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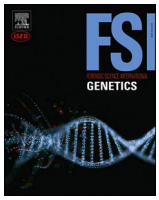

Assessing the consistency of shedder status under various experimental conditions

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ABSTRACT

Shedder status is defined as the propensity of an individual to leave DNA behind on touched items or surfaces and has been suggested as one of the major factors influencing DNA transfer. However, little is known about whether shedder status is a constant property of an individual across multiple measurements or when the environmental conditions are changed. We have assessed DNA depositions of six males on 20 occasions to acquire a reference data set and to classify the participants into high, intermediate, or low shedders. This data set was also used to investigate how the probability of a correct shedder status classification changed when the number of DNA deposition measurements increased. Individual sweat rates were measured with a VapoMeter and data regarding hygiene routines were collected through a questionnaire on each sampling occasion. Next, we investigated how changes in the experimental conditions such as seasonal variation, hygiene routines, the temperature of the touched object, and repeated handling of an object influenced the DNA shedding. Additionally, we assessed DNA collected from the face and from T-shirts worn by the six participants to explore whether shedder status may be associated with the relative amount of DNA obtained from other body parts. Our results indicate that shedder status is a stable property across different seasons and different temperatures of handled objects. The relative DNA amounts obtained from repeatedly handled tubes, worn T-shirts, and from faces reflected the shedder status of the participants. We suggest that an individual's shedder status is highly influenced by the DNA levels on other body parts than hands, accumulating on the palms by frequently touching *e.g.*, the face or previously handled items harboring self-DNA. Assessing physiological differences between the participants revealed that there were no associations between DNA shedding and individual sweat rates.

1. Introduction

Short Tandem Repeat (STR) profiling of trace levels of DNA obtained from handled or touched items is routinely performed at forensic DNA laboratories and has proven useful in forensic examinations [1,2]. The increasing sensitivity of the applied techniques allows for generation of STR profiles from low DNA levels and minute contributions in samples containing DNA from more than one individual [3,4]. This, together with a growing recognition of the complexity of transfer, persistence, prevalence, and recovery of DNA (DNA-TPPR), has led to a substantial increase in the number of potential scenarios explaining the route of DNA deposition [3,5–9]. In court, forensic scientists are often asked to evaluate the evidence on activity level, *i.e.*, reasoning about the possibility of the likelihood of direct *versus* indirect contact between the person of interest (POI) and the object from which the DNA was recovered [10,11]. More knowledge on the different factors behind transfer probabilities is needed to ameliorate the estimation of activity level likelihood ratios [9,12,13].

One of the major factors that influence transfer probability is the shedder status of an individual, defined as the propensity to leave DNA on an object upon contact [9,14–16]. A growing body of research shows that individuals can be categorized as "low/poor", "intermediate" and "high/good" shedders based on the relative DNA amounts they deposit [14,17–21]. The first studies addressing DNA shedder differences included only two categories, *i.e.* good and poor shedders [17,22,23], while the intermediate shedder class was introduced later [14,18,20,

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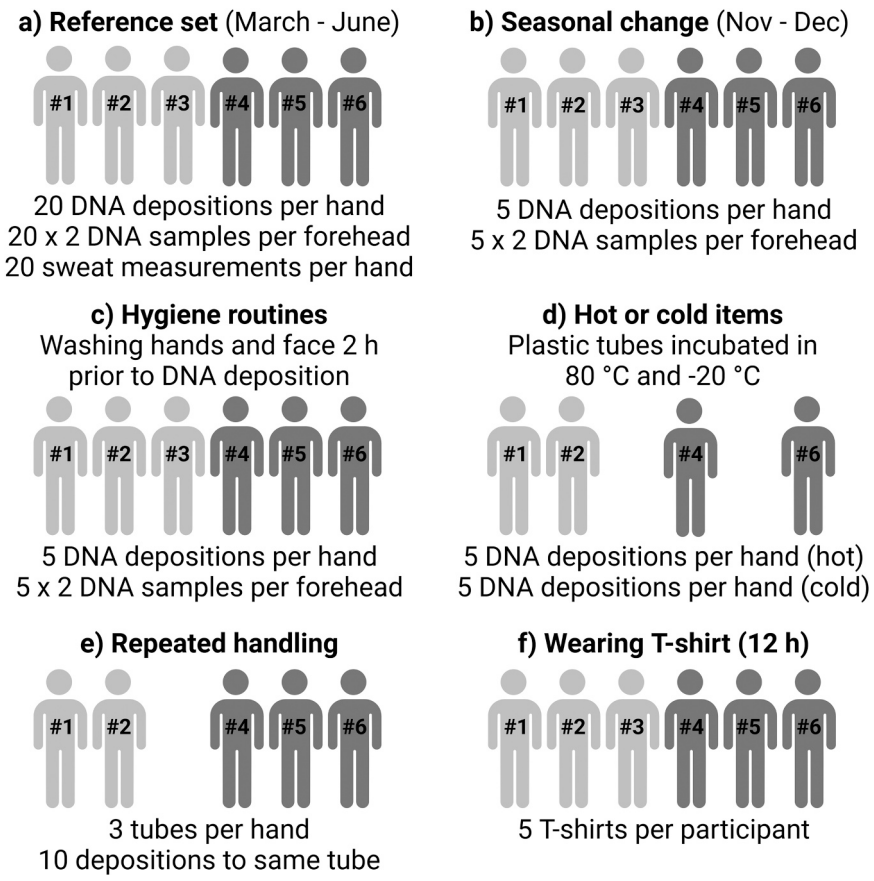

Fig. 1. Overview of the six separate experiments that were conducted on the six participants. The participants were chosen based on their level of DNA shedding, as participants #1–3 (light grey) were seen to deposit lower amounts of DNA in comparison to participants #4–6 (dark grey) in pre-studies. The experiment where DNA was deposited onto hot and cold items included four participants, and the experiment where tubes were handled repeatedly included five participants. Both right and left hands were used in all experiments. Created with BioRender.com.

24]. It has been shown that DNA from high shedders may be found on an object they never touched *via* secondary DNA transfer, especially if a low shedder is the vector of transfer [8,25,26]. Similarly, when a low shedder touches an object that was previously handled by a high shedder, most of the DNA recovered from the object has been shown to originate from the high shedder [26]. Thus, to know the shedder status of a POI would potentially improve forensic evaluations by giving data to assess activity level propositions, for example by applying Bayesian networks [9,27].

