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Prolonged and localized sweat stimulation by iontophoretic delivery of the slowly-metabolized cholinergic agent carbachol

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ABSTRACT

Background: Continuous non-invasive sampling and sensing of multiple classes of analytes could revolutionize medical diagnostics and wearable technologies, but also remains highly elusive because of the many confounding factors for candidate biofluids such as interstitial fluid, tears, saliva, and sweat. Eccrine sweat biosensing has seen a recent surge in demonstrations of wearable sampling and sensing devices. However, for subjects at rest, access to eccrine sweat is highly limited and unpredictable compared to saliva and tears.
Objective: Reported here is a prolonged and localized sweat stimulation by iontophoretic delivery of the slowly-metabolized nicotinic cholinergic agonist carbachol.
Methods: Presented here are detailed measurements of natural baseline sweat rates across multiple days, confirming a clear need for localized sweat stimulation. Iontophoresis was performed with either carbachol or pilocarpine in order to stimulate sweat in subjects at rest. Furthermore, improved methods of quantifying sweat generation rates (nL/min/gland) are demonstrated.
Results: In vivo testing reveals that carbachol stimulation can surpass a major goal of 24-h sweat access, in some cases providing more than an order of magnitude longer duration than stimulation with commonly used pilocarpine. Also demonstrated is reduction of the traditional iontophoretic dosage for sweat stimulation ($<5.25–42\ mC/cm^2$). This increases the viability of repeated dosing as demonstrated herein and for carbachol is as much as 100–1000X less than used for other applications.
Conclusion: This work is not only significant for wearable sweat biosensing technology, but could also have broader impact for those studying topical skin products, antiperspirants, textiles and medical adhesives, nerve disorders, the effects of perspiration on skin-health, skin related diseases such as idiopathic pure sudomotor failure and hyperhidrosis, and other skin- and perspiration-related applications.
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1. Introduction

Eccrine sweat has recently been gaining significant attention as a non-invasive method to access many electrolyte, molecule, and protein analytes found in blood [1]. Such attention builds on established commercial use of sweat for cystic fibrosis testing [2] and testing for metabolites of drugs of abuse [3,4]. The recent surge of interest in sweat biosensing has been caused by a demand for wearable technology that can continuously measure chemical analytes, and because sweat has several inherent advantages compared to other non-invasive biofluids [5–9]. Interest in sweat biosensing has also been inspired by numerous technological demonstrations of small and ergonomic tattoos, bands, and patches which can continuously monitor electrolytes and metabolites in sweat [5,7,10–12]. However, despite the increasing interest, rationale, and device demonstrations, there is a major unresolved confounding issue: sweat access is limited and unpredictable for individuals at rest. Although on-demand sweat stimulation using iontophoresis does exist [2,13], it has been limited primarily to acetylcholine and pilocarpine, which produces a localized sweating response of only several minutes or ~90 min, respectively. Similarly, recent demonstrations of integrated sweat stimulation and sensing have also been limited to about an hour or

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less [14,15]. These hour-long stimulations are useful for point-of-care testing, and are used extensively for cystic fibrosis testing [2], but are inadequate for continuous sensing for 24 h or more.

Reported here is a prolonged and localized sweat stimulation by iontophoretic delivery of the slowly-metabolized agent carbachol. In most subjects, in-vivo testing reveals that carbachol stimulation surpasses a major goal of 24-h generation of sweat. This result is more than an order of magnitude longer duration than that achieved with commonly-used pilocarpine stimulation. Also demonstrated are iontophoretic dosages that are dramatically lower (up to 100–1000X) than other medical uses of carbachol. Furthermore, using these lower dosages, feasibility of repeated stimulation at the same stimulation site is shown. The results of this work are only important from an applied perspective, if natural sweating events are too infrequent or too low in sweat generation rate. Therefore, detailed measurements of natural baseline sweat rates are presented across multiple days, confirming a clear need for localized sweat stimulation. Lastly, this work provides demonstration and rationale for improved yet simple methods of accurately quantifying sweat generation rates (nL/min/gland). This work is not only significant for wearable sweat biosensing technology, but could also have broader impact for those studying topical skin products, antiperspirants, textiles and medical adhesives, nerve disorders, the effects of perspiration on skin-health, skin related diseases such as idiopathic pure sudomotor failure and hyperhidrosis, and other skin and perspiration related applications.

2. Rational for choice of carbachol and for dosages

Acetylcholine (ACh), a naturally produced sweat stimulant, is rapidly hydrolyzed in milliseconds via its complementary enzyme, acetylcholinesterase (Table 1) [16]. In the body, a short duration is advantageous as rapid switching from sweating to non-sweating states is required. However, in a sweat stimulating device, large amounts of ACh sweat stimulant would need to be continually dosed to produce a sustained sweating response. For sweat analysis devices, frequently repeated or continuous stimulation is disadvantageous because it can potentially result in skin damage by electroporation [17] or with poor device design, pH damage [18].

Table 1
Structure and activity of cholinomimetic drugs. Adapted from [16].

	Receptor Activity		
	Nicotinic	Muscarinic	AChE Hydrolysis
Acetylcholine	+++	+++	+++
Bethanechol	+++		–
Carbachol	++	+++	–
Methacholine	+++	+	++
Pilocarpine	++	–	–

Cholinomimetic, carbamic acid esters such as carbachol and bethanechol are more resistant to acetylcholinesterase (AChE) hydrolysis, on the order of 10^7 slower than their acetyl counterparts like ACh [19]. For carbachol and bethanechol, resistance to AChE hydrolysis is achieved by replacing the methyl tail group on ACh with a primary amine group. This allows such molecules to remain and interact at the ACh receptors at the base of the sweat gland and therefore produce sweat for extended periods (hours or more). It should be noted that pilocarpine, an alkaloid muscarinic cholinergic agonist, is also weakly hydrolyzed by AChE but is shorter-acting than carbachol. Pilocarpine is likely metabolized by other mechanisms [20]. Due to their hydrophilicity, choline esters also have poor absorption and distribution within the central nervous system [16,21].

Being slowly metabolized is not the only consideration that may matter for sweat stimulation. For example, although bethanechol or methacholine are slowly metabolized, the β-methyl group of such molecules restricts nicotinic activity, producing a lesser nicotinic-induced sweating response (Table 1). Therefore, the full receptor capability of the eccrine gland would not be utilized (i.e. both nicotinic and muscarinic cholinergic receptors). Details of muscarinic vs nicotinic cholinergic pharmacology and sweating response can be found elsewhere [16,22,23]. With this consideration, in this work it was hypothesized that carbachol could be an excellent prolonged sweat stimulant because (a) it is not susceptible to AChE, (b) it has both high nicotinic (c) and muscarinic recptor activity. Having nicotinic activity is also beneficial in a wearable device, because it can facilitate co-location of sweat sensing and stimulation based on a sudo-motor-axon reflex sweating response [14].

It should be noted that since carbamic acid esters such as carbachol are longer lasting, non-selective, and parasympathomimetic molecules, great care should be taken with their use. There are multiple previous reports for localized delivery of carbachol in the literature, including by iontophoresis, intradermal/intracutaneous injections and for intravenous infusion. For iontophoretic applications, reports of iontophoretic dosages of 300 mC or ~600 μg carbachol (1 mA for 5 min) have been reported [24–27]. These values are ~4–40 x larger than those utilized here (Table 2). Comparatively, reported dosages from two papers via intradermal [28] and intracutaneous [29] injections tallied approximately 15 μg and 0.01 μg, respectively. Furthermore, two studies [30,31] have reported use of 3 μg/kg of carbachol over 25 min via a venous catheter with one subject out of 12 experiencing side effects (dizziness) at infusions of 2 μg/kg [30] and 9 of 12 healthy patients experienced headaches at infusions of 3 μg/kg [31]. Furthermore, most subjects exhibited headaches at 3 μg/kg and more severe side effects (urges to void) at 4 μg/kg [30]. Carbachol is also prescribed for post-operative patients to relieve intraocular pressure by injection of 0.5 mL 0.01% solution (50 μg) into the anterior chamber [32], and to relieve urinary retention by oral dosing at 2 mg [33]. Lastly, for glaucoma, 2 drops of a 0.75-3% carbachol solution can be administered up to 4 times daily which is 0.75–3 mg total daily [34].

