JMIRx Bio

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Volume 3 (2025) ISSN 2819-2044 Editor in Chief: Edward Meinert, MA (Oxon), MSc, MBA, MPA, PhD, CEng, FBCS, EUR ING

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Original Paper

Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation

Markie Peroutka[1], BS; Ignacio Rivero Covelo[1], PhD

Department of Psychology, Professional Counseling, and Neuroscience, University of Wisconsin–Parkside, Kenosha, WI, United States

Corresponding Author:
Ignacio Rivero Covelo, PhD
Department of Psychology, Professional Counseling, and Neuroscience
University of Wisconsin–Parkside
900 Wood Rd
Kenosha, WI, 53144
United States
Phone: 1 8478680045
Email: riveroco@uwp.edu

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Abstract

Background: The neural control of food intake involves interactions between homeostatic and nonhomeostatic systems. The nucleus accumbens shell (AcbSh) and ventral pallidum (VP) play key roles in regulating ingestive behavior and project to each other. Previous studies have shown that these projections influence food consumption, with sex differences reported in the modulation of sucrose intake by VP projections.

Objective: This study aimed to investigate the effects of chemogenetic activation or inhibition of projections from the VP to the AcbSh on sucrose consumption and the motivation to work for sucrose in female rats.

Methods: Chemogenetic tools (DREADD [designer receptors exclusively activated by designer drugs]) were used to selectively activate or inhibit VP projections to the AcbSh in female Sprague-Dawley rats (Gi [inhibitory G protein] DREADD: n=11; Gq [excitatory G protein] DREADD: n=10; and no DREADD: n=12). Rats were trained on a progressive ratio operant task to assess motivation to work for sucrose. Additionally, free-access sucrose consumption tests were conducted using a 20% sucrose solution. The effects of chemogenetic modulation were analyzed using two-way ANOVA.

Results: Chemogenetic manipulation of VP projections to the AcbSh did not significantly affect the motivation to work for sucrose in the progressive ratio task ($F_{2,31}$=1.780; P=.18). However, a significant interaction between DREADD type and drug administration was observed in the sucrose consumption test. Activation of the VP-AcbSh projection (using Gq DREADD) decreased sucrose intake, while inhibition (using Gi DREADD) increased sucrose intake ($F_{2,31}$=18.891; P=.001). No significant changes in sucrose consumption were observed in the control group without DREADD expression (P=.50).

Conclusions: This study shows that projections from the VP to the AcbSh modulate sucrose intake but do not affect the motivation to work for sucrose. Chemogenetic activation reduced sucrose consumption, while inhibition increased it, suggesting that distinct neural circuits within the VP-AcbSh pathway may differentially regulate feeding behaviors. These findings highlight the role of this pathway in the consumption of palatable foods and indicate that future research should consider factors such as sex, food macronutrient composition, and specific neural subpopulations to better understand their role in feeding behavior.

(JMIRx Bio 2025;3:e68519) doi:10.2196/68519

KEYWORDS

ventral pallidum; nucleus accumbens shell; chemogenetics; sucrose; feeding behavior; food motivation; palatable food; DREADD; designer receptors exclusively activated by designer drugs

Introduction

The neural control of food intake and energy balance involves interactions between homeostatic and nonhomeostatic systems. Traditionally, homeostatic regulation was attributed to hypothalamic and brainstem circuits responding to metabolic signals [1].

Critically, ventral striatopallidal structures, including the nucleus accumbens shell (AcbSh) and ventral pallidum (VP), exert a major influence on ingestive behavior by acting on some of these structures, mainly the lateral hypothalamus (LH). Inhibition of AcbSh neurons through gamma-aminobutyric acid (GABA) agonists or glutamate antagonists elicits intense feeding responses and activates LH neurons, as evidenced by increased *Fos* expression [2]. The AcbSh projects to both the LH and VP, with unilateral lesions of either structure attenuating AcbSh-induced feeding [3]. The LH also modulates AcbSh activity directly through neurotransmitters like orexin and melanin-concentrating hormone, and indirectly via subcortical relay regions such as the VP [4,5]. Relatedly, blockage of GABA receptors in the VP elicits food intake in satiated rats [2], and this feeding presents a clear fat preference [6].