Today, there is no widely established method to determine shedder status, and the assessment differs between studies in several aspects, including DNA deposition methods, number of measurements, workflows, and criteria for shedder classification [28]. Such discrepancies complicate comparisons between studies and may explain the divergent results on the consistency of a person's shedder status. While some studies show that shedder status is stable over time [21,24,29], others have described that deposited DNA amounts from a person can vary substantially from one time to another [20,23,27,29,30]. Samie et al. [31] proposed that an individual's shedding ability should be characterized by statistical means based on the distribution of an individual's deposited DNA quantities, rather than assigning an individual to a fixed shedder category. Although this approach has not yet been adapted in the forensic community, most studies conclude several measurements of deposited DNA amounts are required to determine a person's shedder status. Environmental circumstances, such as seasonal change, may further increase the variation in the amounts of deposited DNA from one individual.

Moreover, the underlying factors dictating the shedder status of a person have not yet been fully elucidated. It has been shown that men, in particular young men, tend to shed more DNA than women [18,20,21, 32]. Others have shown that the number of touched items, activities of self-contact and mobile phone usage prior to DNA deposition increase the amount of deposited DNA [20,27,33]. This is most likely explained by the transfer of a person's own DNA from other body parts and personal items to the hands [12,20,33]. A previous study described that the amounts of DNA deposited from hands were correlated to the DNA amounts collected from foreheads [33], although others find no clear association between shedder status and relative DNA amounts collected from other body parts [24,34]. Hygiene routines, *i.e.,* personal habits of showering and washing face and hands, have been suggested as a factor influencing shedder status [14,35]. It has been shown that DNA on hands accumulates within two minutes after hand-washing [36] and that no substantial increase in DNA levels on hands are seen after five to fifteen minutes up to a couple of hours post hand-washing [21,25,26, 37], likely due to that hands constantly pick up DNA from other body parts and items [33]. Whether there is an association between washing/shower routines and DNA accumulation on the rest of the body, is not known. Another potential physiological mechanism behind shedder status is inter-individual propensities to sweat [38,39], and it has been suggested that the condition of the hands (*e.g.,* dry or sweaty) at the time of DNA deposition influences the amount of DNA available on the hand [31].

In this study, we first investigated the intra-individual DNA shedding variation of six male participants including both low and high shedders as known from pre-studies, by allowing them to deposit DNA onto plastic tubes on 20 occasions. We classified the participants as low, intermediate, and high shedders based on a previously published model [14]. In addition, we used the data to analyze how many measurements

Table 1
DNA amounts and detected STR alleles obtained from six male participants under different conditions. Deposited DNA amounts (ng, row 1–6), percentage of self-DNA (row 7–12), and number of detected STR alleles (out of maximum 30, row 13–18) are shown as median and interquartile range (IQR) within parentheses. The median DNA yields against which all participants were measured to assess the shedder status were as follows: Reference set: 0.47 ng, Nov-Dec: 0.44 ng, Washing of hands: 0.35 ng, Hot items: 0.55 ng, Cold items: 0.52 ng, Repeated handling: 11.65 ng. N/A, not available. Asterisk (*) indicates the participants (#) to whom there was a statistically significant difference in DNA deposition.

Row	Experimental setting	Participant #1	Participant #2	Participant #3	Participant #4	Participant #5	Participant #6
Median DNA amount (ng), (IQR)							
1	Reference set (March-June) (n = 40)	0.13 (0.24) * (#3-6)	0.17 (0.24) * (#4-6)	0.32 (0.38) * (#1,4-6)	0.78 (0.97) * (#1-3)	1.1 (0.95) * (#1-3)	1.4 (1.8) * (#1-3)
2	Nov-Dec (n = 10)	0.17 (0.17)	0.13 (0.16)	0.25 (0.37)	0.70 (0.57)	1.6 (1.7)	1.8 (3.1)
3	Washing of hands/face (n = 10)	0.02 (0.28)	0.10 (0.04)	0.17 (0.20)	0.72 (1.6)	0.72 (1.9)	1.1 (0.90)
4	Handling hot item (n = 10)	0.17 (0.19)	0.09 (0.16)	-	2.0 (3.7)	-	1.2 (0.25)
5	Handling cold item (n = 10)	0.05 (0.13)	0.05 (0.04)	-	1.4 (1.8)	-	1.7 (2.4)
6	Repeated handling (n = 6)	2.4 (1.4)	0.98 (0.90)	-	72 (44)	14 (6.5)	14 (3.9)
Median fraction of self-DNA (%), (IQR)							
7	Reference set (March-June)	99 (31)	100 (0)	89 (19)	92 (4)	100 (0)	97 (5)
8	Nov-Dec	100 (19)	100 (0)	100 (7)	100 (5)	100 (0)	100 (3)
9	Washing of hands/face	62 (38)	100 (0)	91 (12)	92 (9)	100 (0)	100 (3)
10	Handling hot item	100 (16)	100 (0)	-	96 (6)	-	99 (2)
11	Handling cold item	86 (32)	100 (0)	-	100 (4)	-	99 (3)
12	Repeated handling	96 (5)	98 (4)	-	100 (0)	100 (0)	95 (3)
Median number of detected self-alleles, (IQR)							
13	Reference set (March-June)	19 (12)	18 (17)	28 (6)	30 (0)	30 (0)	30 (0)
14	Nov-Dec	15 (17)	20 (7)	21 (18)	28 (6)	30 (1)	30 (4)
15	Washing of hands/face	10 (8)	17 (8)	22 (6)	30 (1)	30 (0)	30 (2)
16	Handling hot item	13 (17)	12 (10)	-	30 (0)	-	30 (0)
17	Handling cold item	26 (11)	6 (8)	-	30 (0)	-	30 (0)
18	Repeated handling	30 (3)	30 (1)	-	30 (0)	30 (0)	30 (1)

of DNA deposition are theoretically required to get a consistent shedder status. On the 20 occasions, we also measured sweat rates on the palms of the participants to investigate whether shedder status is associated with sweating. To do this, a Vapometer instrument that measures trans-epidermal water loss per skin surface area and time was applied. Next, we investigated whether environmental conditions such as seasonal variation and the temperature of the touched item influenced the amounts of deposited DNA and the shedder status classification for the six participants. A face-washing prior to DNA deposition was introduced to explore if shedder status could be explained by the hygiene routines of the participants. We hypothesized that if deposition of higher DNA levels were due to infrequent or less thorough showers and face washes, the high shedders would deposit lower DNA amounts following the face-washing. The participants also handled the same plastic tubes ten times in one week to investigate to what extent deposited DNA may accumulate onto an item. Lastly, the inner collars of worn T-shirts were sampled, to explore whether shedder status could be associated with the relative amount of DNA deposited from the neck. The results provide knowledge on whether different factors and conditions influence an individual's shedder status or not, which may be valuable in future investigations of the probability of different DNA transfer scenarios.