Consider the 100%, 50%, 25% and 12.5% dosages used here where the percentage is in comparison to the commercial Wescor Nanoduct iontophoretic pilocarpine dosage (Table 2). Assume a wearable device that requires a 1 cm^2 collection area for a practical sweat sampling and sensing (~100 active glands, Fig. 2, total sweat sampling rate of 0.1 to 1 μL/min). The total daily dosages at 100% and 12.5% Wescor NanoDuct dosage would only be ~80 and ~10 μg for this 1 cm^2 stimulation area. Even the triple repeated dosing experiments conducted here at 12.5% dosage would not exceed ~30 μg/day. Further assume a minimum requirement of 10 active eccrine glands for a viable sweat biosensing device (~1 mm^2, ~10–100 nL/min sampling rate), the required dosages could be as

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Table 2
Percentages used in this work for carbachol dosing, in comparison to the commercial Wescor Nanoduct dosage for pilocarpine. Each stimulation pad (Fig. 1) had an area of 0.88 cm^2 with two stimulation pads being used per user for a total stimulation area of 1.76 cm^2.

Carbachol Dose	Current (mA/cm^2)	Time (s)	Charge (mC/cm^2)	Carbachol Delivered ($\mu g/cm^2$)[a]
12.5%	0.28	18.75	5.25	<9.94
25%	0.28	37.5	10.5	<19.88
50%	0.28	75	21	<39.76
100%	0.28	150	42	<79.53

[a] Theoretical maximum amount. Realistically less than 30% of the total delivered is actually delivered (see Section 3.3).

little as ~8 μg per day. Lastly, as noted at the end of Section 3.3, the efficiency of the delivery of carbachol into the skin is likely less than 30% of the total iontophoretic dose [35]. This reduction in delivered carbachol is due to reduced iontophoresis efficiency from the competition with other mobile ions that are present. Therefore, the actual required dosages could be even lower (perhaps even less than 2.4 $\mu g/mm^2$).

In summary, although our delivery method and direct proximity to blood circulation are different than existing medical applications, we are generally encouraged, given the lower dosages demonstrated here and the potential for small stimulation areas. Reduced dosage should help alleviate possible side-effects and reduce interactions that carbachol may have with other drugs [16].

3. Materials and methods

3.1. IRB protocol

Human subjects testing was performed under the guidance of the University of Cincinnati's (UC) Human Research Protection Program (ID# 2016-0015 approved by the UC Institutional Review Board).

3.2. Reagents and materials

Carbachol 99% (CAS 51–83-2) was purchased from Professional Compounding Centers of America (PCCA, Houston, TX), 99.9%

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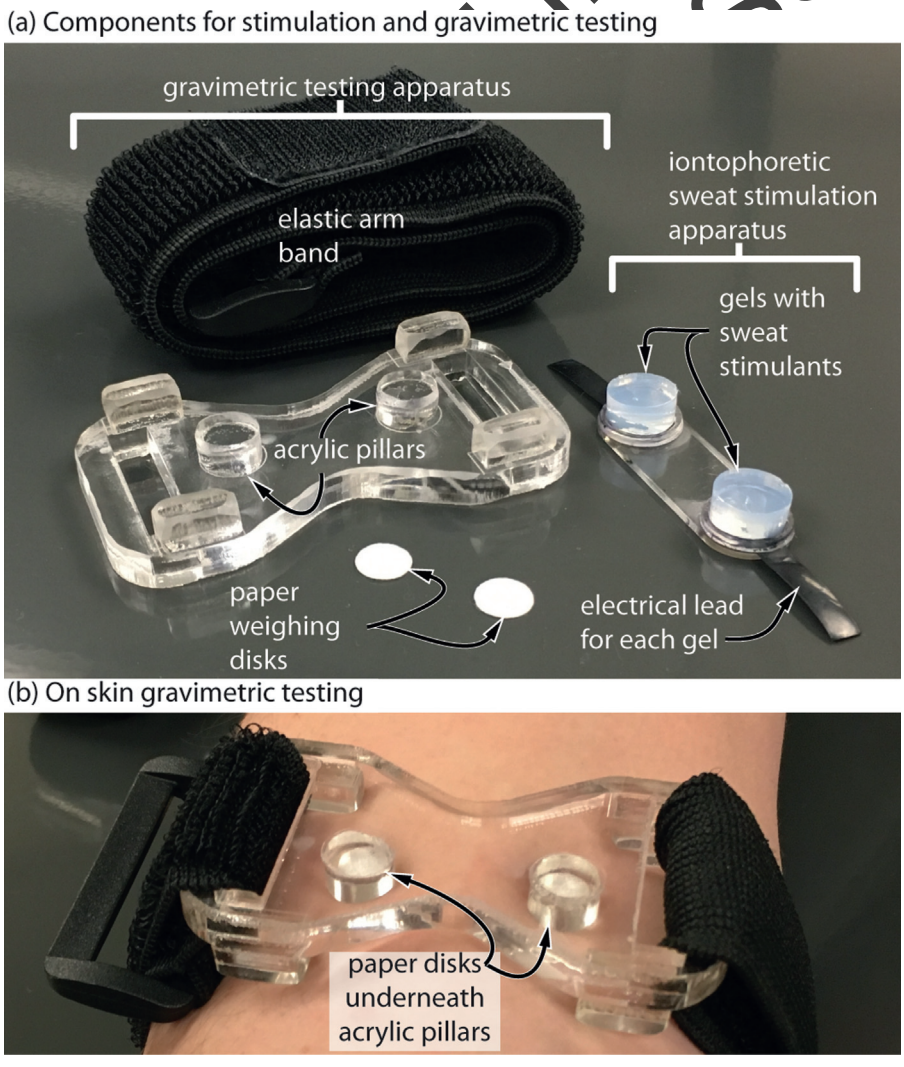


Fig. 1. **(a)** Components for stimulation and gravimetric testing; **(b)** on skin gravimetric test.

agarose (A9539) and bromophenol blue (B0126) were purchased from Sigma Aldrich (St. Louis, MO). Pure 5000 cST and 1000 cST cosmetic-grade polydimethylsiloxane (PDMS) was purchased from ClearCo Products (Willow Grove, PA). 0.25" and 0.06" thick acrylic was purchased from McMaster-Carr (Aurora, OH). Weld-On #3 Acrylic Adhesive was purchased from IPS Corporation (Gardena, CA). Carbon Kapton Film (Kapton 200RS100, 105 Ω/square) was purchased from Dupont (Wilmington, DE).

3.3. Gels for iontophoretic stimulation of sweat

Pilocarpine gel discs were purchased from Wescor, Inc. (South Logan, Utah) and cut down to a smaller size of 0.635 cm thickness and 1.06 cm diameter (~0.88 cm^2 each). Carbachol gel discs of the same dimensions were fabricated using 1% carbachol and 3% agarose, by weight, in deionized water. The carbachol and agar solution was first heated to 150 °C and stirred for 30 min. The aqueous solution of carbachol remains stable even when heated [36]. Next, deionized water lost due to evaporation during the heating/stirring process was added back to the solution and this solution was then again heated at 80 °C for 30 min to ensure the added water was evenly distributed. The carbachol/agarose solution was then cast in an acrylic mold which provided an array of discs of the same dimension as the pilocarpine gel discs. The mold was then placed into a refrigerator at 8 °C where the discs were allowed to solidify. Finally, the carbachol discs were removed from the mold and stored at 8 °C in a plastic bag containing 1% carbachol in deionized water.

