ad libitum with ground fish food (Kreß et al., 2014). Hatching success and larval or pupal mortality were not determined in the present study. The emerged pupae were separated into 2-ml glass tubes and sex and species of emerged adults were identified using 50× magnification (stereomicroscope Motic SMZ168; Kramer et al., 2020). In this way, the specific sorting of mosquito adults according to species and sampling site for further rearing can be achieved. Exclusively *Ae. albopictus* and *Ae. aegypti* larvae hatched from the sampled eggs. Apart from morphological identification, 20 adults (one leg per adult from randomly selected sampling sites) were used for bar-coding via the CO1 marker (Simon et al., 1994; Kumar et al., 2007). For this purpose, the mosquito legs were homogenized in phosphate-buffered saline (PBS). DNA was extracted with the QIAamp DNA Mini Kit (Venlo, The Netherlands). PCR was performed with 0.6 U DreamTaq polymerase (Thermo Scientific, Waltham, MA, USA), 400 μM deoxynucleoside triphosphates, and 0.1 μM of each primer in a total volume of 25 μl. The sequences were edited using Geneious Prime v.2019.2.1 (Auckland, New Zealand) and blasted in NCBI (Altschul et al., 1990; NCBI Resource Coordinators, 2018).

Results

Preference for white cotton sheet as oviposition substrate

Female preference for ovitraps differs between oviposition substrates ($F_{3,596} = 86.06$, $P < 0.0001$). Overall, the most successful substrate for female encountering is the white cotton sheet (COT), followed by wooden stick (WOD), and blue synthetic sheet (BLU), whereas the least preferred oviposition substrate was white filter paper (WHI) (Figure 3A). The GLMM of female preference shows that WHI differs compared to all other methods (WHI vs. WOD: $z = 4.049$; WHI vs. COT: $z = 5.422$; WHI vs. BLU: $z = 3.573$, all $P < 0.001$). WOD and BLU show similar encounters of females, whereas WOD differs from COT (WOD vs. WHI: $z = -4.049$, $P < 0.001$; WOD vs. COT:

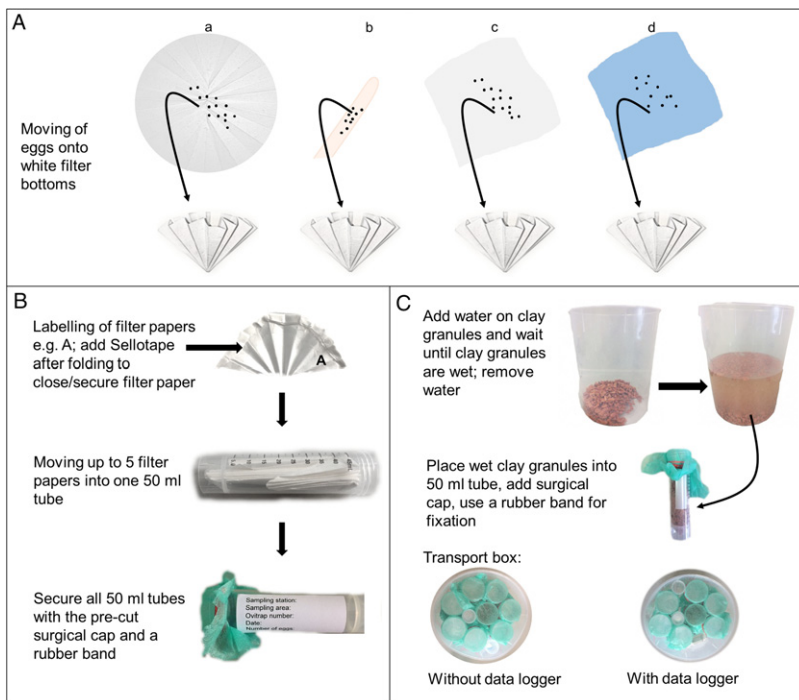


Figure 2 Transport box design and packaging of sampled eggs for further domestic and international transport. (A) Moving eggs from various oviposition substrates – (a) white paper filter, (b) wooden stick, (c) white cotton sheet, or (d) blue synthetic fiber sheet – into transport filters. (B) Labeling and storing of filter papers in 50-ml tubes. (C) Storing of 50-ml tubes in a transport box (1-l vessel). In addition, instruction is given for the construction of a 50-ml tube responsible for high humidity during transport. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Mean (\pm SD, range in parentheses) temperature ($^{\circ}$ C) and relative humidity (%) during the three long-distance transports from Nepal to Germany