2. Materials and methods

2.1. Experimental design

This study was approved by the Swedish Ethical Review Authority (approvals no 2021–00643 and 2021–06891-02). The study was conducted on six male participants aged 25 to 61 years, under written informed consent. The participants were chosen based on their level of DNA shedding in pre-studies, showing that participants #1–3 tended to deposit substantially lower amounts of DNA compared to participants #4–6 (data not shown). To minimize the variation of other parameters, and since men are over-represented as perpetrators of violent crimes, we chose to include only male participants. In total, six separate experiments were performed, some including four or five of the participants (see experimental overview in Fig. 1).

2.1.1. Investigating DNA shedding under everyday conditions

First, DNA shedding of six volunteers was investigated over a four-month period (March-June), when the participants deposited DNA from both hands (each hand was tested separately) onto 50 mL plastic tubes for 30 s at 20 different occasions. This set of data is referred to as the reference set (Fig. 1a). The measurements were randomly distributed during normal working hours, and if a participant was sampled twice on the same day, a minimum of 2 h passed between the deposits. The participants were not aware of the exact time points for the measurements beforehand, to minimize any biased actions such as washing hands or foreheads. On each occasion, the DNA residing on the foreheads of the participants was also measured, since a strong correlation between the DNA amounts deposited from hands and DNA amounts collected from face was found in a previous study [33]. To investigate if there was any correlation between individual sweat rates and deposited DNA amounts, sweat secretions from hands were also determined on each occasion, applying a VapoMeter (Delfin Technologies Oy, Kuopio, Finland) to the palm of the hands. After each occasion, the participants answered a questionnaire stating the time since the last hand wash (< 30 min, 30–60 min, 1–2 h or > 2 h) and the time since the last face wash or shower (the same morning, the day before, 2 days before or ≥ 3 days before).

2.1.2. Effects of external factors and the consistency of shedding

The shedding experiment described in Section 2.1.1 was repeated in November-December of the same year to investigate any effect of seasonal change on DNA shedding and shedder status classification (6 participants, 5 deposits from each hand, giving in total 5 ×2 tubes/samples per participant, Fig. 1b). During this period, the outdoor temperature was 4.1 ± 4.2 °C and the relative humidity was 88.0 ± 8.9% (average ± standard deviations), compared to 10.2 ± 6.2 °C and 71.6 ± 18.1%, respectively, during March – June [40].

To explore the effect of hygiene routines, a study of DNA deposition measurements was performed (6 participants, 5 deposits from each hand, giving in total 5 ×2 tubes/samples per participant), which included thorough washing of hands and face for 15 s each, followed by drying with paper towels, two hours prior to DNA deposition. After the wash, the participants were instructed to carry on with their normal

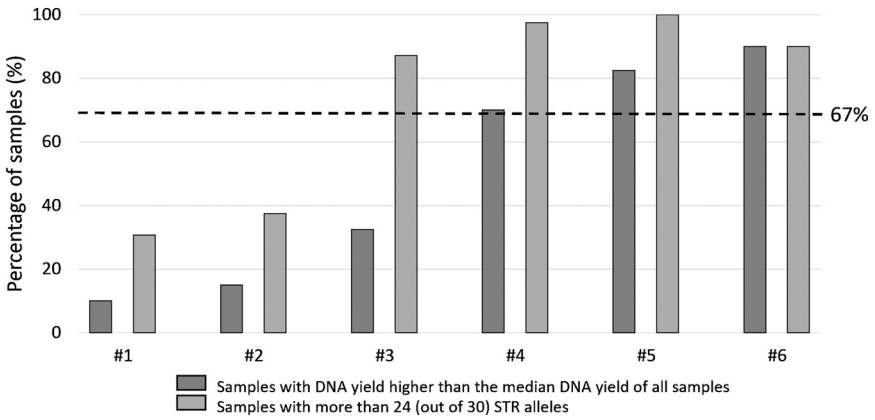

Fig. 2. Shedder classification of the participants as low (#1,2), intermediate (#3) or high shedders (#4–6). High shedders meet both of the following criteria: i) > 67% of the samples should give a higher DNA yield than the median DNA yield of all samples (0.47 ng, n = 240), and ii) > 67% of the samples should give detection of more than 22 (out of 30) STR alleles (n = 40 per participant). Low shedders meet neither of the criteria, and intermediate shedders meet one of the two criteria.

Table 2
Sweat rates (g/m^{2}h) from palms and DNA amounts (ng) collected from the foreheads of the participants at each sampling occasion during March-June (reference set, row 1–2). DNA amounts from foreheads collected in November-December and from the hand/face washing study (row 3–4) are also shown. Next, DNA amounts collected from the inner neck collar of worn T-shirts are presented (row 5) and lastly, DNA amounts deposited from hands in the reference set study are included for comparison (row 6). The interquartile range (IQR) is shown within parenthesis. Asterisk (*) indicates the participants (#) to whom there was a statistically significant difference in DNA deposition amounts.

Row	Experimental setting	Participant #1	Participant #2	Participant #3	Participant #4	Participant #5	Participant #6
Median sweat rates (g/m^{2}h), (IQR)							
1	Reference set (n = 40)	80 (94) * (#2,6)	260 (100) * (#1,3-5)	75 (71) * (#2,6)	90 (60) * (#2,6)	64 (11) * (#2,6)	490 (120) * (#1,3-5)
Median DNA amounts from foreheads (ng), (IQR)							
2	Reference set (n = 40)	1.7 (2.1) * (#3-6)	1.8 (1.1) * (#3-6)	13 (21) * (#1,2)	18 (15) * (#1,2)	21 (27) * (#1,2)	8.9 (8.9) * (#1,2)
3	Nov-Dec (n = 10)	4.8 (3.7)	6.6 (5.8)	17 (15)	22 (32)	8.6 (23)	9.4 (1.4)
4	Washing of hands/face (n = 10)	2.3 (1.9)	3.0 (8.5)	12 (6.7)	17 (15)	15 (26)	7.6 (4.3)
Median DNA amounts from T-shirt (ng), (IQR)							
5	T-shirt (n = 10)	14 (6.3) * (3)	5.5 (4.1) * (3-6)	52 (29) * (1,2)	26 (40) * (2)	28 (12) * (2)	19 (13) * (2)
Median DNA amounts from hands (ng), (IQR)							
6	Reference set (n = 40)	0.13 (0.24)	0.17 (0.24)	0.32 (0.38)	0.78 (0.97)	1.05 (0.95)	1.4 (1.8)

routines, including washing their hands again if needed. In this experiment, DNA was also collected from the foreheads of the participants (Fig. 1c).