Recent studies have suggested a role of sex in the mediation of sucrose consumption. In female rats, optogenetic stimulation of AcbSh projections to the VP decreased sucrose intake and altered its hedonic value [7]. Additionally, increased sucrose intake has been reported in male rats, but not female rats, because of chemogenetic activation of GABAergic projection neurons in the VP [8].

Both the AcbSh and VP regulate food intake. Notably, the relationship between the VP and AcbSh is that of a loop, and the role that projections between the 2 play in feeding remains understudied. The directionality of the circuit is relevant, as projections from the AcbSh to the VP have different effects compared to projections from the VP to the AcbSh [9]. Additionally, as mentioned above, sex differences have been reported when modulating the projections of the VP [8]. Here, we aim to study the role that chemogenetic activation or inhibition of projections from the VP to the AcbSh have on the motivation to work for sucrose and on the consumption of sucrose in female rats. We hypothesize that chemogenetic modulation of the VP-AcbSh pathway, either inhibition or excitation, will alter the motivation to work for sucrose and sucrose consumption.

Methods

Subjects

A total of 36 female Sprague-Dawley rats (Envigo) were used for these studies; they were 75 days old and weighed 250-300 g (at the time of arrival). After all the procedures described in this section were completed, the final number of rats per group were as follows: Gi (inhibitory G protein) DREADD (designer receptors exclusively activated by designer drugs), n=11; Gq (excitatory G protein) DREADD, n=10; and no DREADD, n=12. All rats were pair-housed in temperature- and humidity-controlled rooms with a 12:12 light-dark cycle. In their home cages, rat pairs had access to chewing bones and a polyvinyl chloride pipe hut. After arrival at the facility, the rats were allowed to acclimate to the colony room for at least 1 week before starting behavioral testing; during this time, the rats were handled once a day by researchers. The rats were also handled regularly for the duration of the behavioral experiments. All rats had ad libitum access to food and water for the duration of the experiments. Behavioral testing took place during the light cycle between 10:00 AM and 5:00 PM.

Ethical Considerations

The experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Parkside and were in accordance with the guidelines on animal care and use of the National Institutes of Health.

Surgeries

Surgeries were performed using standard, aseptic, flat-skull stereotaxic techniques under isoflurane anesthesia (5% induction and 2% maintenance) delivered by a precision vaporizer. Once a stable plane of anesthesia was achieved, a sterile eye ointment was applied to both eyes (to prevent corneal desiccation), the analgesic was administered, the scalp was prepped for an incision (hair trimming with alcohol and iodine scrub), an incision was used to expose the skull, and burr holes were created above the target structures for the injection of adeno-associated viruses (AAVs).

An AAV, double-floxed inverse open reading frame (DIO) construct containing an inverted form of either Gi (AAV5 AAV-hSyn-DIO-hM4D(Gi)-mCherry; Addgene) or Gq (AAV5 AAV-hSyn-DIO-hM3D(Gq)-mCherry; Addgene) DREADD was injected into the VP (from bregma: anterior posterior: –0.2 mm; medial lateral: ±1.8 mm; and dorsal ventral: –8.7 mm). A retrograde AAV-Cre viral vector (AAVrg pENN.AAV.hSyn.HI.eGFP-Cre.WPRE.SV40; Addgene) was injected into the AcbSh (from bregma: anterior posterior: 1.6 mm; medial lateral: ±0.8 mm; and dorsal ventral: –8.1 mm). Injections were performed using a Harvard micropump, Hamilton microsyringes connected to fluid-filled flexible tubing, and Plastics One injectors for a final volume of 1 uL at an injection rate of 300 nL per minute.

For pain management, meloxicam (2 mg/kg, subcutaneous) was administered during the surgery and 24 hours later. Triple antibiotic was applied around the incision after closure using wound clips. Clips were removed 7 to 10 days after the surgery.

The rats were allowed to recover for 2 weeks before behavioral testing.