Transport	Temperature ($^{\circ}$ C)	Relative humidity (%)
A	27.0 \pm 1.8 (31.0–24.3)	85.4 \pm 0.9 (87.6–81.8)
B	23.9 \pm 2.0 (28.2–17.4)	83.2 \pm 8.2 (94.2–58.9)
C	21.2 \pm 2.0 (25.4–18.3)	86.0 \pm 4.5 (92.6–76.4)

$z = 2.082$, $P < 0.05$) and not from BLU (WOD vs. BLU: $z = -0.658$, $P > 0.05$). The most successful COT method differs from all other methods (COT vs. WOD: $z = -2.082$, $P < 0.05$; COT vs. WHI: $z = -5.422$, $P < 0.001$; COT vs. BLU: $z = -2.706$, $P < 0.01$) and BLU differs from WHI and COT but not from WOD (BLU vs. COT: $z = 2.706$, $P < 0.01$; BLU vs. WOD: $z = 0.658$, $P > 0.05$; BLU vs. WHI: $z = -3.573$, $P < 0.001$).

The temporal analysis of 5 weeks data shows that the COT is the overall preferred substrate for oviposition, except for the last sampling week (Figure 3B). The percentage of eggs varies between substrates ($F_{3,596} = 465.2$, $P < 0.0001$), with the most eggs on the substrates COT > WOD = BLU > WHI (Figure 3C). The differences in numbers of eggs over the sampling weeks are the same as described for total positive female choice (female preference), with the highest sampling success of COT

compared to other methods except in the last sampling week (Figure 3D). In the 1st week of installation, due to heavy rainfall compared to the other weeks, the ovitraps were lost at two of the five clusters analyzed.

Transportation of eggs and transportation effect on egg hatching

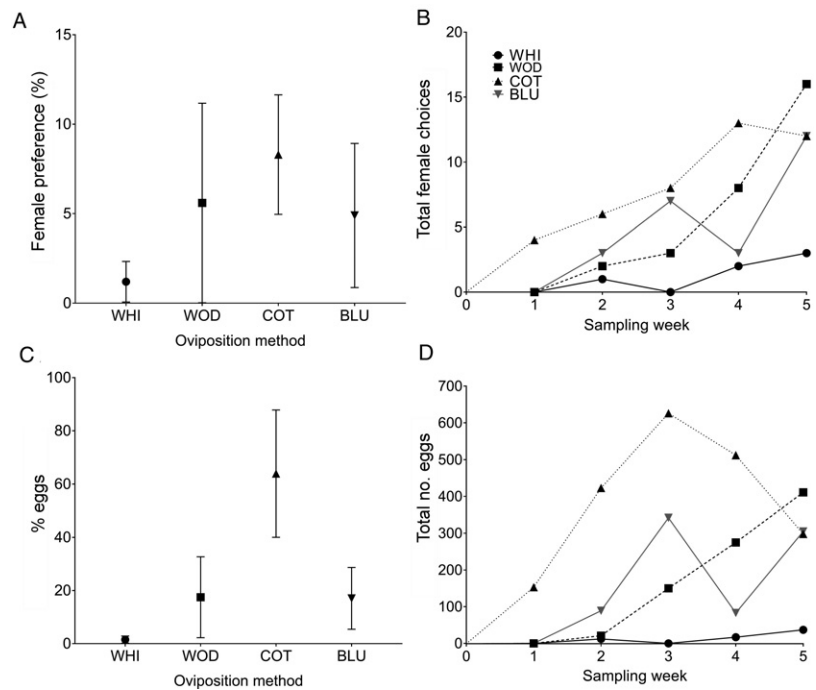
Altogether, 9 060 *Aedes* eggs were transported from Nepal to Germany. Temperature and relative humidity during all transports ranged from 17.4 to 31.0 $^{\circ}$ C and from 58.9 to 94.2% (Table 2, Figure 4). Transport (A) of eggs to Germany reduced the hatching success of *Aedes* eggs, when compared to egg aliquots tested under equal conditions in Nepal via a bilateral hatching experiment (26.0 ± 7.7 vs. $9.5 \pm 4.9\%$; $n = 4$; $t = 36.28$, d.f. = 798, $P < 0.0001$).

Domestically transported eggs (within Nepal) hatched below 50%. Of the eggs related to transports B and C, 7.3–37.3% developed to adulthood (Table 3); hence, in general hatching/emergence success was quite low. Most of the emerged adults in trial 2 (Table 1) were *Ae. aegypti*; of those originating from Naubise, Dharke, or Kakani most were *Ae. albopictus*. CO1 barcoding of 10 randomly chosen adults per species confirmed that morphological identification was accurate.