The effect on the DNA deposition of the temperature of the handled object was explored by heating and cooling the plastic tubes prior to deposition (4 participants, 5 deposits from each hand, giving in total 5 ×2 tubes/samples per participant, Fig. 1d). The tubes were incubated in a heat cabinet at 75 °C or in a − 20 °C freezer for a minimum of 1 h. Within 10 s after removing the tubes from the heat cabinet or freezer, the participants held the tubes for 30 s. The study included five occasions of DNA deposition onto both heated and cooled tubes. To ensure that no contamination occurred during tube incubation, five negative controls, *i.e.* tubes that were not handled, were included in each trial.

Next, we investigated if the relative amounts of DNA depositions among the participants were affected by repeated handling of objects (Fig. 1e). This was done by instructing the participants to hold and handle the same two plastic tubes, one for each hand, ten times in one working week, with a contact of 30 s each time (5 participants, 3 ×2 tubes/samples per participant). The instructions were to handle the tubes over five consecutive days, with at least three active days of tube handling, with a maximum of four handlings per day and a minimum of one hour between each handling. The plastic tubes were positioned in a piece of polystyrene foam with the conical part down, at a shelf above the desk in the participants' offices during this time. The sides of the tubes were not in contact with anything except when handled by the participants, but no cover was used to protect the tubes from the surroundings. To assess contamination from the surroundings, one tube that the participants were instructed not to touch or handle (negative control) was placed in a corner of the same piece of polystyrene foam as the handled tubes (n = 3 per participant).

Lastly, we investigated if the established shedder status from the participants' hands could be associated with the DNA amounts collected from T-shirts that had been worn on different days for 12 h each by the participants (6 participants, 5 T-shirts per participant, Fig. 1f).

2.2. DNA sampling procedure

DNA depositions from hands and DNA from foreheads were collected as previously described [33]. Briefly, the participants were instructed to firmly hold a sterile 50 mL plastic tube (Sarstedt, Helsingborg, Sweden) in each hand for 30 s, without touching the lower conical part or the lid. The tubes were then placed standing with the lids down in a secured area of the lab space. The tube sides were not in contact with anything during this time. Within 1 h, the deposited DNA was collected from the tubes applying a single cotton swab (Selefa, OneMed Sverige AB, Malmö, Sweden) moistened with 60 μL 0.9% NaCl (Nordkrim, Helsingborg, Sweden). The entire surface of each tube was sampled except for the lower conical part and the lids. Five negative process controls (plastic tubes that had not been touched) were swabbed and analyzed for the presence of any contaminating DNA. After DNA collection, the tip of the cotton swab was cut off just above the shaft, with a clean pair of scissors, placed into a microfuge tube and stored at − 20 °C until further processing.

DNA collection from foreheads was performed in parallel to DNA

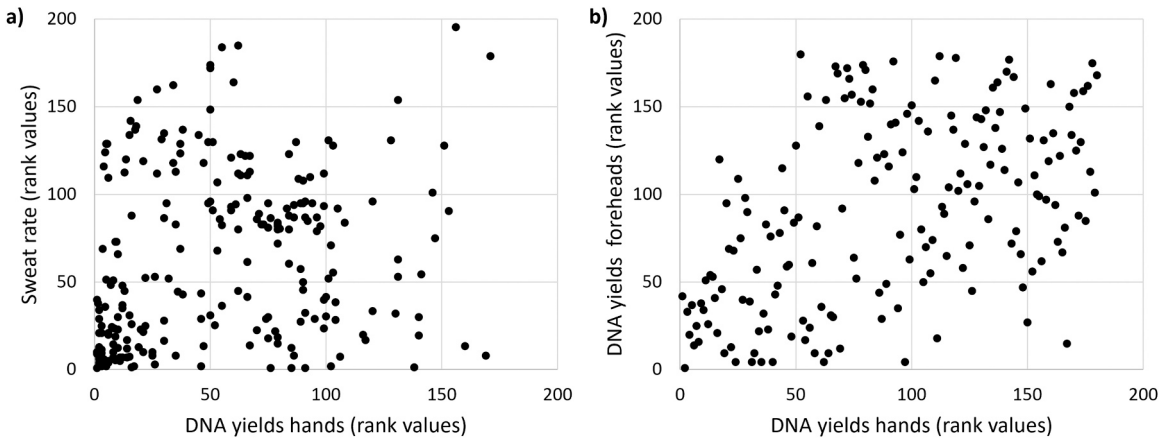

Fig. 3. Correlation plots of a) sweat rates and DNA yields deposited from hands (n = 240), and b) DNA yields from foreheads and DNA yields from hands (n = 180).

deposition in two sets of experiments (reference set: n = 20 ×2 swabs per participant and hygiene routines experiment: n = 5 ×2 swabs per participant, Fig. 1a and c). Two 2 cm × 2 cm squares on each side of the forehead defined by a template cut from a DNA free cloth were swabbed, applying one cotton swab (Selefa) moistened with 100 μL 0.9% NaCl per side [41]. After DNA collection, the swabs were cut off and stored as described above.