Clozapine-N-Oxide Preparation

Clozapine-N-oxide (CNO) was obtained from the National Institute on Drug Abuse Drug Supply Program. CNO was administered intraperitoneally 20 minutes before behavioral testing at a dose of 3.0 mg/kg. CNO was freshly prepared daily by dissolving it in 100% dimethyl sulfoxide (DMSO) and then diluting it with sterile water to a final concentration of 6% DMSO. A 6% DMSO solution in sterile water was used as the vehicle control.

Sucrose Access Under a Progressive Ratio Operant Task

The rats were trained in a progressive ratio (PR) operant task using identical, standard, twin-lever operant chambers (Med-Associates) housed within sound-attenuating chambers. First, the animals got 2 daily, 30-minute, magazine training sessions in the operant boxes, during which reinforcers (45-mg, sucrose, banana-flavored Dustless Precision Pellets; BioServe) were presented at 1-minute intervals, with a “click” generated at the same time as food delivery. Next, the rats were shaped to press the lever and then placed on a fixed ratio (FR) 1 reinforcement schedule for 2 days. The rats got one session of training on an FR2 schedule, followed the next day by one on an FR4 schedule. The rats were then switched to a PR6 schedule, which continued for the remainder of the experiment. Each day, the rats were placed into operant chambers with the house light on and both levers extended; only one lever was associated with the sucrose reward, although presses on both levers were recorded. The first response on the correct lever was followed by a sucrose pellet reward, paired with the operation of the clicker. The number of responses required to earn each subsequent sucrose pellet was increased by 6 after each reinforcer, so that 7 responses were required to earn the second pellet, 13 to earn the third, and so on. The time of each lever press was recorded. Each session continued until a 3-minute pause in responding occurred—a cutoff value that has been used in other studies [10,11]—or 60 minutes had elapsed, at which time the house lights were turned off, the levers were retracted, and the rats were removed from the chambers. The animals ran for 5 days on the PR6 schedule prior to drug treatment. After that, and 20 minutes before behavioral testing, the rats were injected with either CNO (3.0 mg/kg) or the vehicle. All rats were administered 2 injections of CNO on 2 different days and 2 injections of the vehicle, also on 2 different days.

Free-Access Sucrose Consumption Test

The rats were placed in individual home cages with wired bottoms and given access to a 20% sucrose solution for 60 minutes. This procedure was repeated over 2 consecutive days to acclimate the rats to the sucrose solution and minimize neophobia. After these 2 days, the rats were administered with either CNO (3.0 mg/kg) or the vehicle 20 minutes before being placed in the individual home cages. The sucrose bottles were weighed before and after the experiment to measure consumption. As described before, all rats got 2 CNO and 2 vehicle injections, with each injection on a different day.

