Tattooing as a Phenotypic Gambit

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Tattooing as a Phenotypic Gambit

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Running head: Tattooing as a phenotypic gambit

Abstract

Objectives: Tattooing is not an evolved behavior, but it may be a phenotypic gambit to highlight immunological health. Phenotypic gambits are traits or behaviors that appear costly but occur at high rates as a honing process of natural selection not constrained by genetics. Tattooing is an ancient practice that is increasing in popularity worldwide, but it involves wounding the body, which seems counterintuitive because it challenges the immune system and makes one more susceptible to infection. But tattooing may represent a costly honest signal of fitness by "upping the ante" in an era of hygiene or a means to stimulate the immune system in a way that improves and highlights underlying fitness.
Materials and Methods: We investigated this hypothesis by assessing bacteria killing activity (BKA) in saliva samples collected during two studies of tattooing ($N = 40$). We compared previous tattoo experience (extent of body tattooed and hours spent being tattooed) to BKA before and after getting a new tattoo.
Results: Tattoo experience positively predicts post-tattoo BKA ($\beta = .48$, $p = .01$), suggesting that people with more tattoo experience have a relatively more immediate and active immune response than those with less tattoo experience.
Discussion: Tattoo experience may elevate innate immunological vigilance, which could aid in protecting against future dermal insults.

Keywords: tattooing, bacteria-killing activity, phenotypic gambit, immune function, stress

Introduction

A gambit is the sacrifice of something to gain an advantage. The phenotypic gambit is an assumption that traits or behaviors which may at first appear costly occur at high rates as part of a honing or refining process of natural selection not directly constrained by genetics (Grafen, 1991). Tattooing is a gambit in wounding the body, increasing potential for infection, and stressing the immune system (Lynn & Medeiros, 2017). This purposeful, pigmented wounding is an ancient practice, yet the global popularity of tattooing and accompanying industry have been growing exponentially in recent decades (Blanton, 2015; Harris, 2016; Ibisworld, 2017; Jaworska et al., 2018; Kluger, 2015). Tattooing has been widely practiced as a means of group identity and marker of individuality, to distinguish status or accomplishments, to enhance attractiveness, and for apotropaic (magical or protective) and therapeutic-medical reasons; and there are good reasons to believe these wounds provide both psychological and physiological benefits (Piombino-Mascali & Krutak, 2020).

Carmen et al. (2012) pose two hypotheses to explain how short-term harm could be evolutionarily adaptive: (1) tattooing's popularity is result of sexual selection favoring body ornamentation for symbolic communication ("human canvas" hypothesis), and (2) being tattooed comes with physiological costs that can highlight immunological health ("upping the ante" hypothesis). Previous research indicates that tattooing might reflect fitness advantages by highlighting attractiveness, bilateral symmetry (an indication of developmental vigor), personality features, and life experiences (Heppell et al., 2005; Koziel et al., 2010; Osu et al., 2021; Wohlrab et al., 2007; 2009). Another possibility is that the tattooing process could stimulate adaptive response of the innate immune system in accordance with the principles of allostasis (Sterling, 2004). This latter model has been tested with regard to biomarker levels for

cortisol, secretory immunoglobulin A (sIgA), and C-reactive protein (CRP) and find that when tattooing is novel (i.e., the individual has no or few previous tattoos), a rise in cortisol is associated with immunosuppression (Lynn et al., 2016; 2019; 2022). However, among those with relatively higher tattoo experience, immune response begins immediately, decoupled from cortisol response. We interpret this as allostatic adjustment to a repeated and desirable environmental stressor, as observed in exercise studies (Eöry et al., 2021), but it is not clear if changing levels of biomarker reflect actual immune function. Therefore, we retest this model through investigation of actual bacteria-killing activity before and after receiving a new tattoo.

The bacteria-killing activity (BKA) assay measures innate functional immune responses to a known quantity of *Escherichia coli* bacteria relative to a control (Muehlenbein et al., 2011). Previous studies of tattooing and immune function using sIgA and CRP as proxies of immune functions support the proposed model. However, salivary CRP is arguably a better indicator of oral health than of circulating immune activity (Pay & Shaw, 2019), and sIgA and CRP may be more reflections of inflammation than immune performance (Longman et al., 2018).

We seek to build upon current knowledge of human immune function and the seeming paradox of adaptation through "self-injury" by investigating physiological responses to tattooing. While evolutionary models imply long-term advantages not measured in our study, the phenotypic gambit approach assumes an accrual of benefits to humans through a honing process. Similarly, the allostasis concept refers to "stability through change," "fitness under natural selection," or "predictive regulation" (Sterling, 2004). Thus, novel stressors that cause fear or anxiety should produce a stress response, including temporary suppression of functions not immediately necessary for fight-flight-or-freeze, such as immune activity. But tattoos are a welcome stressor to which people return, indicating that previous tattoo experiences were more

rewarding than painful. Rather than a stress response, people with greater previous tattoo experience may be more influenced by the anticipation of reward than by the fear of pain and thus less likely to experience immunosuppression when receiving a new tattoo (Sterling, 2004). We explored this allostatic process, hypothesizing that BKA would (1) be greater at baseline among those with relatively more tattoo experience (TE; more hours spent being tattooed and larger proportion of body tattooed), (2) increase pre-posttest (i.e., over the course of getting a new tattoo) among those with relatively greater TE, and (3) decrease pre-posttest among those with less TE.

Materials and Methods

We used data collected by our team during two previous studies, including a study of 25 clients of three tattoo artists in American Samoa (Lynn et al., 2019) and 48 clients of 26 different artists at a tattoo festival in Puyallup, WA (Lynn et al., 2022). We removed participants whose saliva samples did not contain enough volume or were visibly contaminated. Participants for current analysis included 12 women and 28 men (N = 40) ages 18-60.