New white T-shirts made of 100% cotton in sizes fitting the volunteers were purchased (Stadium, Norrköping, Sweden), machine washed once in 90 °C without detergents and hung dried prior to the experiment. The participants were given six T-shirts each in a paper bag to bring home. They were instructed to wear five of these for 12 consecutive hours per T-shirt and not to limit their usual activities during this time. After wearing the T-shirts, they were instructed to carefully take them off, minimizing contact between the inner neck of the T-shirt and other parts of the body. The T-shirt was placed in a new clean paper bag (one bag per T-shirt) and transported to the laboratory, where DNA was recovered with adhesive tapes (SceneSafe, Burnham-on-Crouch, UK) within 5 days. The sixth T-shirt remained in the initial paper bag at the participant's home during the experiment and was included as a negative control to assess any background DNA, *e.g.*, from the washing. For each T-shirt, one tape was applied on the left side of the inner neck collar and one tape was applied on the right side, applying ten tape lifts per tape. In total, a surface area of approximately 12 cm^2 was sampled on each side of the inner neck collar.

2.3. Sample processing, DNA quantification and STR analysis

DNA extraction using a Chelex-based protocol and DNA quantification by applying the RB1 qPCR assay [42] were performed as previously described [33]. STR analysis was performed using PowerPlex ESX 16 Fast System (Promega Corporation, Madison, WI, USA), VeritiPro Thermal Cycler, ABI 3500 Genetic Analyzer (36 cm capillary array; POP4; injection parameters: 13 s/1.2 kV; analytical thresholds: blue channel 30 rfu, green 45 rfu, yellow 70 rfu and red 75 rfu) and GeneMapper ID-X Software v1.6 (Thermo Fisher Scientific, Waltham, MA, USA). All DNA samples from hands and T-shirts were STR typed. Stutter alleles below the thresholds set by the manufacturer (Promega Corporation) were removed [43]. Electropherograms were examined for self-DNA (alleles corresponding to the STR DNA profile of the participant) and non-self DNA. In accordance with Hansson and Gill [44], up to three foreign STR alleles were considered as drop-in events and were removed. Electropherograms with more than three foreign alleles were considered mixed. Such electropherograms were further manually analyzed as previously described to determine the fraction of self- and non-self DNA in the samples [33]. The percentage of self-DNA in each sample was calculated as ($TPH_{expected\ alleles}$ / $TPH_{total\ alleles}$) × 100, where TPH is the total sum of STR allele peak heights. Based on the result in a previous study, where we found that 99.6 ± 1.2% of the DNA in forehead samples belonged to the donor [33], we concluded that no additional information would emerge from STR typing the forehead samples.

2.4. Sweat rate measurements

A VapoMeter device was used to measure the sweat rates of the participant's palms by holding the device against the center of the open palm. This was done immediately after DNA deposition onto plastic tubes in the reference set experiment, for both left and right palm at each sampling occasion (n = 20 ×2 measurements per participant). The VapoMeter measured transepidermal water loss per skin surface area (a surface area of approximately 1 cm^2 was measured) and time through a humidity sensor that was mounted in a measurement chamber. The chamber was closed by the skin during the measurement period of a few seconds and the sensor recorded the increase of relative humidity (RH) inside the chamber. The evaporation rate value (g/m^2h) was automatically calculated from the RH increase. Ambient temperature and humidity were recorded by the device using an external room sensor.

2.5. Data presentation and analysis

Initial data analysis of DNA yields showed several outliers and that data fitted poorly with a normal distribution. Therefore, robust summary statistics and tests were used to analyze the data, as described below. DNA yields are given as total DNA amounts (ng per extracted sample) and presented as median values and interquartile range (IQR) for each participant. The total DNA yield, the percentage of self-DNA and number of successfully typed expected STR alleles for each sample are available in the Supplementary Material. The participants were classified as low, intermediate, or high shedders based on a model by Johannessen et al. [14], including two criteria. The first criterion is that more than 67% of the samples from one participant should give a DNA yield higher than the median DNA yield of the samples from all participants taken together. The median DNA yield against which all participants were compared was calculated for each set of experiments. The second criterion is that more than 67% of the samples from one participant should give detection of more than 22 (out of 30) expected STR alleles (over 75%). None, one or both criteria should be met to be classified as a low, intermediate, or high shedder, respectively. Since this relative classification model may be somewhat biased due to the small number of participants in our present study, we also classified the participants according to the same model but including data from a previous study with 15 participants [33]. The data on DNA yield from hands from the reference set (March-June) was used to investigate how the

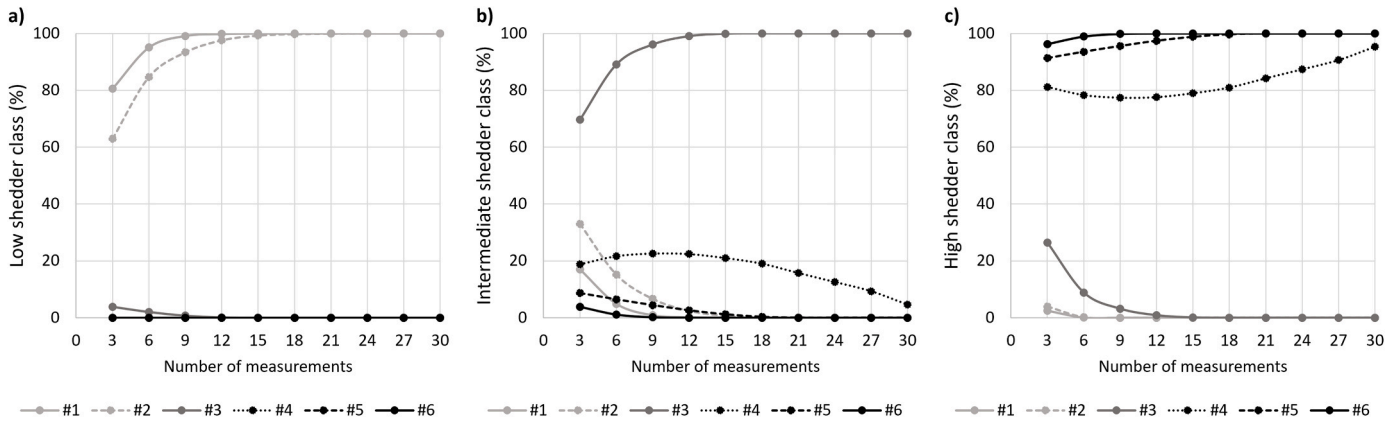

Fig. 4. Plots of the probability of being assigned to low (a), intermediate (b), and high (c) shedder classes (y-axes) as the number of measurements per participant (#1–6) increases from 3 to 30 (x-axes) in steps of 3. The data is based on 5000 random combinations of measurement data for each participant and number of measurements.

number of measurements from each participant influenced the shedder classification. From the 40 data points per participants, 5000 randomly generated combinations per number of measurements, ranging from 3 to 30 measurements in steps of 3, were categorized according to the shedder model described above, applying a script in MatLab (The MathWorks Inc. Natick, MA, USA) described below using pseudo code.

```
For n = 3,6,9, … 30 measurements
      Generate 5000 random combinations of size n per participant in the
      reference data set
      Assign shedder class as high, intermediate, or low for each combination
      according to the model described above
End
```

The results are shown as the percentage of combinations giving rise to high, intermediate, and low shedder classes plotted against the number of measurements.