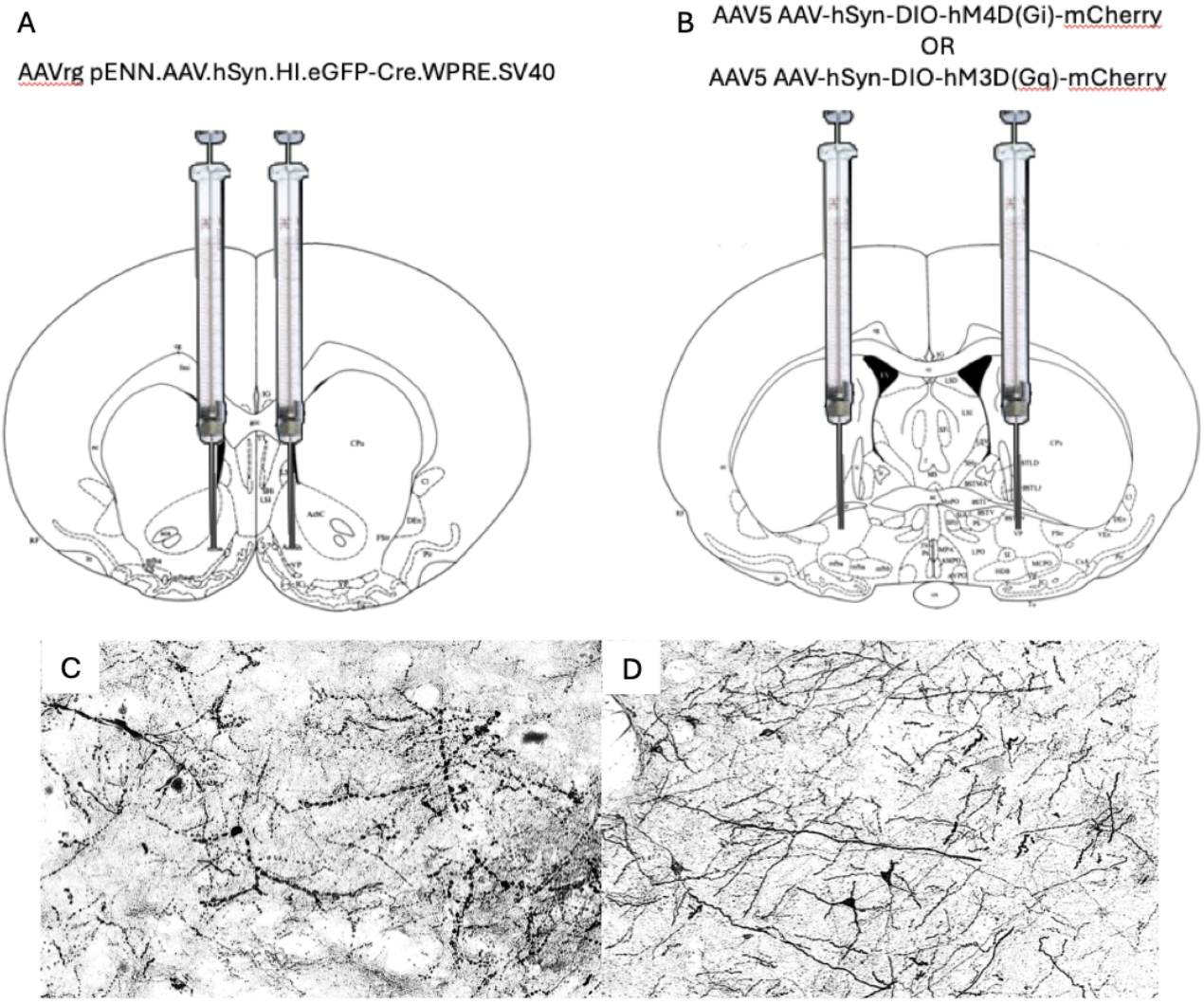
Perfusion and Tissue Processing

After completing the behavioral experiments, the rats were anesthetized with 5% isoflurane and transcardially perfused with 0.9% saline followed by 4% formaldehyde (pH=7.4) for fixation. The brains were extracted, postfixed in 4% formaldehyde for 24 hours at 4°C, and immersed in increasing concentrations of sucrose solutions every 24 hours (10%, 20%, and then 30% sucrose in 0.1 M phosphate-buffered saline [PBS], pH=7.4) at 4°C over the course of 3 days. The brains were then encased in Tissue-Plus O.C.T. (Fisher HealthCare), frozen using dry ice, and subsequently sectioned in the coronal plane (45 µm) using a cryostat.