Procedures for both studies were the same except where noted below. We obtained permission from tattoo artists to recruit their clients for a study of tattooing and health. Study parameters were explained to clients, who agreed that we could use the samples to conduct secondary analysis of immune and endocrine functions related to our study hypothesis (both approved by University of Alabama IRB). We collected baseline demographic information (gender and age), including measures of previous TE (total hours tattooed, extent of body tattooed, and years since first tattoo), and measured weight and height to calculate body mass index. In addition, we collected a saliva sample immediately before beginning the new tattoo, and a second sample at the end of the tattoo session (in the first study) or an hour later (in the

second study). We noted the time each sample was collected and calculated the time between samples (latency).

BKA assays were conducted on samples that had enough saliva volume remaining after primary analyses (Lynn et al., 2019; 2022). A single lyophilized *E. coli* pellet (MicroBiologics Epower Microorganisms #0483E7) was reconstituted in sterile phosphate buffered saline and then diluted into a working solution, which produced approximately 200–300 colonies per 20 µL of aliquot. Aliquots of bacteria working solution were added to diluted saliva in a microcentrifuge tube, vortexed, and incubated for 30 minutes. After incubation, the samples were spread on trypticase soy agar plates (BD BBL #211043) in triplicate and incubated overnight at 37°C. The number of colonies on each plate the next day were counted, and the percent bacteria killed for each sample relative to a positive control (media and bacteria only) was calculated.

Of 73 pre-posttest saliva sample pairs (146 total saliva samples), 15 samples had insufficient saliva remaining to conduct BKA analysis, and 34 were highly contaminated such that *E. coli* didn't grow or grew poorly. Furthermore, we retained samples only if we could conduct BKA analysis on both the pre- and posttest in a pair. Among the 40 participants whose samples were retained for this analysis, 30 samples displayed small amounts of contamination, but *E. coli* colonies appeared healthy, so we have included these samples in the analysis. Contamination was most likely due to participants eating or smoking before providing their samples. Cortisol (µg/dL) and sIgA (mg/dL) were assayed for previous studies with assay details published (Lynn et al., 2020; 2022). All biomarker variables were standardized to account for skewedness.

We created two TE variables. The first explores the importance of extent of body and time being tattooed (TE). The second explores receiving tattoos in a rate of time (TER; i.e., does

it matter if a person gets many tattoos in a short duration of months or years or gets them over a longer timespan?). We created the first TE variable by summing extent of body tattooed and hours tattooed. We created the TER variable by dividing this sum by years since first tattoo. We used the means of each TE variable to create dichotomous variables (e.g., Low/High TE) and compared BKA at pre- and posttest using paired and independent samples *t*-tests to test hypotheses 1 and 3, respectively. We used analysis of covariance (ANCOVA) in separate models to test the influence of TE and TER on the posttest BKA measure (hypothesis 2), controlling for pretest BKA, BMI, age, gender, cortisol change, and latency. Finally, we compared our measures of functional immune response (BKA) to inflammation (sIgA) by running the same model with sIgA in place of BKA. Analysis was conducted using SPSS Version 27 (IBM Corp., Armonk, NY) and statistics considered significant if $p < .05$.

Results

Table 1 displays descriptive statistics for variables used in analysis. BKA is displayed as percent of *E. coli* killed in the sample, with negative numbers indicating that more bacteria grew than were killed. These numbers appear small, since they are percentages and not units, the magnitude of change is large. The mean BKA increase from pre-post is 59%, and the highest rate of bacteria killing observed in the sample was 91%.

Table 1. Descriptive Statistics

		Min	Max	Mean	Median	SD
Age		18.00	60.00	35.80	37.00	11.32
BMI		17.38	43.05	31.62	30.58	6.08
BKA (%)	Pretest	-.32	.67	.11	.14	.25
	Posttest	-.80	.91	.19	.22	.41
Cortisol (μg/dL)	Pretest	.0002	.39	.02	.002	.06
	Posttest	.0003	.20	.01	.002	.04
sIgA (mg/dL)	Pretest	.07	308.48	15.13	.50	64.11
	Posttest	.05	648.65	26.28	.55	117.04
Latency		13.00	299.00	120.35	110.00	47.84
Tattoo experience	Extent	0	21.50	6.19	3.83	5.94

Hours	0	85.00	13.70	8.25	16.53
Years	0	35.00	16.00	16.00	9.66
TE: Extent+Hours	0	100.00	19.89	12.30	20.82
TER: (Extent+Hours)/Years	0	33.33	2.87	.97	6.98

We dichotomized the TE variables using the medians to ensure equal variances (Low TE = 0-12.30, High TE = 12.31+; Low TER = 0-.97, High TER = .98+), split the dataset to compare group pretest BKA, and conducted a paired samples *t*-test on pre- and posttest BKA. We found higher pretest BKA among the Low TE group when using the TE variable ($t = .17$, $p = .87$) but greater pretest BKA in the High TER group using the TER variable ($t = -1.23$, $p = .27$). Neither difference was significant.

We also compared pre-posttest BKA changes using both TE variables. Using the TE variable, BKA decreased pre-posttest for the Low TE group, but the difference was not significant ($t = .08$, $p = .94$). For the High TE group, there was a statistically significant pre-posttest increase ($t = -2.56$, $p = .02$). Using the TER variable, BKA increased pre-posttest for both groups, but neither change was significant (Low TER: $t = -.70$, $p = .49$; High TER: $t = -1.32$, $p = .21$).

We conducted ANCOVA using both TE variables, controlling for age, BMI, gender, latency (time duration between pre- and posttest samples), and pre-posttest change (Δ) in cortisol, but neither the model nor TER were significant. By contrast, the model for TE was significant ($F_{8,26} = 3.63$, $p = .01$, adjusted $r^2 = .38$), and TE was a significant predictor with a moderate effect size (Table 2).