Inter-individual differences in sweat rates (g/m^2h) and DNA yields (ng) obtained from handled plastic tubes, foreheads or T-shirts were tested statistically with Kruskal-Wallis rank sum test followed by Dunn's test with Bonferroni corrections applied with R software [45,46]. Differences were denoted as significant only when Dunn's test confirmed p-values below 0.05. To investigate whether altered experimental conditions (seasonal change, washing hands and face two hours prior to sampling, the temperature of the handled plastic tubes) influenced the amounts of deposited DNA onto plastic tubes compared to the reference set, Kruskal-Wallis rank sum test followed by Dunn's test with Bonferroni corrections were performed for each participant with the experimental conditions/settings as the factor levels. The same statistical approach was applied to investigate whether seasonal change and washing hands and face two hours prior to sampling influenced forehead DNA levels. Correlations between amounts of deposited DNA from hands and sweat rates or DNA obtained from foreheads were tested statistically with Spearman's rank correlation performed with R software [47]. Differences in DNA yields between dominant and non-dominant hands from the reference data set were tested with paired t-test.

Kruskal-Wallis rank sum test followed by Dunn's test with Bonferroni corrections was performed to investigate whether there was a difference in DNA amounts from handled plastic tubes, foreheads or T-shirts depending on the time since last hand or face wash/shower.

3. Results

3.1. Deposited DNA amounts and shedder status under different conditions

Six male participants deposited DNA onto plastic tubes on 20 different occasions over a four-month period (March – June, reference set, $n = 20 \times 2$ depositions per participant). Significant differences in deposited DNA amounts were seen between the participants (Kruskal-Wallis rank sum test: $p < 0.0001$ and Dunn's test: p ranging from $<$ 0.0001 to 0.04, $n = 40$ per participant, Table 1, row 1). We classified the participants as low (#1,2), intermediate (#3) or high shedders (#4–6) based on deposited DNA amounts and the number of detected STR alleles (Fig. 2), using a method modified from Johannesson *et al.* [14]. The median number of detected STR alleles (out of maximum 30) was 19 (#1), 18 (#2), 28 (#3) and 30 (#4–6) (Table 1, row 13). The DNA amounts from low shedders were below 0.2 ng in 67.5% and above 0.5 ng in 12.5% of all samples. In a few samples from the low shedders, no DNA could be detected (#1: 0 – 1.2 ng; #2: 0 – 1.0 ng). Only 2.5% of the samples from the high shedders gave DNA amounts below 0.2 ng while 80% gave DNA amounts above 0.5 ng (#4: 0.15 – 30 ng; #5: 0.16 – 12 ng; #6: 0.22 – 8.1 ng). The intermediate shedder (#3) deposited DNA levels in the lower range (0 – 1.6 ng) although the median DNA amount was twice as high compared to the low shedders (Table 1). When the participants were classified in the context of a larger data set from a previous study including 15 additional participants [33], the shedder classes did not change for any of the six participants (data not shown).

The amounts of deposited DNA were not affected by seasonal change, when hands and face were thoroughly washed two hours prior to sampling, or when DNA was deposited onto hot or cold tubes (Kruskal-Wallis

rank sum test and Dunn's test: $p > 0.05$, Table 1, row 1–5). Consequently, the shedder status of participants previously categorized as low (#1 and #2) and high shedders (#4–6) persisted when the sample data from the studies with changed conditions was analyzed. Participant #3 however, previously categorized as an intermediate shedder, was categorized as a low shedder in the experiment performed during November-December and the experiment with a washing step at 2 h prior to sampling.

When five participants handled plastic tubes repeatedly (10 times for 30 s over 5 days), the amount of accumulated DNA reflected the previously assigned shedder status of each participant (Table 1, row 6). Compared to a single DNA deposition, the median DNA yields increased by a factor of 6 – 18 for participants #1, #2, #5 and #6, and by a factor of 92 for participant #4 (Supplement Tables S1–12). All samples ($n = 6$ per participant) from the low shedders (#1 and #2) gave DNA yields below the median DNA yield of the samples from all participants in this experiment. The percentage of samples with a higher DNA yield than the median for all samples from the high shedders (#4–6) was $\geq 67\%$. This reflects the first criterion of low and high shedder classification. The second criterion for shedder status determination, based on the percentage of samples with > 22 detected alleles, is clearly not applicable for repeatedly handled items. Due to the accumulated DNA, the samples from all participants had more than 22 detected alleles in $> 67\%$ of the samples (Table 1, row 18).

In line with previous studies [18,20,21,48], there were no differences in DNA deposition between dominant and non-dominant hands when the participants were assessed individually (paired t-test, $p = 0.24$ - 0.99, $n = 29$ for #3, $n = 32$ for #5, $n = 42$ for #1, #2, #4, and #6). Neither was there any difference cohort-wide between dominant (median 0.52 ng, IQR 1.0) and non-dominant (median 0.52 ng, IQR 1.5) hands (paired t-test for all participants, $p = 0.32$, reference set; $n = 234$).

Non-self STR alleles were detected in more than 50% of the samples from participants #1, 3, 4, and 6, while fewer samples from participants #2 and #5 contained non-self DNA (18%). Interestingly, participant #2 and #5 lived alone during all experimental trials, but so did participant #1. The amount of non-self DNA in each sample was low compared to the amount of self-DNA (the average percentage of non-self DNA was 26% for #1, 22% for #2, 12% for #3, 9% for #4, 3% for #5, 5% for #6, Supplement Tables S1–6). The low shedders (#1 and #2) had a higher percentage of non-self DNA than the high shedders (#4–6), coinciding with observations reported in other studies [21,24,25,48].