Preliminary calculations were done to estimate the amount of charged drug that would be iontophoretically delivered to the subject. The dose of the charged drug delivered was estimated as the product of the current (mA or mC/s) and the duration of iontophoresis (s), which provides the delivered charge (mC). From this the charge can be used to calculate the moles of electrons utilizing Faraday's constant (96,485 C/mol). Since the drugs delivered are monovalent, an estimation of the mass delivered can be made with no adjustment for valence (Table 2). Importantly, based on previous work [35] we estimate that ~30% of the charge delivered in Table 2 are associated with the stimulant while the remaining ~70% are mainly other smaller ionic species including Cl^- from beneath the skin. These competing ions, as well as drug deterioration could cause lower amounts of stimulant to be delivered. To ensure precision in the iontophoretic dosing, the gel discs were all fabricated at the same time and the skin was cleaned prior to stimulation (Section 3.5).

3.4. Apparatus for sweat stimulation and for gravimetric testing

The stimulation and gravimetric holders were designed in AutoCAD (Autodesk San Rafael, CA). The full dimensions for these holders are provided as AutoCAD files in online Supplementary material for this paper. The various layers for the holders were then laser cut and epoxied using Weld-on #3 acrylic adhesive. An

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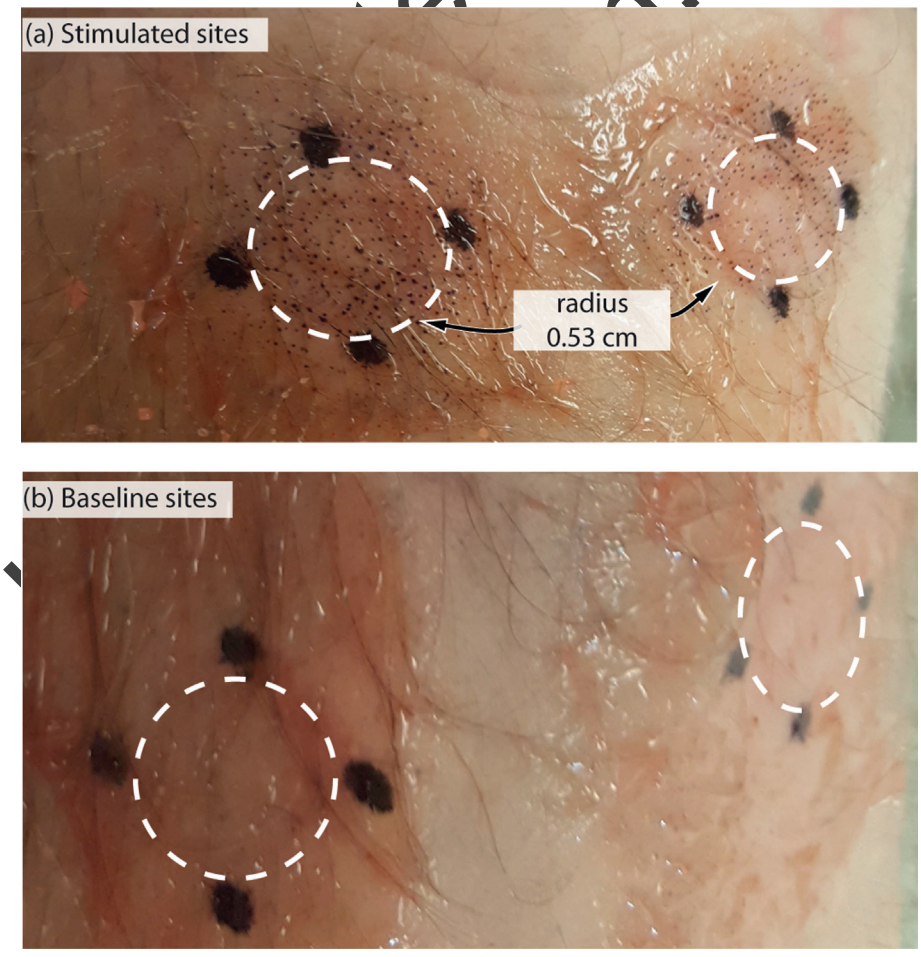


Fig. 2. Example experimental result in sweat pore imaging for both the **(a)** stimulated and **(b)** baseline sites.

additional carbon-coated Kapton film (<105 ohm/square) was epoxied to the stimulation apparatus to provide a driving electrode for iontophoresis of the sweat stimulant. The completed apparatus for sweat stimulation and for gravimetric testing is shown in Fig. 1.

3.5. Iontophoresis stimulation

Iontophoresis was performed utilizing a commercial iontophoresis unit with current and dosage controls (ActivaDose II, ActivaTek, Gilroy, CA). Before stimulation, subjects first had the volar surface of their dominate forearm cleaned with isopropanol (IPA) and deionized water to remove potential contaminants. Then the acrylic stimulation holder and the selected stimulant gel of pilocarpine or carbachol (Fig. 1) were placed on the clean location. A return electrode of the same gel disk was also placed on the clean surface. Iontophoresis was then performed at the carbachol doses listed in Table 2, or at a dose of ~90.65 μg/cm^2 for pilocarpine. The carbachol doses presented in Table 2 are listed as a percent of the standard iontophoretic dose (42 mC/cm^2) used in the commercial Wescor Nanoduct sweat stimulation device.

It is important to note that the two stimulation sites (medial and lateral of the dominant forearm) were delivered individually so that the current dosages for each would be as accurate as possible. Performing iontophoresis on both sites simultaneously could cause errors in current dosage if one site had a different electrical resistance of skin than the other. To perform these stimulations individually, the sweat stimulation holder in Fig. 1 was held in a random fixed location against the forearm, and medial and lateral iontophoretic stimulations were performed serially (one after the other). Once stimulation occurred no additional stimulation was performed for at least 24 h, with exception of the results shown in Fig. 10, which were performed all on the same location once the sweat rate was determined to be below 1 nL/min/gland. Following stimulation, images of the stimulation site were taken to monitor for potential irritation. Because some tests lasted as long as several days, a Sharpie™ marker was applied, and reapplied as needed, to mark the stimulation sites and allow alignment with the testing methods used (Sections 3.6–3.8).

3.6. Basis peak watch and Gamry potentiostat

A Basis Peak™ fitness monitoring watch was attached to the volar surface of the dominate wrist of three subjects in a location that was not iontophoretically stimulated. Continuous data were obtained from this source which includes galvanic skin response (GSR), activity (steps/motion detector), heart rate, and skin temperature. It should be noted that the Basis Peak watch product is no longer available, but other GSR measuring devices can be substituted to perform such measurements, as discussed below.

A Gamry potentiostat Reference 600™ (Gamry Instruments, Warminster, PA) was also used to obtain a GSR signal. This benchtop testing system was utilized on three subjects 3–4 times a day for three days (unlike a watch, continuous data collection is not practical). The built-in single frequency EIS measuring mode was used with the following settings: 2 kHz monitoring frequency, 353 mV AC voltage, 0.6 s repeat time for 1.02 min, 0.636 cm^2 testing area (with gold plated electrodes). From this the electric conductance was obtained and averaged over each minute.