Immunohistochemistry

The accuracy of DREADD expression in the VP and AcbSh was assessed using immunohistochemistry aimed at visualizing mCherry protein in DREADD-expressing neurons using procedures described previously [12]. Free-floating coronal sections from the VP and AcbSh were first rinsed 3 times in 0.1 M PBS (pH=7.4). Endogenous peroxidase activity was blocked by incubating sections in 1% H_2O_2 for 10 minutes, followed by 3 additional rinses. To prevent nonspecific binding of the secondary antibody, sections were incubated in 0.1 M PBS containing 0.4% Triton X-100 (TX) and 2.5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc). Sections were then incubated overnight at room temperature with the primary antibody (rabbit anti-mCherry; Abcam; diluted 1:30,000) in 0.1 M PBS + 0.4% TX + 1% NDS. Then, sections were rinsed again before being incubated for 1 hour in a biotinylated, donkey, anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc; diluted 1:500) in 0.1 M PBS + 0.4% TX + 1% NDS. Peroxidase staining was obtained by using a standard avidin-biotin procedure using the Vectastain Elite ABC Kit (Vector Laboratories, Inc; diluted 1:1000 for A and B). Chromogenic reaction occurred by incubating sections in a 0.1 M PBS solution containing 0.02% 3,3’-diaminobenzidine tetrahydrochloride and 0.012% H_2O_2. Sections were rinsed and stored at 4°C until mounted, air dried, and covered with slips using a toluene-based mounting medium (Permount; Thermo-Fisher Scientific). Bright-field images containing the VP or AcbSh were captured using a Zeiss Axioscan light microscope and were analyzed by an experimenter blinded to the experimental groups. The location of mCherry expression was confirmed using a rat brain atlas [13]. A schematic representation of the approach and representative mCherry pictures can be found in Figure 1 [14].

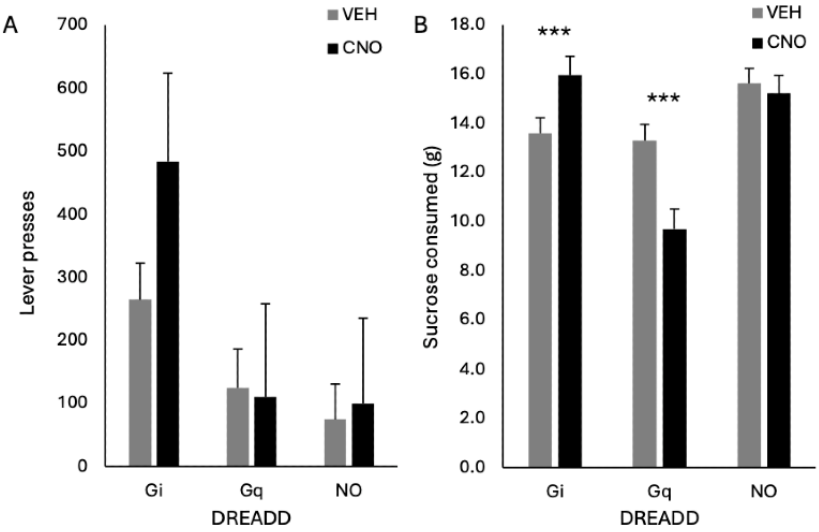
Figure 1. (A) A retrograde AAV-Cre viral vector was injected into the AcbSh. (B) An AAV DIO construct containing an inverted form of either Gi or Gq DREADD was injected into the VP (adapted from Paxinos and Watson [14]). Representative AcbSh (C) or VP (D) 10× microphotograph of mCherry immunohistochemistry. AAV: adeno-associated virus; AcbSh: nucleus accumbens shell; DIO: double-floxed inverse open reading frame; DREADD: designer receptors exclusively activated by designer drugs; Gi: inhibitory G protein; Gq: excitatory G protein; VP: ventral pallidum.



Results

A 2-way ANOVA was performed to evaluate the effects of DREADD type (Gq, Gi, or no DREADD) and drug administered (vehicle or CNO) on lever presses in a sucrose PR task. The results indicated no significant main effect for DREADD type ($F_{2,31}$=2.421; P=.10); no significant main effect for drug administered ($F_{1,31}$=2.004; P=.17); and no significant interaction between DREADD type and drug administered ($F_{2,31}$=1.780; P=.18; Figure 2A).

Figure 2. (A) CNO administration did not affect motivation to work for sucrose, as measured using a progressive ratio task in non–food-deprived, DREADD-expressing rats (Gi and Gq) and control rats (no DREADD). (B) Non–food-deprived rats expressing inhibitory (Gi), excitatory (Gq), or no DREADD were given 1 hour to consume a 20% sucrose solution after being injected with either the vehicle or CNO. CNO-induced chemogenetic inhibition of the VP-AcbSh pathway increased sucrose consumption in rats (*P*=.001), excitation decreased it (*P*=.001) and had no effect on rats not expressing DREADD (*P*=.50). CNO: clozapine-N-oxide; DREADD: designer receptors exclusively activated by designer drugs; Gi: inhibitory G protein; Gq: excitatory G protein; VEH: vehicle.