Negative process controls from the study assessing repeated handling (plastic tubes that were placed next to the handled tubes during the five-day experiment, $n = 3$ per participant), harbored low amounts of DNA in comparison to the repeatedly handled tubes, ranging from 0.09 $\pm$ 0.07 ng (#6) to 0.55 $\pm$ 0.32 ng (#5). The majority of the detected STR alleles on the non-handled tubes originated from the participant himself (around 60% for #6% and 90% for #5). No other negative process controls (swabbed clean plastic tubes, $n = 5$) carried any detectable DNA.

3.2. Potential factors behind inter-individual differences in shedder status

The sweat rates from hands were measured with a VapoMeter on each sampling occasion in the reference set experiment. The sweat rates differed significantly between participants (Kruskal-Wallis rank sum test and Dunn's test: $p < 0.0001$, $n = 40$ per participant, Table 2, row 1), but there was no correlation to the amounts of deposited DNA from hands cohort-wide (Spearman's rank correlation $\rho = 0.009$, $p = 0.90$, $n = 240$, Fig. 3a). Neither was there any clear association between sweat rates and DNA deposition on an individual level (Spearman's rank correlation #1: $\rho = -0.09$, $p = 0.58$, #2: $\rho = -0.13$, $p = 0.43$, #3: $\rho = -0.02$, $p = 0.90$, #4: $\rho = -0.38$, $p = 0.02$, #5: $\rho = -0.04$, $p = 0.80$, #6: $\rho = -0.04$, $p = 0.80$, $n = 40$ per participant).

The DNA yields from foreheads also differed substantially between participants (Kruskal-Wallis rank sum test and Dunn's test: $p < 0.0001$, $n = 40$ per participant, Table 2, row 2). The forehead samples collected in November-December contained similar levels of DNA compared to samples collected in March-June (reference set) except for the low shedders #1 and #2, who gave higher forehead DNA levels in November-December (Kruskal-Wallis rank sum test: $p = 0.04$ (#1) and $p = 0.0001$ (#2) and Dunn's test: $p = 0.03$ (#1) and $p = 0.0001$ (#2), Table 2, row 3). DNA amounts from foreheads did not change for any of the participants when hands and face were washed two hours prior to sampling compared to the reference data set without a controlled washing step (Kruskal-Wallis rank sum test and Dunn's test: $p > 0.05$, Table 2, row 4). In line with our previous findings [33], a correlation was seen between DNA amounts deposited by hands and DNA amounts collected from foreheads (Spearman's rank correlation $\rho = 0.49$, $p < 0.0001$, $n = 180$, Fig. 3b).

To investigate whether the given shedder status reflected the relative amount of DNA obtained from other body parts, the participants wore T-shirts for 12 h. Statistical analysis showed that DNA amounts from the T-shirts differed significantly between participants (Kruskal-Wallis rank sum test: $p < 0.0001$ and Dunn's test: p ranging from < 0.0001 to 0.03, $n = 10$ per participant, Table 2, row 5). The lowest DNA yield was retrieved from participant #2 followed by #1 (both low shedders), #6, #4, and #5 (all high shedders), and the highest DNA yield was obtained from #3 (intermediate shedder). The results from T-shirts are thus concordant for the participants categorized into low and high shedder status, but not for participant #3 who was previously categorized as an intermediate shedder. The negative controls, *i.e.,* T-shirts that were not worn but otherwise handled in the same manner as the worn T-shirts, contained low amounts of DNA ranging from 0.01 $\pm$ 0.02 ng (#2,3) to 0.84 $\pm$ 0.24 ng (#4) compared to the worn T-shirts.

The time since the participants took a shower had no effect on the amount of DNA collected from the T-shirts (Kruskal-Wallis rank sum test: $p = 0.31$, $n = 5$ occasions, Supplement Tables S7–12). Similarly, the time since the last hand or face wash was not associated with the shedder status of the participants as the low shedders deposited less DNA from both hands and foreheads compared to the high shedders regardless of the time since last wash (Supplement Fig. S1 and Tables S1-12). Thus, our data do not support hygiene routines as a major factor behind shedder status classification.

3.3. Effect of increasing the number of DNA deposition measurements

When the data from the reference set ($n = 40$ per participant) was randomly combined in sets of three to 30 in steps of three measurements per participant and subsequently classified as high, intermediate, and low shedder, it was revealed that at three measurements, the probability of being assigned to the "true" shedder class (*i.e.* identical to the classification with 40 measurements) was above 60% for the low shedders, around 70% for the intermediate shedder, and above 80% for all high shedders (Fig. 4). The probability for correct classification increased substantially with six and nine measurements, especially for the low and intermediate shedders. With 12 measurements, all participants except for #4 had a probability above 97% to be assigned to their "true" shedder class.

4. Discussion

We have shown that changes in the experimental settings had no or very little influence on the amounts of shed DNA and thus the shedder status classification of six male participants, known from previous studies to differ in their DNA shedding propensities. We addressed seasonal change, that is, when DNA depositions were performed during November-December instead of March-June. It is known that drier skin, which normally comes with cold weather and low humidity [49], can influence DNA shedding [22]. We found no effect of the seasonal change on DNA deposition, but this may in part be due to the mild climate of

southern Scandinavia where the studies were conducted. The mean temperature for November-December was 6 °C lower than in March-June while the mean humidity was higher, 88% compared to 72% [40]. These differences may be too small to have a clear effect on the skin, but it is also possible that the increase in humidity may counteract the effect of the temperature drop in November-December. The outcome may have been different in other parts of the world, where temperature and humidity differences between seasons may be more extreme. It has been shown that environmental temperature and humidity can impact the persistence of deposited DNA over time, depending on the material of the touched object [50]. When we investigated whether heating or cooling the handled object influenced DNA deposition *per se*, we found no such effects for any of the participants.

The amounts of DNA collected from foreheads correlated to the level of deposited DNA from hands, in line with a previous study [33]. Around ten times more DNA was obtained from the faces of intermediate and high shedders than from the low shedders. On average, people touch their face 23–50 times per hour [51,52], and it has been suggested that DNA accumulates on hands by touching one's face and other body parts as well as personal belongings [3,20,33,38,53]. We also found that the amounts of DNA collected from the inner neck collar of T-shirts were associated with the wearer's DNA shedding propensity, at least for the low and high shedders. These findings suggest that the shedder status of an individual is reflected by the amounts of DNA residing on the skin of the face and neck, and it is tempting to hypothesize that relative DNA levels are correlated also between other body parts. However, Goray and van Oorschot [24] found no such association between the shedder status of their participants and the DNA collected from arms, foreheads, and necks. Another study assessed the amounts of cellular material in lip- and thumbprints, but no correlation was detected between the two [34].