3.7. Gravimetric testing method

Gravimetric collector disks were laser cut (1.06 cm diameter) out of TechniCloth™ nonwoven wipes (TX609, Texwipe, Kernersville, NC). Gravimetric testing occurred in a temperature (70 °F) and humidity (50%) controlled room. Additionally, subjects remained seated (resting) during the test. These cloth collection disks were pre-weighed by an analytical balance and then placed on both stimulation and baseline sites of the volar surface of the dominant forearm. Baseline sites were placed roughly two or more inches distally from the stimulation sites. Next, the gravimetric testing apparatus was placed on top of the collector disks (Fig. 1b). The acrylic posts on the gravimetric testing apparatus were 0.635 cm thick and 1.06 cm in diameter. The purpose of these posts is to hold the cloth collector disks in intimate contact with the stimulation and baseline sites, while also allowing evaporation from surrounding areas of skin. This allows capture of the sweat generated directly under the collector disks, eliminating evaporation at these sites, and reduces otherwise unpredictable amounts of sweat collection from neighboring areas. This is important for accurate sweat rate measurement, as will become apparent in the next paragraph. Collector disks were left on the skin for 10 to 30 min depending on previous sweat rates and the need for an adequate sample volume (initial gravimetric test lasted for 10 min). Following each test the samples were removed from the skin and immediately weighed again. The difference in weight (Δw) was then entered in the following equation to determine the total sweat rate (Q) in terms of nL/min: $Q(\mathrm{nL/min}) = \left(\Delta w(\mathrm{g}) \times 10^6(\mathrm{nL/g})\right)/(t(\mathrm{min}))$. The total sweat rate were then divided by the average number of active sweat glands (see Section 3.8) to obtain the sweat generation rate in nL/min/gland. Sweat generation rate is the metric reported throughout this paper.

Before this preferred technique was utilized, an inferior technique was attempted where the disks were held in place by simply wrapping the forearm with Saran™ wrap. While it was determined that this technique was satisfactory for very low baseline (natural) sweat rate measurements, it was inaccurate for stimulated and high natural sweat rate measurements. It was observed that the Saran™ wrap against the skin would wick sweat to the collector disks from areas outside the collector disk. This created an unpredictable collection area, and motivated the implementation of use of posts as described above and illustrated in Fig. 1.

3.8. Sweat pore imaging

Sweat pore imaging was performed to count the number of activated sweat glands due to iontophoretic stimulation of sweat. Sweat pores were imaged following the first gravimetric testing of sweat generation rate, and periodically through the rest of the testing for each subject. For each photograph, a suspension of bromophenol blue dye in cosmetic-grade silicone oil was placed on the arm for one minute while sitting (resting) in the temperature and humidity controlled room as detailed above. Images were then taken and ImageJ was used to count the number of pores. This oil/dye technique has been previously utilized and reported by our group [37] and by others [38]. The oil/dye mixture used here consisted of pure 5000 cSt and 100 cSt cosmetic grade PDMS oil which was mixed in a 1:3.5 ratio. For mixing, bromophenol blue dye (powder form) was added to obtain a final concentration of 7% (w/w) in the oil and dispersed with a vortex mixer and ultrasonication (the dye does not dissolve in the oil).

With the oil/dye approach, the oil prevents evaporation and the pH-sensitive dye partitions from the oil into the sweat emerging from the sweat ducts and stains the sweat blue. The result is that only locations of sweat are stained dark blue (the oil remains fairly clear). A photograph of the represented sweat pore result is shown in Fig. 2. Experimental results for observed active gland densities for all tests are provided in Tables 3–4 in the online Supplementary material.

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3.9. Statistical analysis

Eight subjects participated in this study. Three of the participants were female with ages ranging between 21 and 47. The remaining five subjects were male with ages between 23 and 41. The right arm was the dominant arm and the testing arm for all subjects. All of the subjects besides one female (Asian) were Caucasian.

Unless otherwise specified, sweat rates were obtained on two sites per subject (medial and lateral sites, Fig. 1). This procedure limited the total iontophoretic dose (both sites combined) to be less than or equal to the commercial Wescor Nanoduct sweat stimulation product. The data from medial and lateral sites were analyzed using Welch's *t*-test to determine for significant difference ($p < 0.05$). Since there was no significant difference ($p < 0.05$) between the two-sample location (15 of 15 comparisons), the data from the two sites was combined for further analysis. Consequently, the data reported are the average of the medial and lateral sampling sites.

The initial sweat generation magnitude and duration (above 1 and 0.1 nL/min/gland) of sweating responses were obtained from two repeat experiments per case, and were analyzed separately using ANOVA to determine if the charge-dose means were different ($p < 0.05$). There was deemed significant differences in means in 3 of the 3 comparisons ($p < 0.05$). Since this is true, a Tukey two-sample *t*-test, assuming unequal variance and differences deemed significant for $p < 0.05$, could be ran. Initial sweat generation magnitude showed 1 of 10 comparisons were significantly different, while 6 of 10 comparisons for duration above 1 nL/min/gland and 4 of 10 comparisons for duration above 0.1 nL/min/gland were deemed significantly different ($p < 0.05$). These significant differences are detailed in Figs. 4–6.

Finally, sex dependency on the initial sweat generation magnitude and duration (above 1 and 0.1 nL/min/gland) of sweating responses was analyzed using a Welch's *t*-test to determine if there was a significant difference ($p < 0.05$). Initial sweat generation magnitude and duration above 1 nL/min/gland showed 4 of 5 comparisons were significantly different, while 2 of 5 comparisons for duration above 0.1 nL/min/gland were deemed significantly different ($p < 0.05$). These results are detailed in Figs. 7–9.

4. Results

In this section, the data and analysis techniques are described in detail. The majority of the discussion of the data is reserved for the Discussion in Section 5. Please see the online Supplementary files for individual subject data (Figs. 11–16).