A 2-way ANOVA was performed to evaluate the effects of DREADD type (Gq, Gi, or no DREADD) and drug administered (vehicle or CNO) on 20% sucrose consumption in non–food-deprived rats. The results indicated a significant main effect for DREADD type ($F_{2,31}$=11.170; *P*=.001); no significant main effect for drug administered ($F_{1,31}$=3.148; *P*=.09); and a significant interaction between DREADD type and drug administered ($F_{2,31}$=18.891; *P*=.001; Figure 2B).

Post hoc testing using Bonferroni correction for multiple comparisons indicated that sucrose consumption was significantly higher for rats expressing Gi DREADD when CNO was administered than when the vehicle was administered (*P*=.003). Additionally, sucrose consumption was significantly lower for rats expressing Gq DREADD when CNO was administered than when the vehicle was administered (*P*=.001). There was no significant difference between the sucrose consumption of rats expressing no DREADD administered with either CNO or the vehicle (*P*=.50; Figure 2B).

Discussion

In female rats, chemogenetic excitation or inhibition of projections from the VP to the AcbSh influenced consumption of a 20% sucrose solution but had no effect on the motivation to work for a sucrose pellet, as measured using a PR task. Specifically, chemogenetic activation of projections from the VP to the AcbSh in non–food-deprived female rats decreased consumption of the 20% sucrose solution. Conversely, chemogenetic inhibition of the same projection increased consumption of the 20% sucrose solution.

In contrast, Scott et al [8] reported that chemogenetic activation of VP projection neurons resulted in no significant changes in rat chow or sucrose consumption. This apparent discrepancy between the 2 studies can be explained by multiple reasons. Possibly the most crucial difference between the 2 studies is that, here, we used a dual vector approach to express DREADD in VP neurons that project to the AcbSh, while Scott et al [8] used a single vector approach, leading to all GABAergic VP projection neurons expressing DREADD. Thus, here, chemogenetic manipulations affected a small subset of VP projection neurons, namely those that project to the AcbSh, while in the study conducted by Scott et al [8], all VP projections were affected by chemogenetic modulation. It is nonetheless informative that we observed different behavioral effects, as this suggests that different VP efferents might have a variety of behavioral effects. This matter could be addressed by future studies dissecting the role of each VP efferent. Additional studies should also consider the sex differences noted by Scott et al [8].

Other differences to consider between the 2 studies include the concentration of sucrose used in the free-access test, as we used a 20% concentration while Scott et al [8] used 10%; the fact that our rats remained pair housed as opposed to single housed; and the differences in rat strain, as they used Long-Evans rats and we used Sprague-Dawley rats. Additionally, there were also differences in the DREADD agonist used: JHU37160 versus CNO in our experiment. While all these differences possibly contributed to some extent to the different behavioral results between the 2 studies, we consider that the most likely difference stems from the targeting of all GABAergic VP projecting neurons in Scott et al [8] versus only VP neurons projecting to the AcbSh in this study.

The directionality of the VP-AcbSh pathway has also been studied by Smedley et al [9]. Interestingly, this group saw no effect on free feeding on male rats when the projections from the VP to the AcbSh were chemogenetically inhibited. Besides the sex differences in the subjects, it is also notable that Smedley et al [9] measured the intake of standard rat chow. In contrast, here, we measured the consumption of a 20% sucrose solution. It is then possible that either or both factors, sex and food stuff,

might contribute to the different behavioral results observed. Thus, it appears that projections from the VP to the AcbSh mediate sucrose consumption but not motivation to work for sucrose. Future studies looking at other VP effects might be able to dissect which projections are involved in the motivation to work for sucrose and other palatable foods.