Table 2. ANCOVA of tattoo experience (TE) on bacteria-killing activity (BKA)

	Standardized β	t	*P*
(Constant)		-2.21	.04
$BKA_{Pretest}$	.41	2.69	.01
Tattoo experience[a]	.48	2.90	.01
Age	-.12	-.71	.48
BMI	.23	1.36	.19

Gender	-.29	-1.69	.10
Latency	.24	1.39	.18
$Cortisol_{Pre\text{-}posttest\ \Delta}$	-.23	-1.48	.15

[a] TE = Extent of body + Hours tattooed

Finally, we compared BKA to sIgA as a proxy of immune function by substituting sIgA for BKA in the model. The sIgA models were significant, but neither TE nor TER was a significant predictor. In these sIgA models, only latency between sample collection times was significant (Table 3). This latter finding was unexpected, as tattoo experience has been a significant predictor of posttest sIgA in previous tattoo studies and because sIgA is one among numerous immunofactors active in killing bacteria.

Table 3. ANCOVA of tattoo experience (TE) on secretory immunoglobulin A (sIgA)

	Standardized β	t	*P*
(Constant)		-.38	.71
$sIgA_{Pretest}$	.82	11.09	< .01
Tattoo experience[a]	.04	.54	.58
BMI	-.01	-.16	.88
Age	.09	1.25	.22
Gender	-.09	-1.38	.18
Latency	.21	2.81	.01
$Cortisol_{Pre\text{-}posttest\ \Delta}$	-.07	-1.14	.26

[a] TE = Extent of body tattooed + Hours tattooed

Discussion

We explored BKA in saliva samples collected from people receiving new tattoos to determine if their previous TE influences how their immune system responds to the new tattoo. Consistent with allostasis theory of stress response (Goldstein & McEwen, 2002), we predicted that BKA would be suppressed or limited in tattoo neophytes (hypothesis 1) but would increase in response to a new tattoo among those with relatively more TE (hypothesis 2). Furthermore, we compared pretest BKA of Low and High TE groups to determine if an immunological priming effect from previous TE persists over time as evidenced by relatively higher baseline BKA among the High TE group (hypothesis 3).

There was a decrease pre-posttest in BKA for the Low TE group, which is consistent with our third hypothesis, but that change was not statistically significant. Our second hypothesis was supported, as TE was a significant predictor of posttest BKA when controlling for pretest BKA, BMI, age, gender, and cortisol. However, the amount of time since one's first tattoo was neither a predictive factor, nor did we find greater pretest BKA among the High TE group.

We thought BKA might be elevated in the high TE group relative to the lower experience participants if tattooing produced an immunological priming effect that persists over time. This hypothesis derives from one our team's previous studies in which mean sIgA at pretest was significantly greater for that study's high tattoo experience participants relative to the low tattoo experience group (Lynn et al., 2020). We also predicted that BKA would decrease in the low TE group, though previous results for sIgA on this count are also mixed (Lynn et al., 2019; 2022). We divided tattoo experience by years since the participant's first tattoo. This enabled us to develop a refined rate-of-tattoo-experience variable similar to one used in our first study (Lynn et al., 2016). We thought that if a priming effect of tattooing on the immune system exists, it might decay over time, which we could detect by calculating this rate variable. However, similar to our Puyallup study (Lynn et al., 2022), we did not detect any effects for TER. An alternative explanation that may explain this lack of a TER effect is that memory of the tattoo experience may fade over time, and early first tattoo experiences may play little bearing on later appraisal of a new tattoo after years have passed.

This was a proof-of-concept study to determine the efficacy of BKA as a biomarker of actual immune activity and not simply levels. The failure to replicate previous findings with regard to sIgA is puzzling, but it is possible that there are other mechanisms of innate immune response are more important in response to *E. coli* than sIgA and represents a better model going

forward than studies of select antibodies or hormones. We find support for the utility of the BKA salivary assay in field studies of immune activity, but we do not know if these findings reflect immunological benefits that are clinically significant.

This study has limitations, including the sample size and condition of the samples. As this was secondary analysis of samples assayed previously for multiple biomarkers, many samples did not have sufficient saliva volume left to assay BKA. Others were contaminated and disposed of. A subset of samples included in analysis had some contamination and were retained because the *E. coli* colonies appeared unaffected by the contamination, but future research should include a larger participant cohort and screen participants for oral health and contamination. A complete analysis of immune response to tattooing would include measures of adaptive immunity as well as innate responses. Future study using the rate of tattooing experience might consider the repetition of tattooing experiences over time spans and not simply the timespan since a first tattoo. Finally, there are many other factors that influence physiological responses to tattooing, including type of equipment used and cleaning protocols, site and tattooist hygiene, ink quality and composition, and other factors (Farley et al., 2019; Rademeyer et al., 2020), some of which we controlled for but that will require a larger sample to assess in a full analytic model.

Conclusions

We examined bacteria killing activity relative to tattooing to determine if immune responses are adaptively honed through repeated cultural practices that stress the body. We found that dermal stress of tattooing results in increased innate immune activity that is influenced by previous TE, supporting the model of tattooing as a phenotypic gambit. Tattooing may therefore enhance evolutionary fitness signals. However, we did not detect immunosuppression necessarily associated with being a tattoo novice, and we failed to replicate previous findings for the

biomarker sIgA. Tattooing can enable researchers to explore the dynamics of the interacting endocrine and immune systems, but more research is necessary. Fortunately, the popularity of tattooing and the variability in how tattoos are administered continue to grow. Future research should explore the intervening mechanisms that mediate these dynamic activities and to determine if such fitness signals also have clinical value for health.

Acknowledgements

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Data Availability

Files used in this analysis are available from The University of Alabama Institutional Repository at _____.

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Figure Captions

Graphical Abstract

Photo of in-progress tattoo taken at the Northwest Tatau Festival, Puyallup, WA, July 2018. Photo by Christopher D. Lynn.