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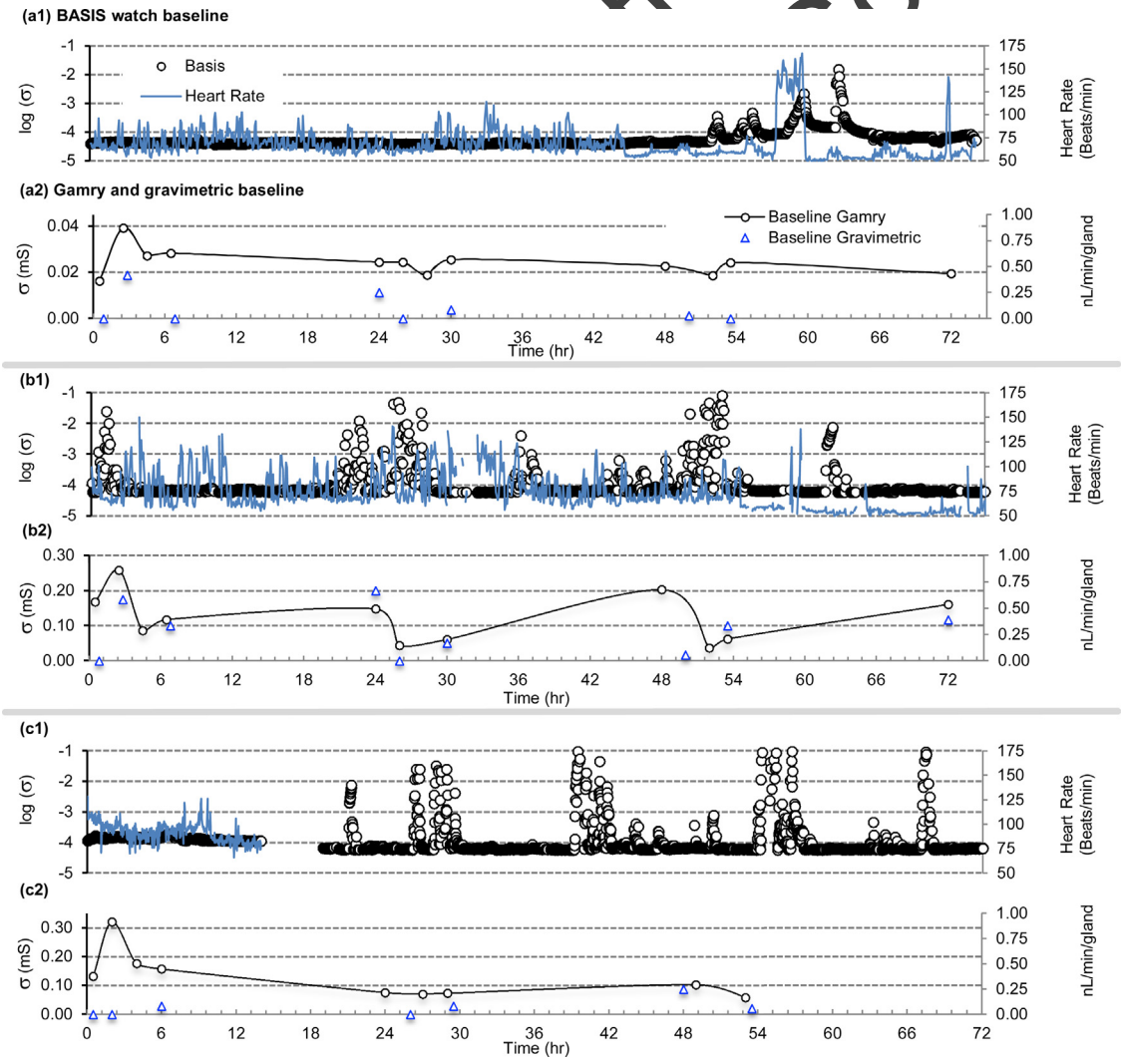


Fig. 3. For subjects a, b, and c: (1) Basis relative skin conductance (σ), heart rate (beats/minute) (2) Gamry quantitative skin conductance (mS), gravimetric sweat generation rate (nL/min/gland).

4.1. Baseline-only data (natural sweating events)

Continuous monitoring of baseline (natural) sweating is presented for three subjects in Fig. 3. The subjects were measured during normal work week which included primarily non-manual labour in an air-conditioned environment. In this data set, the Basis Peak watch captures a galvanic skin response (GSR) signal which is measured as relative changes in skin conductance (σ). The skin conductance is plotted logarithmically along with the heart rate of the subject (beats/min). Additionally, for each subject skin impedance was also measured using a benchtop Gamry potentiostat system. The Gamry skin impedance is more sensitive measurement and is quantitative (σ=mS), but is not wearable or easily portable, so less data was gathered. Gravimetric sweat generation rate data was also gathered using cloth collector disks as described previously in Section 3.7. As will be discussed in greater detail in Section 5, these results show that natural sweat events are very low, intermittent, and therefore unreliable for sweat sampling for non-athletes and non-manual labourers in a controlled environment (air-conditioning).

4.2. Stimulated sweat results

To ensure that the stimulated results reported here were not due to natural sweating events, natural baseline sweat generation rate measurements were also taken for all subjects. The raw data showing both natural baseline and stimulated sweat generation rates can be found in the online Supplementary file (see Fig. 8). In addition, to enable more accurate calculation of sweat generation rates the number of active sweat glands for all subjects was photographed and analysed (see online Supplementary file, Tables 3–4).

Figs. 4 and 7 show the bar and whisker graphs of the average initial sweating responses for all subjects due to stimulations using pilocarpine (100%) and carbachol (100-12.5%), in terms of dose vs. initial sweat rate (nL/min/gland). Statistically all initial sweat rate medians were consistent in Fig. 4 for all doses besides 12.5% carbachol which, was only slightly lower than the other doses. The differences in the initial sweat rate depending on gender in Fig. 7

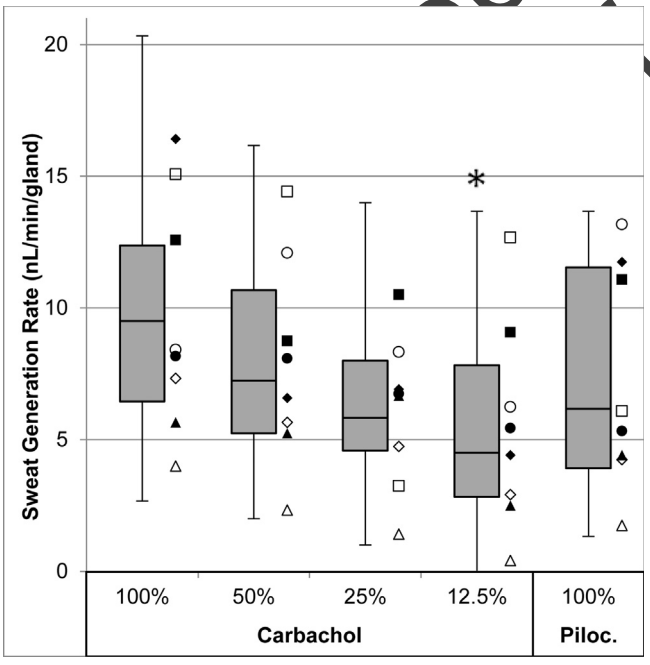


Fig. 4. Bar and whisker graph of the initial sweat generation rates for all subjects at specific doses. Scatter plot for the average initial sweat rate subject is to the right of the bar and whisker for each dose. Symbols indicate individual subject's medial and lateral average values. * Indicates significant difference of means of that dose to 100% carbachol ($p < 0.05$).

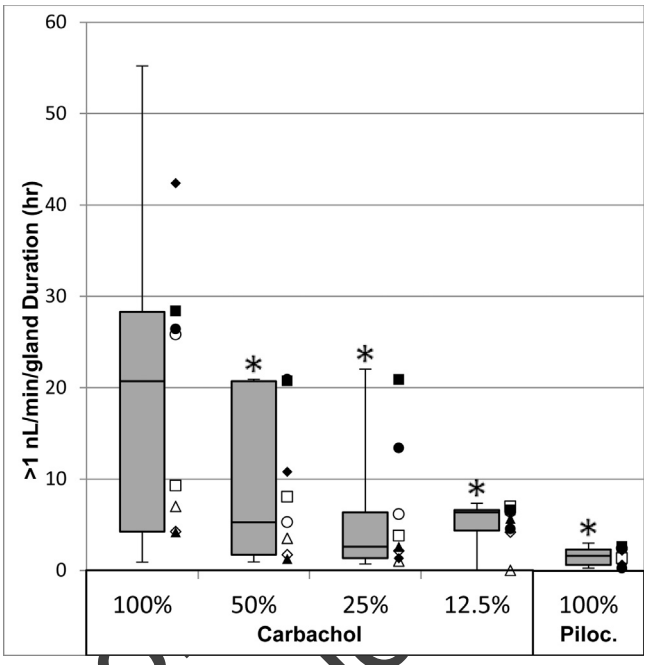


Fig. 5. Bar and whisker graph of the duration of sweating response with a 1 nL/min/gland cutoff for all subjects at specific doses. Scatter plot for the average duration between medial and later locations for each subject is to the right of the bar and whisker for each dose. Symbols indicate individual subject's medial and lateral average values. * Indicates significant difference of means of that dose to 100% carbachol ($p < 0.05$).