Additionally, it has been reported that pharmacological activation of the VP leads to increased preference for fat consumption [6]. In contrast, the food used in this study contained mainly carbohydrates, 94% in the case of the sucrose pellets used in the PR task and 20% in the case of the free-access task. It is then plausible that identical manipulations of the VP-AcbSh pathway could result in different behavioral effects if fats instead of carbohydrates were used as food rewards. Future studies should consider the possibility that different behavioral effects might be observed by using fats or offering a choice of different macronutrients.

Further, it has been described that arkypallidal neurons located in the VP inhibit AcbSh neurons and increase consumption of a 5% sucrose reward in mice [15]. In contrast, in this study, activation of the VP-AcbSh pathway led to a decrease in the consumption of the 20% sucrose reward in rats. This discrepancy could be caused, at least in part, by the difference in the nature of the projection neurons recruited and their putative roles, as we targeted all VP neurons projecting to the AcbSh, while Vachez et al [15] specifically targeted ventral arkypallidal neurons. It is then possible that the behavioral effects of modulating the whole VP-AcbSh pathway, as done here, differ from that of specific neural subpopulations. Also intriguing is the possibility that the VP-AcbSh pathway underlies different behavioral outcomes depending on the timing of the stimulation applied. Vachez et al [15] used phasic optogenetic stimulation, while we used more tonic chemogenetic manipulations. Future studies should contemplate the examination of phasic versus tonic stimulation in this pathway.

In conclusion, our findings indicate that the VP-AcbSh pathway mediates the consumption of a palatable sucrose solution. Chemogenetic manipulation of VP projections to the AcbSh selectively influenced sucrose intake without affecting motivation to work for sucrose pellets, suggesting that distinct VP efferents play differential roles in feeding behavior versus food-seeking motivation. Additionally, the findings indicate a nuanced role for the VP-AcbSh pathway in modulating the intake of specific macronutrients. Future studies that dissect the role of the VP-AcbSh pathway should consider variables such as macronutrient profile, sex, and neural subpopulations as well as their possible interactions.

Acknowledgments

The authors would like to thank the College of Natural and Health Sciences for the support; the National Institute on Drug Abuse Drug Supply Program for providing the clozapine-N-oxide; and the following students for their help with this work: Megane Beaupre, Ari Hjelmeseth, Miranda Johnson, Hedi Morris, Zach Wilson, Aryan Patel, Mark Huckeby, and Dylan Jensen.

Conflicts of Interest

None declared.

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Abbreviations

AAV: adeno-associated virus
AcbSh: nucleus accumbens shell
CNO: clozapine-N-oxide
DIO: double-floxed inverse open reading frame
DMSO: dimethyl sulfoxide
DREADD: designer receptors exclusively activated by designer drugs
FR: fixed ratio
GABA: gamma-aminobutyric acid
Gi: inhibitory G protein
Gq: excitatory G protein
LH: lateral hypothalamus
NDS: normal donkey serum
PBS: phosphate-buffered saline
PR: progressive ratio
TX: Triton X-100
VP: ventral pallidum

Edited by O Singh; submitted 07.11.24; peer-reviewed by D Wirtshafter, J Grimm; comments to author 01.01.25; revised version received 08.01.25; accepted 18.01.25; published 08.03.25.

Please cite as:
Peroutka M, Rivero Covelo I
Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation
JMIRx Bio 2025;3:e68519
URL: https://bio.jmirx.org/2025/1/e68519
doi:10.2196/68519
PMID:

Original Paper

Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study

Ebrahim Abbasi[1], PhD

Department of Medical Entomology and Vector Control, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran

Corresponding Author:
Ebrahim Abbasi, PhD
Department of Medical Entomology and Vector Control
School of Health
Shiraz University of Medical Sciences
Zand Street
JGHF+XFG
Shiraz, 3761833650
Iran
Phone: 98 9124338389
Fax: 98 9124338389
Email: abbasie.ebrahim@gmail.com

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Abstract

Background: Ticks are well-known ectoparasites of domestic animals, causing significant economic losses and playing a crucial role in the transmission of pathogens within the livestock industry worldwide, including in Iran. Understanding the distribution and diversity of ticks is essential for effective control strategies, especially in regions like Tehran province, where livestock plays a vital role in the economy.