Table 1. Descriptive Statistics

		Min	Max	Mean	Median	SD
Age		18.00	60.00	35.80	37.00	11.32
BMI		17.38	43.05	31.62	30.58	6.08
BKA (%)	Pretest	-.32	.67	.11	.14	.25
	Posttest	-.80	.91	.19	.22	.41
Cortisol (μg/dL)	Pretest	.0002	.39	.02	.002	.06
	Posttest	.0003	.20	.01	.002	.04
sIgA (mg/dL)	Pretest	.07	308.48	15.13	.50	64.11
	Posttest	.05	648.65	26.28	.55	117.04
Latency		13.00	299.00	120.35	110.00	47.84
Tattoo experience	Extent	0	21.50	6.19	3.83	5.94
	Hours	0	85.00	13.70	8.25	16.53
	Years	0	35.00	16.00	16.00	9.66
	TE: Extent+Hours	0	100.00	19.89	12.30	20.82
	TER: (Extent+Hours)/Years	0	33.33	2.87	.97	6.98

Table 2. ANCOVA of tattoo experience (TE) on bacteria-killing activity (BKA)

	Standardized β	t	*P*
(Constant)		-2.21	.04
$BKA_{Pretest}$	.41	2.69	.01
Tattoo experience[a]	.48	2.90	.01
Age	-.12	-.71	.48
BMI	.23	1.36	.19
Gender	-.29	-1.69	.10
Latency	.24	1.39	.18
$Cortisol_{Pre\text{-}posttest\ \Delta}$	-.23	-1.48	.15

[a] TE = Extent of body + Hours tattooed

Table 3. ANCOVA of tattoo experience (TE) on secretory immunoglobulin A (sIgA)

	Standardized β	t	*P*
(Constant)		-.38	.71
$sIgA_{Pretest}$	.82	11.09	< .01
Tattoo experience[a]	.04	.54	.58
BMI	-.01	-.16	.88
Age	.09	1.25	.22
Gender	-.09	-1.38	.18
Latency	.21	2.81	.01
$Cortisol_{Pre\text{-}posttest\ \Delta}$	-.07	-1.14	.26

[a] TE = Extent of body tattooed + Hours tattooed

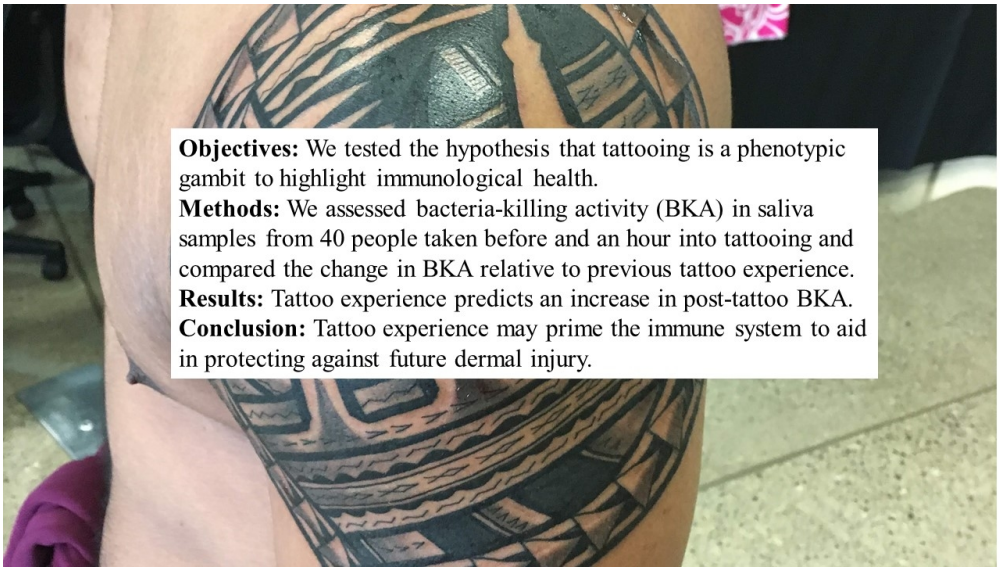

Photo of in-progress tattoo taken at the Northwest Tatau Festival, Puyallup, WA, July 2018. Photo by Christopher D. Lynn.

338x190mm (96 x 96 DPI)

Tattooing as a Phenotypic Gambit

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Running head: Tattooing as a phenotypic gambit

Abstract

Objectives: Tattooing is not an evolved behavior, but it may be a phenotypic gambit to highlight immunological health. Phenotypic gambits are traits or behaviors that appear costly but occur at high rates as a honing process of natural selection not constrained by genetics. ~~Tattooing involves wounding the body, which challenges the immune system and opens the body to infection and other potential health complications.~~ Tattooing is an ancient practice that is increasing in popularity worldwide~~. With hygiene innovations that make~~, but it involves wounding the body, which seems counterintuitive because it challenges the immune system and makes one more susceptible to infection. But tattooing ~~safer, the current popularity~~ may ~~be~~represent a ~~means~~costly honest signal of fitness by "upping the ante~~"; stimulating~~" in an era of hygiene or a means to stimulate the immune system ~~to heal~~ in a ~~uniquely visible~~ way ~~to highlight~~that improves and highlights underlying fitness.
Materials and Methods: We investigated this hypothesis by assessing bacteria killing activity (BKA) in saliva samples collected during two studies of tattooing ($N = 40$). We compared previous tattoo experience (extent of body tattooed and hours spent being tattooed) to BKA before and after getting a new tattoo.
Results: Tattoo experience positively predicts post-tattoo BKA ($\beta = .48$, $p = .01$~~).~~
~~**Discussion:** Tattoo experience may~~), suggesting that people with more tattoo experience have a relatively more immediate and active immune response than those with less tattoo experience.
Discussion: Tattoo experience may elevate innate immunological vigilance, which could aid in protecting against future dermal insults.