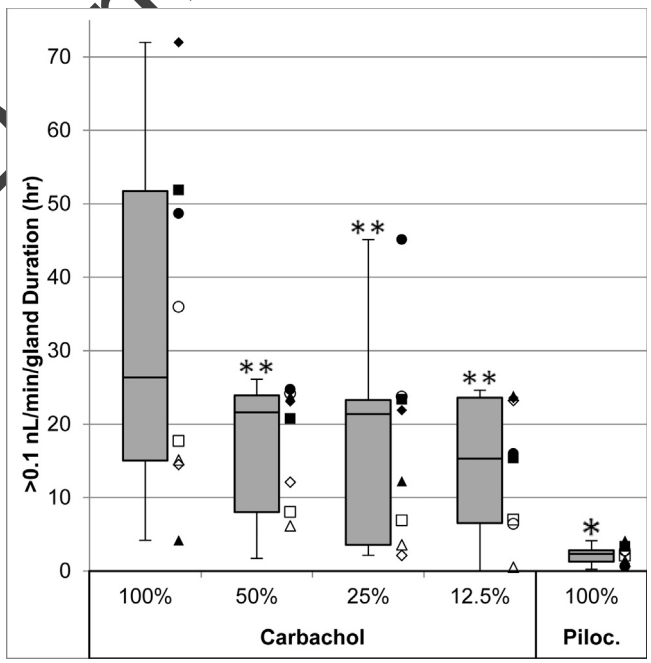


Fig. 6. Bar and whisker graph of the duration of sweating response with a 0.1 nL/min/gland cutoff for all subjects at specific doses. Scatter plot for the average duration between medial and later locations for each subject is to the right of the bar and whisker for each dose. Symbols indicate individual subject's medial and lateral average values. ** Indicates significant difference of means of that dose to 100% carbachol and pilocarpine ($p < 0.05$). * Indicates significant difference of means of that dose to 100% carbachol ($p < 0.05$).

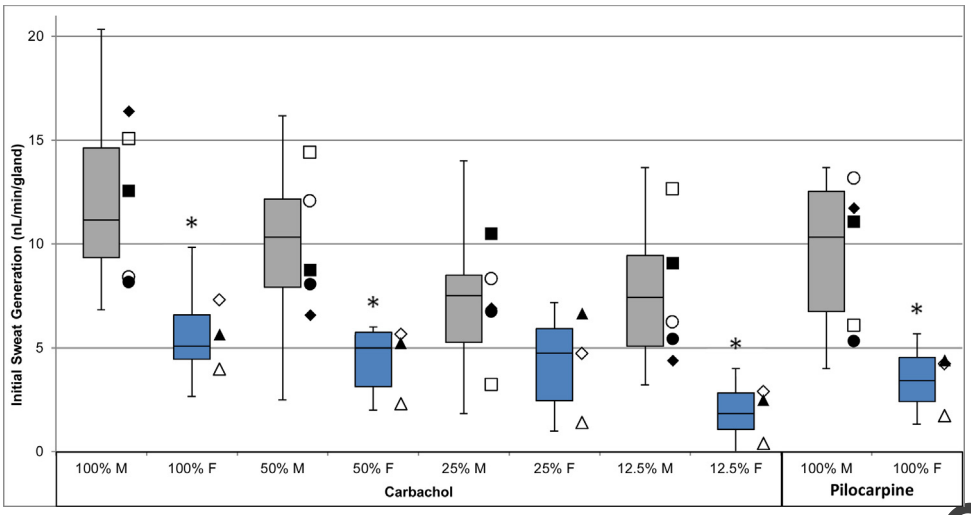


Fig. 7. Bar and whisker graph of the initial sweat generation rates for male and female subjects at specific doses. Scatter plot for the average initial sweat rate between medial and later locations for each subject is to the right of the bar and whisker for each dose. Symbols indicate individual subject's medial and lateral average values. * Indicates significant difference of means of that dose for male and female ($p < 0.05$).

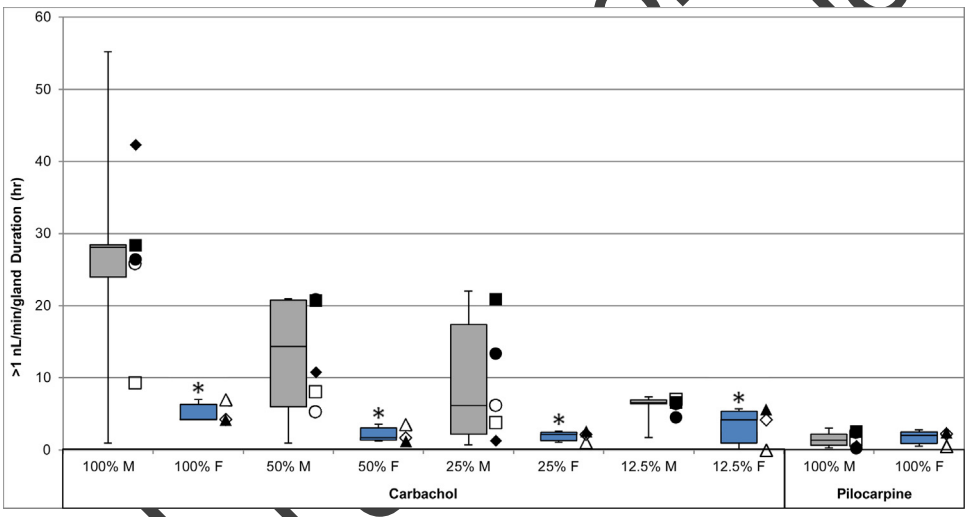


Fig. 8. Bar and whisker graph of the duration of sweating response with a 1 nL/min/gland cutoff for all male and female subjects at specific doses. Scatter plot for the average duration between medial and later locations for each subject is to the right of the bar and whisker for each dose. Symbols indicate individual subject's medial and lateral average values. * Indicates significant difference of means of that dose for male and female ($p < 0.05$).

were all statistically different in male and females for all doses besides 25% carbachol.

Figs. 5–6 and Figs. 8–9 show the bar and whisker graphs of the sweat generation durations for 1 and 0.1 nL/min/gland cutoffs. Figs. 5 and 6 show all the data for all combined study participants while Figs. 8 and 9 compare the durations of stimulation for male and female participants. Fig. 5 shows the duration of stimulation above 1 nL/min/gland, where all doses have statistically the same medians besides the duration stimulated by 100% carbachol. Fig. 6 shows the duration of stimulation greater than 0.1 nL/min/gland, where all doses have statistically the same medians besides the duration stimulated by 100% carbachol (much larger) and 100% pilocarpine (much smaller). The differences in the duration of sweat stimulation depending on gender with a 1 nL/min/gland cutoff are shown in Fig. 8, and statistically details that females have a lesser response to all doses of carbachol than males. With a cutoff of 0.1 nL/min/gland (Fig. 9) the only doses that showed a decrease in sweat stimulation duration between male and female subjects were 100% and 25% carbachol with all other doses showing no significant decrease in stimulation duration. The online Supplementary files provide plots for individual subject sweating responses in terms of sweat generation rate (nL/min/gland vs. time).

Following testing of the single-dose experiments discussed above, repeated stimulation experiments were performed utilizing the lowest dose of carbachol (12.5%). The repeated simulation was performed at the same locations three times for each of three different subjects. Once the subject's sweat generation rate decreased to below 1 nL/min/gland another stimulation was implemented until a total of three stimulations had been delivered. The results from this test are presented in Fig. 10. The total (cumulative) durations achieved for each subject was 32.47, 18.78, and 17.82 h.