Discussion

An operation procedure for *Aedes* egg sampling for monitoring and surveillance in a low-resource setting and their

Figure 3 Oviposition on ovitraps with one of four substrates: white paper filter (WHI), wooden stick (WOD), white cotton sheet (COT), or blue synthetic fiber sheet (BLU). (A) Mean (\pm SD; $n = 30$ ovitraps) preference of females for oviposition substrates (positive choice: occurrence of eggs deposited = 1; negative choice: no eggs = 0). (B) Positive choice of females over time. (C) Distribution of eggs over the four substrates [mean (\pm SD) % eggs]. (D) Total number of eggs sampled over time.



domestic and international transport is presented. The sampling substrates used in this study for oviposition choice tests could easily be obtained. The oviposition substrate using a black cup with COT was the most successful sampling substrate based on the total number of eggs laid, as well as the females' preferences. The substrates and egg batches could be safely transported within the country of origin using a normal suitcase even though sampling was in part conducted at remote places that were difficult to access. However, international transport damaged the *Aedes* eggs. Therefore, we recommend for international transport by airplane with high air pressure the investment in specifically designed packaging for shipment of biohazardous substrates.

Oviposition cups with a volume of 180 ml were used because they were easy to purchase and to transport, although larger cups with a bigger surface area might increase the sampling success of the substrate as shown in laboratory experiments (Gunathilaka et al., 2018). As oviposition substrate, COT and WOD were chosen because of prior studies conducted with similar materials and they are also easy to purchase in low-resource settings (Fay & Perry, 1965; Fay & Eliason, 1966; Chadee et al., 1995; Arunachalam et al., 1999; Chanampa et al., 2018). In the laboratory, successful egg sampling was obtained on coffee filters (Kreß et al., 2014). However, thin paper like that of coffee filters is easily damaged especially in the field under the influence of environmental stressors (such as

heavy rainfall). Therefore, we decided to test thicker laboratory filter papers for WHI instead. BLU was used because previous egg sampling campaigns in Nepal using it were successful (I Gautam, pers. comm.; Kramer et al., 2020). For reasons of transport, we did not work with hay infusion (Reiter et al., 1957; Velo et al., 2016), oak leaf infusion (Trexler et al., 1998), or drain water (Colton et al., 2003).

Oviposition behavior is complex, as it is influenced by a range of factors, such as pre-existing eggs (Chadee, 2009), larvae (Wong et al., 2011; Davis et al., 2015), and abiotic factors such as temperature, humidity (de Almeida Costa et al., 2010), and rainfall (Hoeck et al., 2003; Micieli & Campos, 2003; Vezzani et al., 2004; Anália et al., 2010). Heavy rainfall may disturb egg-laying females, which may explain the reduced number of eggs during the 1st week of our egg sampling campaign. This is why for surveillance it is important to observe the number of eggs as well as the encountering of female mosquitoes with the ovitraps. An increase of eggs and female encounterings over our sampling period with all oviposition substrates was observed. In parallel, relative humidity in the evening increases over the sampling weeks and onset of monsoon in general at the beginning of the survey had been observed (Department of Hydrology and Meteorology, Nepal). Several studies correlated an increase of female encounters or the number of eggs with an increase in rainfall and relative humidity (Hoeck et al., 2003; Micieli & Campos, 2003;