Keywords: tattooing, bacteria-killing activity, phenotypic gambit, immune function, stress

Introduction

A gambit is the sacrifice of something to gain an advantage. The phenotypic gambit is an assumption that traits or behaviors which may at first appear costly occur at high rates as part of a honing or refining process of natural selection ~~that is~~ not directly constrained by genetics (Grafen, 1991). Tattooing is a gambit in wounding the body, increasing potential for infection, and stressing the immune system (Lynn & Medeiros, 2017). ~~Tattooing~~This purposeful, pigmented wounding is an ancient practice, yet the global popularity of tattooing and accompanying industry have been growing ~~rapidly for several~~exponentially in recent decades (Blanton, 2015; Harris, 2016; Ibisworld, 2017; Jaworska et al., 2018; Kluger, 2015). Tattooing has been widely practiced as a means of group identity and marker of individuality, to distinguish status or accomplishments, to enhance attractiveness, and for apotropaic (magical or protective) and therapeutic-medical reasons; and there are good reasons to believe these wounds provide both psychological and physiological benefits (Piombino-Mascali & Krutak, 2020).

Carmen et al. (2012) pose two hypotheses to explain how short-term harm could be evolutionarily adaptive: (1) tattooing's popularity is result of sexual selection favoring body ornamentation for symbolic communication ("human canvas" hypothesis), and (2) being tattooed comes with physiological costs that can highlight immunological health ("upping the ante" hypothesis). Previous research indicates that tattooing might reflect fitness advantages by highlighting attractiveness, ~~underlying~~bilateral symmetry~~,~~ (an indication of developmental vigor), personality features, and life experiences (Heppell et al., 2005; Koziel et al., 2010; Osu et al., 2021; Wohlrab et al., 2007; 2009). ~~Tattoos may also improve fitness by enhancing the immune system (Lynn et al., 2016; 2019; 2022). We test this hypothesis through investigation of~~Another possibility is that the tattooing process could stimulate adaptive response of the innate

immune system in accordance with the principles of allostasis (Sterling, 2004). This latter model has been tested with regard to biomarker levels for cortisol, secretory immunoglobulin A (sIgA), and C-reactive protein (CRP) and find that when tattooing is novel (i.e., the individual has no or few previous tattoos), a rise in cortisol is associated with immunosuppression (Lynn et al., 2016; 2019; 2022). However, among those with relatively higher tattoo experience, immune response begins immediately, decoupled from cortisol response. We interpret this as allostatic adjustment to a repeated and desirable environmental stressor, as observed in exercise studies (Eöry et al., 2021), but it is not clear if changing levels of biomarker reflect actual immune function. Therefore, we retest this model through investigation of actual bacteria-killing activity before and after receiving a new tattoo.

The bacteria-killing activity (BKA) assay measures innate functional immune responses to a known quantity of *Escherichia coli* bacteria relative to a control (Muehlenbein et al., 2011). Previous studies of tattooing and immune function using ~~secretory immunoglobulin A (~~sIgA~~)~~ and ~~salivary C-reactive protein (~~CRP~~)~~ as proxies of immune functions support the proposed model. However, salivary CRP is arguably a better indicator of oral health than of circulating immune activity (Pay & Shaw, 2019), and sIgA and CRP may be more reflections of inflammation than immune performance (Longman et al., 2018).

We seek to build upon current knowledge of human immune function and the seeming paradox of adaptation through "self-injury" by investigating physiological responses to tattooing. While evolutionary models imply long-term advantages not measured in our study, the phenotypic gambit approach assumes an accrual of benefits to humans through a honing process. ~~We explored that~~Similarly, the allostasis concept refers to "stability through change," "fitness under natural selection," or "predictive regulation" (Sterling, 2004). Thus, novel stressors that

cause fear or anxiety should produce a stress response, including temporary suppression of functions not immediately necessary for fight-flight-or-freeze, such as immune activity. But tattoos are a welcome stressor to which people return, indicating that previous tattoo experiences were more rewarding than painful. Rather than a stress response, people with greater previous tattoo experience may be more influenced by the anticipation of reward than by the fear of pain and thus less likely to experience immunosuppression when receiving a new tattoo (Sterling, 2004). We explored this allostatic process, hypothesizing that BKA would (1) be greater at baseline among those with relatively more tattoo experience (TE; more hours spent being tattooed and larger proportion of body tattooed), (2) increase pre-posttest (i.e., over the course of getting a new tattoo) among those with relatively greater TE, and (3) decrease pre-posttest among those with less TE.

Materials and Methods

We used data collected by our team during two previous studies, including a study of 25 clients of three tattoo artists in American Samoa (Lynn et al., 2019) and 48 clients of 26 different artists at a tattoo festival in Puyallup, WA (Lynn et al., 2022). We removed participants whose saliva samples did not contain enough volume or were visibly contaminated. Participants for current analysis included 12 women and 28 men (N = 40) ages 18-60.

Procedures for both studies were the same except where noted below. We obtained permission from tattoo artists to recruit their clients for a study of tattooing and health. Study parameters were explained to clients, who agreed that we could use the samples to conduct secondary analysis of immune and endocrine functions related to our study hypothesis (both approved by University of Alabama IRB). We collected baseline demographic information (gender and age), including measures of previous TE (total hours tattooed, extent of body

tattooed, and years since first tattoo), and measured weight and height to calculate body mass index. In addition, we collected a saliva sample immediately before beginning the new tattoo, and a second sample at the end of the tattoo session (in the first study) or an hour later (in the second study). We noted the time each sample was collected and calculated the time between samples (latency).

BKA assays were conducted on samples that had enough saliva volume remaining after primary analyses (Lynn et al., 2019; 2022). A single lyophilized *E. coli* pellet (MicroBiologics Epower Microorganisms #0483E7) was reconstituted in sterile phosphate buffered saline and then diluted into a working solution, which produced approximately 200–300 colonies per 20 µL of aliquot. Aliquots of bacteria working solution were added to diluted saliva in a microcentrifuge tube, vortexed, and incubated for 30 minutes. After incubation, the samples were spread on trypticase soy agar plates (BD BBL #211043) in triplicate and incubated overnight at 37°C. The number of colonies on each plate the next day were counted, and the percent bacteria killed for each sample relative to a positive control (media and bacteria only) was calculated.