5. Discussion

In this discussion section, we will first review the general importance of this type of study, and then discuss the data such

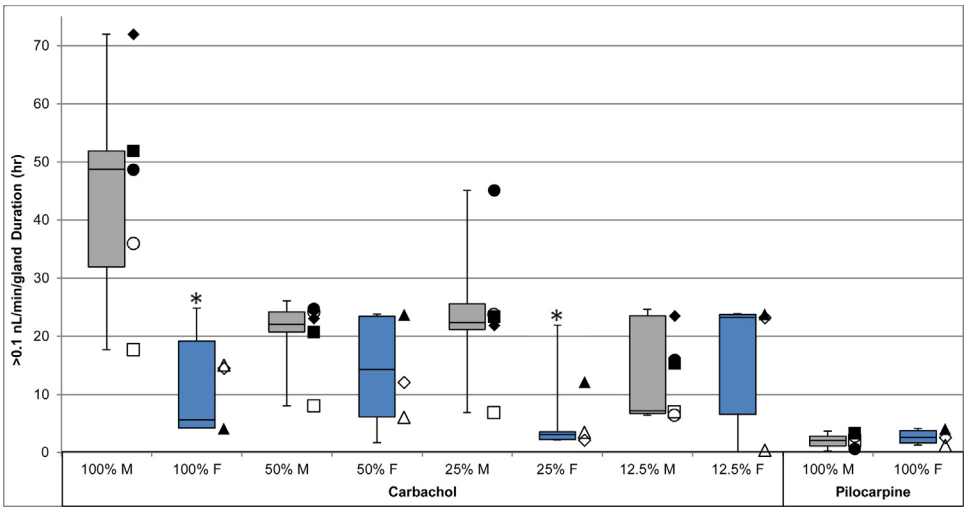


Fig. 9. Bar and whisker graph of the duration of sweating response with a 0.1 nL/min/gland cutoff for all male and female subjects at specific doses. Scatter plot for the average duration between medial and later locations for each subject is to the right of the bar and whisker for each dose. Symbols indicate individual subject's medial and lateral average values. * Indicates significant difference of means of that dose for male and female ($p < 0.05$).

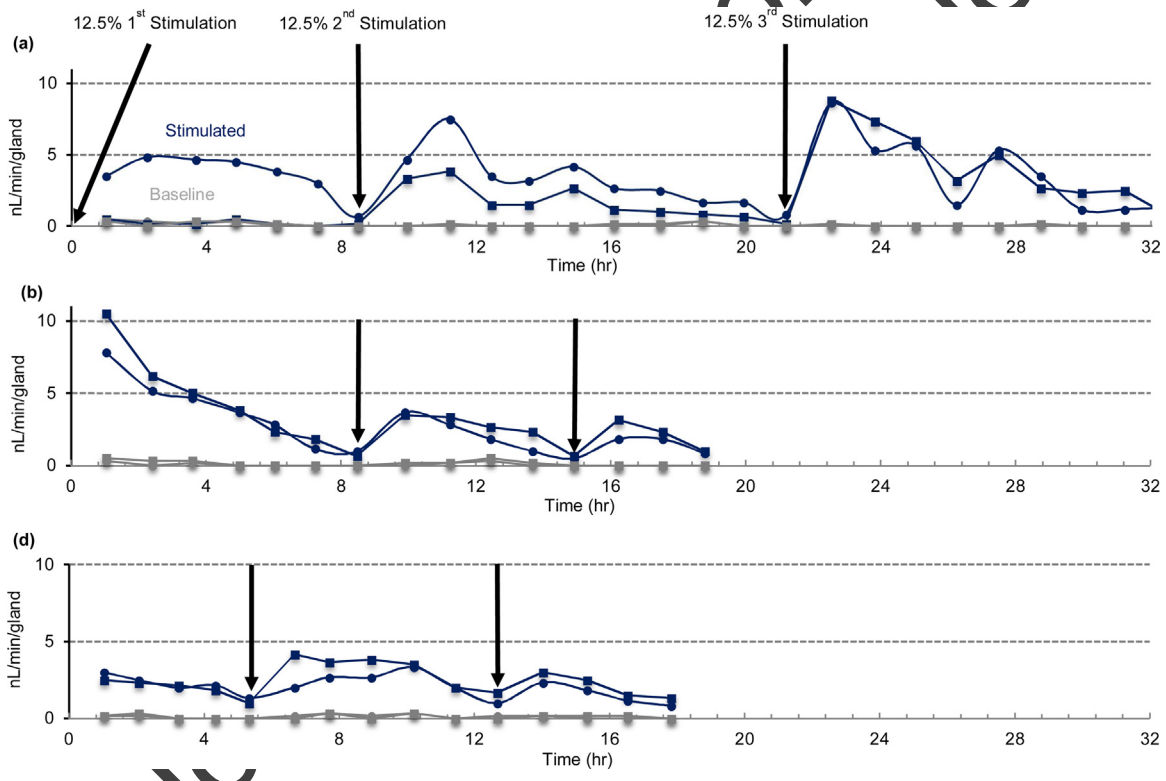


Fig. 10. Repeated 12.5% carbachol stimulations three times for subjects a, b, and d. Stimulations were performed at time = 0 and as noted by the arrow points.

that it can be appreciated in the appropriate context. Lastly, we will discuss the feasibility of possible alternate methods for achieving prolonged sweat stimulation.

5.1. Fundamental and applied importance

From a fundamental perspective, this study provides insights into the long duration and inter-subject variability for sweating induced by a cholinergenic agent that is weakly or not susceptible to metabolism by AChE. In particular, this study shows sustained sweating following a single iontophoretic dose of carbachol that is much longer than commonly observed for pilocarpine. To the best of our knowledge, no previous study has reported durations for sweat stimulation lasting greater than 24 h, including even previous carbachol studies [24–27].

From an applied perspective, the duration and magnitude of the sweating responses achieved here are important for enabling wearable sweat biosensing for individuals at rest [1,14]. Carbachol now presents a potential option for applications where it is desired to measure analytes in sweat continuously for 24 h or more.

The results in this study are also clinically useful in at least three ways. First, sweat rate is typically reported in the literature as a relative measure, and this study provides improved methods for inexpensively, ergonomically, and accurately quantifying sweat

generation rate. Second, when working on clinical discovery and correlation of the many analytes in sweat [39], small sample volumes can be an issue. Therefore, the results of the present study are important because they allow greater volumes of sweat sample with less stress on the subject (e.g. no need for a prolonged thermal load). Third, larger molecular weight and hydrophilic analytes undergo dilution that depends on sweat generation rate [1]. Therefore, the results of the present study delineate collection windows where the sweat generation rate can be fairly stable and therefore analyte dilution can be predicted.

Many of these same benefits discussed above are also useful for idiopathic pure sudomotor failure and hyperhidrosis (see next subsection) as well as other types of studies including skin topicals, cosmetics, antiperspirants, adhesives, textiles, etc. Again, not only is the convenience to the test subject potentially improved, but it is possible that data can be more consistently collected. Lastly, as utilized here, prolonged localized stimulation has the advantage that on the same body region, any sort of testing can be performed with a control experiment nearby where sweating is not occurring.

5.2. Disease diagnostic improvements

The methods detailed in this work improve upon the current quantifiable systems used to diagnose idiopathic pure sudomotor failure, which include a semi-quantitative sudomotor axon reflex test (sweat rate). As mentioned previously, the stimulation methods we report here can resist rapid decomposition by AChE hydrolysis providing a longer and less temporally dependent test (QSART acethylcholine [40], Table 1). The results here can also more accurately quantify the sweat responses (actual quantitative nL/min/gland data).