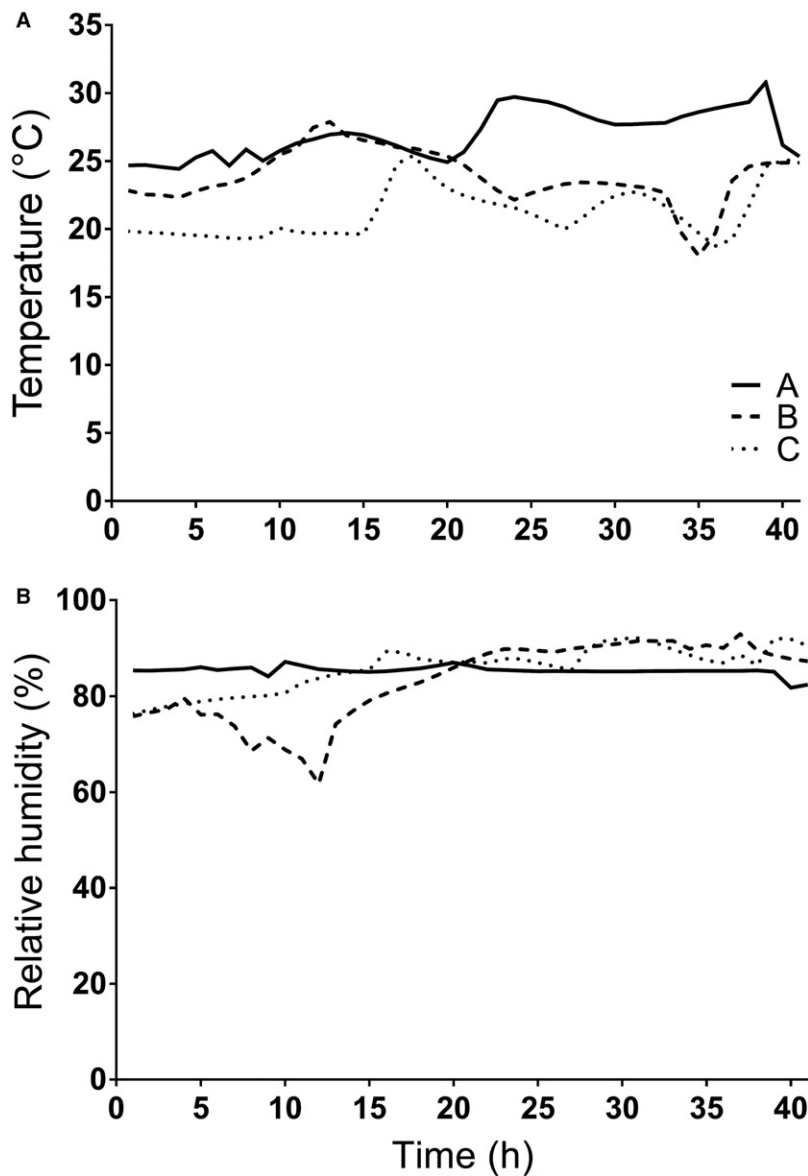


Figure 4 Average temperature (°C) and relative humidity (%) during three transports (h) from Nepal to Germany, recorded at 15-min intervals by data loggers stored near the mosquito eggs. Transport A: 7–8 August 2018; B: 28–29 September 2018; C: 28–29 October 2018.

Vezzani et al., 2004; Anália et al., 2010). However, with increasing rainfall, breeding sites of mosquitoes increase, leading to population growth in general which also explains the increase in the number of eggs in the ovitraps over the sampling period during this study. According to the literature, eggs sampled per trap belong most likely to only one or a few other females. *Aedes aegypti* prefer to lay eggs in ovitraps that are free of eggs (Chadee, 2009), but they also prefer to lay eggs in containers where other immature stages are present (Wong et al., 2011). Moreover, *Ae. aegypti* lays about half of their eggs in one site, and the remaining ones are distributed over various places, in batches of 1–30 eggs (Oliva et al., 2014). Molecular

evidence confirmed oviposition at multiple sites by testing the relationship between larvae in different containers: families of *Ae. aegypti* clustered as groups across multiple containers (Colton et al., 2003). Species comparison showed that *Ae. aegypti* is more likely to skip oviposition than *Ae. albopictus* (Rey & O'Connell, 2014). *Ae. albopictus* prefers to lay eggs in uncrowded compared to crowded larval conditions, and distributes eggs among multiple uncrowded options (Davis et al., 2015). Accordingly, eggs sampled per ovitrap belong most likely to only one or a few *Aedes* females.

More than 20 *Aedes* species and other mosquito genera such as *Culex*, *Malaya*, or *Armigeres* are present in Nepal

Table 3 Emergence success (%) and number of eggs sampled per *Aedes* species and sampling site. All eggs were sampled using a black 180-ml cup and white cotton sheet as oviposition substrate. Percentage of females is given in parentheses

Sampling site ¹	No. sampled eggs	Emergence (%)	<i>Ae.</i>	
			<i>albopictus</i> (%)	<i>aegypti</i> (%)
Chitwan	2296	14.5	2.8 (50.8)	11.7 (45.1)
Dhading	905	12.3	1.2 (9.1)	11.1 (61.0)
Dharke	1004	7.3	4.6 (37.0)	2.7 (48.1)
Naubise	665	9.5	9.3 (54.8)	0.2 (0)
Kathmandu	1450	37.3	12.3 (59.2)	25.0 (48.9)
Kakani	40	12.5	12.5 (80.0)	0 (-)

¹Sampling time and number of ovitraps differ per site (see Table 1).