Of 73 pre-posttest saliva sample pairs (146 total saliva samples), 15 samples had insufficient saliva ~~left~~ remaining to conduct BKA analysis, and 34 were highly contaminated such that *E. coli* didn't grow or grew poorly. Furthermore, we retained samples only if we could conduct BKA analysis on both the pre- and posttest in a pair. Among the 40 participants whose samples were retained for this analysis, 30 samples displayed small amounts of contamination, but *E. coli* colonies appeared healthy, so we have included these samples in the analysis. Contamination was most likely due to participants eating or smoking before providing their samples. Cortisol (µg/dL) and sIgA (mg/dL) were assayed for previous studies with assay details

published (Lynn et al., 2020; 2022). All biomarker variables were standardized to account for skewedness.

We created two TE variables. The first explores the importance of extent of body and time being tattooed (TE). The second explores receiving tattoos in a rate of time (TER; i.e., does it matter if a person gets many tattoos in a short duration of months or years or gets them over a longer timespan?). We created the first TE variable by summing extent of body tattooed and hours tattooed. We created the TER variable by dividing this sum by years since first tattoo. We used the means of each TE variable to create dichotomous variables (e.g., Low/High TE) and compared BKA at pre- and posttest using paired and independent samples *t*-tests to test hypotheses 1 and 3, respectively. We used analysis of covariance (ANCOVA) in separate models to test the influence of TE and TER on the posttest BKA measure (hypothesis 2), controlling for pretest BKA, BMI, age, gender, cortisol change, and latency. Finally, we compared our measures of functional immune response (BKA) to inflammation (sIgA) by running the same model with sIgA in place of BKA. Analysis was conducted using SPSS Version 27 (IBM Corp., Armonk, NY) and statistics considered significant if $p < .05$.

Results

Table 1 displays descriptive statistics for variables used in analysis. BKA is displayed as percent of *E. coli* killed in the sample, with negative numbers indicating that more bacteria grew than were killed. These numbers appear small, since they are percentages and not units, the magnitude of change is large. The mean BKA increase from pre-post is 59%, and the highest rate of bacteria killing observed in the sample was 91%.

Table 1. Descriptive Statistics

	Min	Max	Mean	Median	SD
Age	18.00	60.00	35.80	37.00	11.32

BMI		17.38	43.05	31.62	30.58	6.08
BKA (%)	Pretest	-.32	.67	.11	.14	.25
	Posttest	-.80	.91	.19	.22	.41
Cortisol (µg/dL)	Pretest	.0002	.39	.02	.002	.06
	Posttest	.0003	.20	.01	.002	.04
sIgA (mg/dL)	Pretest	.07	308.48	15.13	.50	64.11
	Posttest	.05	648.65	26.28	.55	117.04
Latency		13.00	299.00	120.35	110.00	47.84
Tattoo experience	Extent	0	21.50	6.19	3.83	5.94
	Hours	0	85.00	13.70	8.25	16.53
	Years	0	35.00	16.00	16.00	9.66
	TE: Extent+Hours	0	100.00	19.89	12.30	20.82
	TER: (Extent+Hours)/Years	0	33.33	2.87	.97	6.98

We dichotomized the TE variables using the medians to ensure equal variances (Low TE = 0-12.30, High TE = 12.31+; Low TER = 0-.97, High TER = .98+), split the dataset to compare group pretest BKA, and conducted a paired samples *t*-test on pre- and posttest BKA. We found higher pretest BKA among the Low TE group when using the TE variable ($t = .17$, $p = .87$) but greater pretest BKA in the High TER group using the TER variable ($t = -1.23$, $p = .27$). Neither difference was significant.

We also compared pre-posttest BKA changes using both TE variables. Using the TE variable, BKA decreased pre-posttest for the Low TE group, but the difference was not significant ($t = .08$, $p = .94$). For the High TE group, there was a statistically significant pre-posttest increase ($t = -2.56$, $p = .02$). Using the TER variable, BKA increased pre-posttest for both groups, but neither change was significant (Low TER: $t = -.70$, $p = .49$; High TER: $t = -1.32$, $p = .21$).

We conducted ANCOVA using both TE variables, controlling for age, BMI, gender, latency (time duration between pre- and posttest samples), and pre-posttest change (Δ) in cortisol, but neither the model nor TER were significant. By contrast, the model for TE was significant ($F_{8,26} = 3.63$, $p = .01$, adjusted $r^2 = .38$), and TE was a significant predictor with a moderate effect size (Table 2).

Table 2. ANCOVA of tattoo experience (TE) on bacteria-killing activity (BKA)

	Standardized β	t	*P*
(Constant)		-2.21	.04
$BKA_{Pretest}$	.41	2.69	.01
Tattoo experience[a]	.48	2.90	.01
Age	-.12	-.71	.48
BMI	.23	1.36	.19
Gender	-.29	-1.69	.10
Latency	.24	1.39	.18
$Cortisol_{Pre\text{-}posttest\ \Delta}$	-.23	-1.48	.15

[a] TE = Extent of body + Hours tattooed

Finally, we compared BKA to sIgA as a proxy of immune function by substituting sIgA for BKA in the model. The sIgA models were significant, but neither TE nor TER was a significant predictor. In these sIgA models, only latency between sample collection times was significant (Table 3). This latter finding was unexpected, as tattoo experience has been a significant predictor of posttest sIgA in previous tattoo studies and because sIgA is one among numerous immunofactors active in killing bacteria.