Little is known about the accumulation of DNA following repeated handling of an item, which is the case for regularly used personal items. There have been speculations that the relative accumulation of deposited DNA may decline with repeated handling and eventually lead to "saturation" of the handled item due to an equilibrium between deposition and removal of DNA [6]. If this was the case, the DNA levels on a regularly used item would not reflect the shedder status of the individual. To address the question, the participants in our study deposited DNA onto tubes ten times during five days. The obtained DNA amounts did reflect the shedder status of the participants, with substantially higher DNA amounts accumulated from the high shedders. These results give no indication of a DNA saturation effect when objects are handled up to ten times. Whether longer handling periods may eventually lead to a state of saturation of DNA on an object remains to be elucidated.

Both physiological and behavioral mechanisms have been suggested as potential factors governing shedder status [14,35,54]. Individual ability to secrete sebaceous fluid on the skin surface has been proposed as a possible influence as it was shown that most of the deposited DNA originated from sebaceous skin areas [55,56]. When assessed, there were no indications that sebum production was associated with DNA levels residing on the skin or DNA shedding from hands [33]. Melanin production has also been implicated as a possible physiological agent affecting DNA shedding, but no correlation between the two could be established [35]. Individual sweat rates have frequently been suggested as a potential determinant behind shedding [12,14,55], as sweat contains cell-free DNA which has been found to be a component of touch DNA [39,57]. In the present study, we assessed the sweat rates of the six participants by measuring trans-epidermal evaporation rates from palms on each sampling occasion. Indeed, we found significant differences in sweat rates between the participants, which is in line with other studies reporting inter-individual variations in the function of the human eccrine sweat glands [58]. However, we found no association between DNA shedding and sweat rates. The results may have been different if female participants were included, as sweat gland activity is regulated by hormones and known to be different in men and women [59–61].

Personal hygiene habits have also been proposed as a potential factor influencing DNA shedding [14], but conclusive data are lacking. Cedillo-Cruz *et al.* reported a positive correlation between the amounts of deposited DNA and the time since last shower [35], while others found no association between DNA shedding levels and hygiene routines [48]. We wanted to assess the impact of hygiene routines further and asked the participants to wash their faces and hands thoroughly two hours prior to DNA sampling. We hypothesized that if hygiene habits influenced shedding levels, we would observe lowered DNA amounts following the thorough face wash, particularly for the high shedders. However, no significant differences in obtained DNA amounts were seen between the experiments with and without washing, for any of the participants. We can conclude that a thorough wash of the face, applying plenty of soap and washing for 15 s, is either not sufficient to remove cells and DNA residing on the skin, or that cells and DNA indeed is removed but accumulate again in the two hours that passed between the wash and DNA sampling. Either way, this suggests that differences in washing routines are not sufficient to explain inter-individual differences in shedder status. This is further supported by the data on the washing habits of the six participants. On each sampling occasion, the participants stated the last time they washed their hands and face or took a shower, but there was no correlation between this information and the deposited DNA amounts. It has previously been reported that no association between deposited DNA amounts and time since the last hand wash can be established [21,25,26,37] unless hand wash was performed immediately prior to the DNA sampling, when a decrease in deposited amounts was seen [38,62]. The quick DNA accumulation on hands is bound to be very different from how DNA accumulates on the skin of the rest of the body. While most DNA on hands originate from touching one's own body and personal belongings [33], DNA may accumulate on the skin of other body parts more slowly and presumably due to the turnover of keratinocytes and other cells [12].

We found extensive fluctuations in intra-individual measurements, and some high shedder samples had DNA levels in line with the low shedder samples. The fact that the amount of deposited DNA can vary heavily for one individual from one time to another has previously been shown by others [20,23,27,29,30,63]. Performing only one or few measurements to estimate shedder status may thus give skewed results, meaning that multiple measurements are required for a proper estimation of shedder status. When we used our reference data set to randomly generate 5000 combinations of different numbers of measurements for each participant, we found that the probability of estimating the correct shedder class increased substantially when six measurements were used instead of three. For all but one participant (#4, a high shedder), the probability of being assigned to the correct shedder class was close to 100% when 12 measurements were used per participant. In most research on DNA shedding, including ours, shedder classification relies on the relative DNA amounts that are obtained within each study and are thus dependent and may be limited by both the shedding ability and the number of participants included [9,14,21,28]. Our study included a rather small number of participants, but when we performed an additional shedder classification applying a data set from a previous study with 15 participants, we were able to confirm their assigned shedder classes. One participant initially categorized as an intermediate shedder was classified as a low shedder under changed experimental settings but gave high DNA levels from face and T-shirts. Thus, the intermediate shedder deviates from the observed correlation between DNA amounts on face and hands, but since only one of our six participants was an intermediate shedder, we do not have adequate data to suggest that this is an intermediate shedder trait. However, others have shown that intermediate shedders are more prone to alternate between shedder categories when classified more than once [14]. Whether intermediate shedders are a product of relative definitions of shedder classes or a population that truly vary more in their shedding propensities, requires further investigation.

5. Conclusions

All our results taken together suggest that low and high shedder status are rather stable properties across time and different seasons, the contact surface temperature of the handled object, and individual washing habits. The relative DNA levels obtained from repeatedly handled tubes and worn T-shirts reflected the shedder status of the participants, as did the amounts of DNA collected from the foreheads. We further assessed whether individual sweat rates and hygiene routines may dictate shedder classification but found no support for this. The importance of multiple measurements of DNA deposition for an increased probability of correct shedder classification is highlighted, and we conclude that around 12 measurements are needed to approach close to 100% probability, at least for our data set. The results from this study add knowledge to how individual shedder status may or may not vary across environmental settings and may be valuable for experts when interpreting DNA results on activity level.

CRediT authorship contribution statement

Linda Jansson: Conceptualization, Investigation, Methodology, Supervision, Visualization, Writing – original draft. **Chiara Siti:** Investigation, Methodology, Writing – review & editing. **Ronny Hedell:** Methodology, Writing – review & editing. **Christina Forsberg:** Conceptualization, Writing – review & editing. **Ricky Ansell:** Conceptualization, Writing – review & editing. **Johannes Hedman:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2023.103002.

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