(Darsie et al., 1990; IM Kramer, S Baral, I Gautam, M Braun, A Magdeburg, P Phuyal, M Dhimal, B Ahrens, DA Groneberg & R Müller, unpubl. data), but our set-up using a black cup and COT oviposition substrate collected exclusively *Ae. albopictus* and *Ae. aegypti* eggs. Species specificity of ovitraps cannot be concluded from these data, as the ovitraps were installed at hotspots of these two species. Ovitrap were often in competition with optimal breeding sites of *Aedes* females which are especially discarded vehicle tires (Gautam et al., 2009, 2015). However, installation of ovitraps at hotspots of two vectors species is an advantage for effective vector surveillance.

The temperature recorded during the international transports ranged from 17.4 to 31.0 °C. As indicated by the bilateral hatching experiment, international transport reduces the hatching success of *Aedes* species. The adverse effects on the eggs may be due to the fluctuating temperature ($\Delta 13.6$ °C) and/or humidity ($\Delta 35.3\%$ r.h.) during the transports. According to the literature, however, those environmental conditions should not negatively affect the survival of pharate larvae in eggs. In *Ae. aegypti*, the lowest developmental zero temperature is 10–14 °C with a near linear relationship until 30 °C, that is, the higher the temperature the better the development (Eisen et al., 2014). The temperature during the international transport of *Ae. albopictus* should also not have harmed their hatching success, as the lower temperature threshold for development is 10.4 °C, and between 20 and 30 °C, the survival of all stages (L1-adult) is comparable. Relative humidity varied between 58.9 and 94.2% during all international transports of 40 h duration, but this humidity range should also not reduce the hatching success – *Ae. aegypti* still shows >80% hatching success after 1 month exposure of a relative humidity of 98, 86, 75, or 43% (Luz et al., 2008). Decrease in the hatching success of *Ae. aegypti* with an increase in

exposure time to a range of relative humidities (90, 75, 60, 30, 0%) was also observed by Kliewer (1961). Survival in diapausing and non-diapausing *Ae. albopictus* eggs decreased with decreasing relative humidity from 90 to 73 and 44%: overall mean survival time dropped from 116.8 to 30.7 days in non-diapausing eggs and from 205.3 to 64.8 in diapausing eggs (Sota & Mogi, 1992). Hence, the effect of $\Delta 13.6$ °C and $\Delta 35.3\%$ r.h. for transport durations of 40 h should be of minor importance.

In the bilateral hatching experiment, the higher hatching success in Nepal compared to Germany might be due to the higher test temperature in Nepal (29.1 vs. 26 °C in Germany). In contrast, the hatching success of *Ae. aegypti* was reported to decrease with temperature increasing from 24 to 35 °C (Rueda et al., 1990; Mohammed & Chadee, 2011). Farjana et al. (2012) did not demonstrate any significant difference in hatching between 20 and 30 °C in both species and Delatte et al. (2009) showed no significant difference in hatching success of *Ae. albopictus* at 25 and 30 °C (49.2 vs. 51.4%). Hence, different temperatures during the bilateral hatching experiment in Nepal and Germany should not have had an effect on subsequent egg hatching.

Therefore, we hypothesize that the decrease in egg hatching success after international transport could have been caused by air pressure. In modern airplanes, due to the aircraft pressurization systems, the cruising altitude is between approx. 7 and 12.5 km, whereas the cabin pressure corresponds roughly to an altitude of approx. 2 km (Hinninghofen & Enck, 2006). However, the rapid take-off and landing of the airplane may affect the hatching success after transport. Future sampling campaigns should take the detrimental effect of long flights on egg hatching success of the *Aedes* species into account and sample extra eggs. We also recommend the use of a transport box especially designed for shipment by airplane in pressurized and non-pressurized cargo bays (Biopack 2 – 1.5L UN Combination Packaging). Worldwide international collaborations in the field of tropical medicine and experimental entomology are increasing and rely on transportation of sampled materials to specifically equipped laboratories. Therefore, effective national and international transport methods of vector eggs are required. The transportation method described here provides good results with regard to survival temperatures and relative humidity, and can be used further when coupled with the Biopack 2 packaging.

In conclusion, detection of the two dengue vectors was effective by sampling eggs using a white cotton sheet even though effective surveillance of the two vector species *Ae. aegypti* and *Ae. albopictus* also includes larvae/pupae or adult sampling. The methodology described here for the sampling and domestic transportation of *Aedes* eggs in low-resource settings could be used later for the

subsequent successful rearing and breeding of *Ae. aegypti* and *Ae. albopictus* in a BSL3 laboratory, whereas the international transport of eggs needs further methodological improvement by additionally using a pressure-regulated transport box. Hence, we encourage *Aedes* researchers to follow the proposed operation procedure for sampling and transport of *Aedes* eggs.

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