Table 3. ANCOVA of tattoo experience (TE) on secretory immunoglobulin A (sIgA)

	Standardized β	t	*P*
(Constant)		-.38	.71
$sIgA_{Pretest}$	.82	11.09	< .01
Tattoo experience[a]	.04	.54	.58
BMI	-.01	-.16	.88
Age	.09	1.25	.22
Gender	-.09	-1.38	.18
Latency	.21	2.81	.01
$Cortisol_{Pre\text{-}posttest\ \Delta}$	-.07	-1.14	.26

[a] TE = Extent of body tattooed + Hours tattooed

Discussion

We explored BKA in saliva samples collected from people receiving new tattoos to determine if their previous TE influences how their immune system responds to the new tattoo. Consistent with allostasis theory of stress response (Goldstein & McEwen, 2002), we predicted that BKA would be suppressed or limited in tattoo neophytes (hypothesis 1) but would increase

in response to a new tattoo among those with relatively more TE (hypothesis 2). Furthermore, we compared pretest BKA of Low and High TE groups to determine if an immunological priming effect from previous TE persists over time as evidenced by relatively higher baseline BKA among the High TE group (hypothesis 3).

~~Contrary to~~There was a decrease pre-posttest in BKA for the Low TE group, which is consistent with our third hypothesis, ~~BKA increased from pre-posttest for both groups, though differences were~~but that change was not statistically significant. Our second hypothesis was supported, as TE was a significant predictor of posttest BKA when controlling for pretest BKA, BMI, age, gender, and cortisol. ~~The~~However, the amount of time since one's first tattoo was ~~not~~neither a ~~significant~~ predictive factor~~. We~~, nor did ~~not~~we find greater pretest BKA among the High TE group.

We thought BKA might be elevated in the high TE group relative to the lower experience participants if tattooing produced an immunological priming effect that persists over time. This hypothesis derives from one our team's previous studies in which mean sIgA at pretest was significantly greater for that study's high tattoo experience participants relative to the low tattoo experience group (Lynn et al., 2020). We also predicted that BKA would decrease in the low TE group, though previous results for sIgA on this count are also mixed (Lynn et al., 2019; 2022). We divided tattoo experience by years since the participant's first tattoo. This enabled us to develop a refined rate~~-~~of~~-~~tattoo~~-~~experience variable similar to one used in our first study (Lynn et al., 2016). We thought that if a priming effect of tattooing on the immune system exists, it might decay over time, which we could detect by calculating this rate variable. However, similar to our Puyallup study (Lynn et al., 2022), we did not detect any effects for TER. An alternative explanation that may explain this lack of a TER effect is that memory of the tattoo experience

may fade over time, and early first tattoo experiences may play little bearing on later appraisal of a new tattoo after years have passed.

~~This study was limited by~~ This was a proof-of-concept study to determine the efficacy of BKA as a biomarker of actual immune activity and not simply levels. The failure to replicate previous findings with regard to sIgA is puzzling, but it is possible that there are other mechanisms of innate immune response are more important in response to *E. coli* than sIgA and represents a better model going forward than studies of select antibodies or hormones. We find support for the utility of the BKA salivary assay in field studies of immune activity, but we do not know if these findings reflect immunological benefits that are clinically significant.

This study has limitations, including the sample size and condition of the samples. As this was secondary analysis of samples assayed previously for multiple biomarkers, many samples did not have sufficient saliva volume left to assay BKA. Others were contaminated and disposed of. A subset of samples included in analysis had some contamination and were retained because the *E. coli* colonies appeared unaffected by the contamination, but future research should include a larger participant cohort and screen participants for oral health and contamination. ~~Finally, a~~A complete analysis of immune response to tattooing would include measures of adaptive immunity as well as innate responses. Future study using the rate of tattooing experience might consider the repetition of tattooing experiences over time spans and not simply the timespan since a first tattoo. Finally, there are many other factors that influence physiological responses to tattooing, including type of equipment used and cleaning protocols, site and tattooist hygiene, ink quality and composition, and other factors (Farley et al., 2019; Rademeyer et al., 2020), some of which we controlled for but that will require a larger sample to assess in a full analytic model.

Conclusions

~~Our study~~We examined bacteria killing activity relative to tattooing to determine if immune responses are adaptively honed through repeated cultural practices that stress the body. We found that dermal stress of tattooing results in increased innate immune activity that is influenced by previous TE, supporting the model of tattooing as a phenotypic gambit. Tattooing may therefore enhance evolutionary fitness signals. However, we did not detect immunosuppression necessarily associated with being a tattoo novice, and we failed to replicate previous findings for the biomarker sIgA. Tattooing can enable researchers to explore the dynamics of the interacting endocrine and immune systems, but more research is necessary. Fortunately, the popularity of tattooing and the variability in how tattoos are administered continue to grow. Future research should explore the intervening mechanisms that mediate these dynamic activities and to determine if such fitness signals also have clinical value for health.

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Data Availability

Files used in this analysis are available from The University of Alabama Institutional Repository at _____.

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Dear Dr. Turner,

Thank you for the thorough review. I am delighted to resubmit our manuscript titled "Tattooing as a phenotypic gambit" with the revisions suggested by reviewers, editorial board members, and associate editors. Below we explicitly address these comments:

Please also note that the American Journal of Biological Anthropology and its publisher, Wiley, have recently implemented a data sharing policy. Officially, AJBA now expects, but does not absolutely require, that all data be publicly available. Authors are required to include a Data Sharing Statement in their manuscript stating whether the data underlying the manuscript are publicly available or not, and if so, where. Explicit instructions are now part of the Instructions for Authors. Any manuscript submitted or resubmitted to AJBA will now be required to follow these procedures. They will be incorporated into the Manuscript Central submission protocols as well.

We have made the SPSS dataset used in the analysis available via The University of Alabama Institutional Repository and are awaiting the completion of screening and assignment of a DOI.

I concur with the editorial board member and the reviewers that this manuscript will be of interest to our readership. To make it stronger and clearer, I ask the authors to consider the comments of the reviewers, with which I agree.
I would like to ask the authors to consider relaxing the reliance on statistical significance (p-values) for reporting and interpreting the results. Can you comment on the magnitude of the pre-post differences? What can we say about the biological significance of these differences?

We have added explanations of the BKA statistics and the magnitude of the change being discussed to results and discussion so the meaning of these data are more clear. Thank you for this helpful suggestion.