Another disease worthy of discussion is hyperhidrosis. Hyperhidrosis is commonly diagnosed in a semi-quantifiable method similar to the gravimetric method described here [40], through evaporimetry [42], or by qualitative assessment of sweating through a starch-iodine test [43]. The gravimetric tests previously used for hyperhidrosis diagnosis use a method similar to the Saran™ wrap method described in this paper which can be inaccurate because sweat can wick and be collected from neighboring areas to the collection sample could provide a false positive by going above the threshold for hyperhidrosis. The improved gravimetric testing techniques reported here resolve this issue. Additionally, the sweat pore imaging technique described here can provide more information than the qualitative starch-iodine test that just shows sweating areas and not sweat pore densities. Furthermore, because carbachol has the potential to maximally stimulate the sweat glands, it can allow hyperhidrosis testing to measure the percent of max sweat generation or pore activation per individual. This is important because it allows the hyperhidrosis testing to correct for aerobic conditioning and other person-to-person variances in sweat generation [44].

5.3. Achieving >24 h of sweat generation

Our primary objective was to achieve a localized sweat stimulation response greater than 24 h in duration. Figs. 4–6 show a wide range of sweat rates for the different stimulation dosages and across different subjects. The lower doses had shorter durations (Figs. 5 and 6) and slightly reduced initial sweat generation rates (Fig. 4). This raises the question as to whether or not the 100% dose used here is adequate to occupy all the receptor sites of the sweat gland for sweat stimulation. For example, if the data for 50% and 100% stimulation were nearly identical, it would have suggested that all the receptor sites were occupied for these dosages. Such a trend is not seen in the data, and it could be that even higher dosages than 100% could produce even longer durations of sweat generation and greater initial sweat generation rates. One could also speculate that the data should not be interpreted solely in terms of receptor site occupation, because the increased duration for the larger doses could also represent a "reservoir effect" with additional carbachol being in the epidermis and slowly diffusing to receptor sites over time. With no information on carbachol drug clearance it is difficult to assess this potential effect. However, we speculate, that given the very long durations of sweating, a strong effect due to diffusion of carbachol is unlikely, especially since the receptor sites are in the dermis and interstitial fluid is refreshed quickly in the dermis (~<30 min) [45]. Furthermore, the capillary density around sweat glands is larger than that found across most of the dermis, and therefore the local refresh rate for introduction of new interstitial fluid could be even faster near the receptor sites. Although half of the subjects exhibited >24 h of sweating at >1 nL/min/gland with a single 100% dose, several subjects fell well short of this target. Four of the subjects exhibited sweating responses >24 h and three subjects exhibited sweating responses that lasted for >48 h (online Supplementary file, Fig. 10). We found a >48 h sweating response to be surprising, but equally surprising was just how short the response was for some of the other subjects.

5.4. Repeated stimulations

Less surprising and perhaps more encouraging from an applied perspective are the repeated stimulation results. The triple repeated 12.5% dosage stimulation data shown in Fig. 10 represent a possible technological solution to achieving >24 h localized stimulation even for the subjects who had short stimulation responses. In particular, examining the single dosage experiments, the 12.5% stimulation (Fig. 6) shows that 5 out of 8 subjects exhibit a stimulated sweating response of >0.1 nL/min/gland for >8 h. The exceptions (subjects e, g, h) at least showed >1 nL/min/gland for >7 h at 100% dosage (Fig. 5). Therefore, it is possible (but not proven) that all subjects in this study could achieve >24 h sweat stimulation if the stimulant is dosed 3 to 4 times at 100% per dose or less. As discussed at the end of Section 2, smaller stimulation and sweat sampling areas could be utilized to offset the increased dose of carbachol due to repeated dosing. It could also be that subjects with a shorter duration of sweating response to carbachol may have a greater systematic tolerance to carbachol, potentially alleviating possible concerns of sweating response to carbachol may have a greater systematic tolerance to carbachol, potentially alleviating possible concerns of repeated dosing.

Repeated stimulations could show a weaker response after each subsequent dosage. However, the limited set of results of Fig. 7 are not adequate to determine if there is a localized reduction of sweat generation rate following each stimulation. Simply, the subject could locally or systemically develop increased tolerance to carbachol. In addition, reduced sweat generation with repeated iontophoresis utilizing only tap water has been reported elsewhere [46].

Lastly, we note that technology reported elsewhere [14] can allow side-by-side integration of stimulant and sensors, making such repeated stimulation and sensing at least theoretically possible within a single wearable device.

5.5. Gender & other factors

As seen in Figs. 7–9 there is a wide difference of sweating results for the different stimulations between male and female subjects with the women of this study experiencing shorter durations and reduced initial sweat generation rates compared to male counterparts. Differences between sweating events in male and female have been previously studied [44,47] with men having a sweat

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generation rate up to two times greater than females. However, Notley et al. shows that the main significant factor in sweating response differences is not due to gender specific variability (5%) but is due to mass-specific surface area (10–48%) [47]. Figs. 7–9 are generally consistent with these previous findings. Additionally, Figs. 7–9 show the differences in the sweat generate rates and duration of sweat stimulation between the two stimulants for male and female. While males show a large fluctuation between the two stimulants, females response is not as strong. For instance, Figs. 8 and 9 show on average a 18 to 23 times increase in duration between a 100% pilocarpine and 100% carbachol stimulations for males while females show only 3 to 4 times increase.

Potentially more influential on the data is a measure of an individual subject's aerobic capacity as subjects with greater aerobic capacity show greater sweating responses [44]. However, in our study we did not record individual subjects' aerobic capacity. Therefore, further understanding of the nature of the causes in variances of sweat responses is not possible with the data presented herein. Future studies are suggested that compare thermally stimulated sweat generation rates to those generated by carbachol and other stimulants. In particular, attention could be paid to stimulants that target both or mainly just one of the muscarinic or nicotinic receptor sites. Such studies could also examine the same subjects in a state where they have reduced aerobic capacity (sedentary lifestyle) and for the same subject after a lifestyle period of regular aerobic conditioning [13].

5.6. Stability of sweat generation rate

Fig. 10 suggests that sweat generation rates can be fairly stable over periods of several hours, which is long enough to collect multiple samples of sweat with potentially predictable dilution rates for large analytes [1].

5.7. Current dosage and iontphoretic stress on skin

As discussed in Section 2, dramatically lower dosages are potentially possible when using carbachol for localized sweat stimulation. The reduced current dosage is also important because less iontophoresis stress is required, potentially reducing known concerns for repeated iontophoresis on skin [17,18]. Furthermore, because of the long duration of the sweat response, the need to repeat the iontophoresis can be infrequent and this allows more time for the epidermis to recover following iontophoresis [48].

5.8. Potential alternate approaches

A final question is what alternative strategies could be considered for prolonged sweat stimulation. One could alternatively consider utilizing AChE inhibitors such as neostigmine [49] to prolong sweat generation following cholinergic activitation, but generally this approach is less desirable for at least two reasons. *First,* generation of sweat still requires release of AChE and therefore sweat generation is not guaranteed. Furthermore, with inhibitors the increase in sweat generation rate is only moderate at best [49]. *Second,* such inhibitors would need to be continually dosed since they would not be locally bound. This could be highly undesirable due to continued stress on the skin for delivery of the inhibitors. The inhibitors could also cause systemic side effects. Carbachol stimulation is different than use of an inhibitor because our results clearly demonstrate that it stimulates the eccrine sweat gland for prolonged periods, such that continual dosing is not required.

Conflicts of interest

Corresponding author Dr. Jason Heikenfeld has an equity interest in Eccrine Systems, Inc., a company that may potentially benefit from the research results, and also serves on the company's Board. The terms of this arrangement have been reviewed and approved by the University of Cincinnati in accordance with its conflict of interest policies. All other authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.jidermsci.2017.10.013.

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