Abstract
The abstract currently reads a little like the authors ran out of space. It would be nice to see a little more detail given in the results and discussion subsections.

We have fleshed out the results and discussion sections of the abstract.

Introduction
The introduction feels a little brief and could do with providing a little more information. For example:
1) As the authors explain, tattooing – an act of self-wounding – is very popular. The introduction would benefit from a couple of sentences explaining (in more detail than at present) just why many people do this.
2) More importantly, why do the authors suppose that an 'Tattooing may also improve fitness by enhancing the immune system'? This is a very interesting point and is well referenced with the lead author's recent work. However, it is not explained, and is central to the logic of the proposed hypothesis. Could you expand on this a little, for the benefit of readers unfamiliar with these previous papers?
3) Why might you expect BKA to
decrease pre to post-tattoo in participants with low TE (hypothesis 3)?

Thank you for highlighting this logic gap. We have now explained the apparent contradiction of tattooing as a wound in more detail, which some historical context. Furthermore, we have introduced the allostasis theory that is implied by our hypotheses, explained it, and made it clear that our hypotheses derive theoretically from said work. We point out that another definition of allostasis is "regulation under selection" to emphasize the theoretical connections being made between "phenotypic gambit" and "allostasis" as theoretical models.

Methods

The baseline measure was taken immediately before the beginning of the new tattoo. Might there be any kind of anticipatory effect on BKA levels?

The authors note that the time of measurement was detailed. How might time affect BKA, and how was this factored into the analysis?

TER variable – it seems as though this variable may assume a consistent rate of tattooing. It could be that the same value is assigned to participants who, for example, obtained their first tattoo many years ago, then no more for a long time, and then many tattoos recently and someone who received the same number / area coverage of tattoos but at a more regular rate. Might this be expected to confound the results of this particular analysis?

There might be an anticipatory effect for BKA. We have considered anticipatory effects for cortisol and possible immunosuppressive effects of this anticipation. We plan to ask about participant fear or anxiety re the tattooing experience in the pretest surveying in future research but did not collect those data for the current analysis.

We collect time data to calculate the duration between measures and time it takes to complete the tattoo. While there are diurnal patterns for some biomarkers (like cortisol), it has not been established that all elements involved in bacteria killing follow the same patterns. Furthermore, to determine if time of day is important, we would need to collect samples for a full day. We have data from another study that may relate to this question, but those data have not been sufficiently analyzed yet to address this question.

The variable implies a consistent rate of tattooing, but that is not the intent. The intent is to determine if a single instantiation of tattooing earlier in life has a persistent effect, and it appears that it does not. The reviewer is correct is assuming that the giant gaps likely confound the utility of the variable, so we go back to the drawing board in constructing a useful variable that accounts for time.

Results

Could you please add units to Table 1.

Done, thank you!

Discussion

3rd hypothesis: Line 44 – 'BKA increased from pre-posttest for both groups'. This isn't what the results says (Lines line 13) – 'Using the TE variable, BKA decreased pre-posttest for the Low TE group'

It would be good to consider the construction of the TER variable (see my comment above), for which no significant results were found.

The second paragraph refers to hypothesis 3 and 2 in brackets. For clarity, it would be useful to do the same for hypothesis 1.

Thank you for catching that. We have modified the Discussion to reflect the actual findings.

We agree that the construction of the TER variable is problematical and will work on an alternative means of exploring tattooing experience as a rate in future research designs.

Paragraph one of the discussion has all three hypotheses bracketed, so I think this might be a reviewer oversight?

Conclusions
It seems, from the results section, as though the findings are rather mixed. I think this could be reflected in the conclusions, which at present don't really summarise the entirety of the findings,
BKA would
(1) be greater at baseline among those
with relatively more tattoo experience (TE; more hours spent being tattooed and larger proportion of body tattooed),
(2) increase pre-posttest (i.e., over the course of getting a new tattoo) among those with relatively greater TE, and
(3) decrease pre-posttest among those with less TE.

Thank you for this. We have fleshed out the conclusion, noting both the mixed nature of the findings.

The process of tattooing is far from uniform. Because these study subjects were recruited in 2 different locations (Samoa and a tattoo festival in Washington state), and because at least 29 different tattoo artists were responsible for the body art, the potential for skewed results are enormous. Variables include type of equipment used and the cleaning protocols for this equipment; hygiene of the room in which the tattoos were performed; PPE worn by the tattoo artist; attention to aseptic technique by the tattoo artist; composition of the ink used; age and storage of the ink; and combination of ink types used. Other important variables include personal health information about the subjects, including: history of infectious diseases, history of immune-suppressing conditions, surgical history, use of substances (alcohol, recreational drugs, tobacco), number of previous tattoo experiences and intervals of time between each one, and history of any infectious or inflammatory events after previous tattoos.

For a review of why these types of factors are relevant and important, this reviewer recommends
Farley, C., Van Hoover, C., & Rademeyer, C. A. (2019). Women and tattoos: Fashion, meaning, and implications for health. Journal of Midwifery and Women's Health, 64(2), 154-169. doi.org/10.1111/jmwh.1293
Rademeyer, C. A., Farley, C., & Van Hoover, C. (2020). Body art: Health implications and counseling considerations for individuals with piercings and tattoos. Nursing for Women's Health, 65(3), 210-227. doi.org/10.1016/j.nwh.2020.03.006

This manuscript would be strengthened by a more thorough discussion of these other

variables. It is, however, a valuable first look at an intriguing physiological marker of innate functional immune response following a tattoo experience.

We appreciate these points and hope to address more of them in future research. This was a proof-of-concept analysis, so we have sought to test first if we could detect a signal of immune response through the noise of all this variation. In previous work, we have controlled for tattoo artist or style to account for some of the variation different methods introduce, as well as medications, drug/alcohol/cigarette/marijuana use, medical issues, allergies, and other relevant data. In future work, we will focus on collecting volume of participants over longer periods of time using single or few tattoo studios to reduce the variability.

Sincerely,

Christopher Lynn