Objective: This study aimed to determine the frequency and distribution of livestock ticks across different seasons and climatic zones in Tehran province.

Methods: In 2019, a total of 1623 domestic animals infested with ticks were examined, including chickens, sheep, camels, cows, pigeons, and dogs. A total of 806 ticks were collected, comprising 121 (15%) soft ticks and 685 (85%) hard ticks. Tick species were identified and categorized based on their occurrence in mountainous and plain climate regions.

Results: Out of the 806 collected ticks, 44.8% (n=361) were found in the mountainous region and 55.2% (n=445) were found in the plain region. The most abundant species was *Rhipicephalus sanguineus* (n=307, 38.1%), while *Rhipicephalus (Boophilus) annulatus* was the least common (n=3, 0.4%). Seasonal variation indicated peak infestation in the spring (n=486, 60.3%) and the lowest infestation in the winter (n=77, 9.6%).

Conclusions: The study highlights the significant diversity and abundance of both soft and hard ticks in livestock across various regions of Tehran province. These findings emphasize the need for targeted tick control measures, considering the differences in tick distribution between mountainous and plain climate regions.

(JMIRx Bio 2025;3:e69542) doi:10.2196/69542

KEYWORDS

impact of climate; seasonal change; frequency; livestock; ticks; Tehran

Introduction

Ticks are of outstanding medical and veterinary importance because they transmit severe and dangerous diseases to humans and animals [1]. In humans, most diseases caused by spirochetes and rickettsia are transmitted by ticks. In addition, these arthropods cause severe diseases such as paralysis, encephalitis, and tularemia [2]. Moreover, ticks cause livestock financial losses worldwide and in Iran, where the annual impairment caused by ticks is estimated at US $13.9 to US $18.7 billion [3].

Ticks on livestock cause localized bite-site lesions and systemic effects. They can lead to anemia, paralysis, and even death by transmitting diseases like theileriosis and babesiosis [4]. On the other hand, global climate change has significantly impacted the stability and distribution of their life cycles [5], and climate conditions are the most critical factor determining tick distribution [6]. The tick-borne diseases associated with wildlife and climate change favor the re-emergence of diseases and the possible risk of the emergence of new ones [7-9].

Although the parasitic fauna of *Argasidae* and *Ixodidae* ticks and some of their ecological characteristics have been studied in a few areas of Iran [10], the various species from all regions of Iran in general and Tehran province in particular and especially the seasonal activity are not thoroughly studied. Tehran province is located between mountainous and plain regions [11]. Three factors—humid westerly winds, the province's extent, and the Alborz mountain range—play an essential role in Tehran province's climate. The Alborz mountain range has tempered the climate of Tehran province. It is mountainous: temperate in the north and semiarid in the lowlands. Tehran province features a diverse geography, encompassing mountainous and plain areas with distinct climates. In the mountainous region, such as Shemiranat, the average annual temperature ranges from 10 °C to 12 °C, with increased precipitation of 350 to 400 mm annually. Winters are cold, with substantial snowfall, while summers are mild. Conversely, the plain areas like Varamin have hotter conditions, averaging from 18 °C to 20 °C annually, with lower rainfall of 150 to 250 mm. These plains experience hot summers and milder winters, characterized by a semiarid climate [12,13].

This study was conducted to determine the seasonal and climatic frequency of tick species in Tehran province on the body surface of livestock, involving chickens, camels, cattle, dogs, pigeons, and sheep in different areas. The importance and current status of tick control in this region will become more evident when we know the distribution of ticks, their location, and presence in each region, and the epidemiological situation can be determined. In Tehran province, so far, not much research has been done on the climatic and seasonal distribution of different species of ticks.

Also, the published information about livestock infested with ticks in this area is not complete, so the purpose of this study is to provide an accurate scientific report of the situation of livestock infested with hard and soft ticks in different climates and during different seasons of the year; this information can be used in macroplanning to combat foreign parasites.

Methods

Geographical Area

The study was conducted in two different environments: plain and mountainous regions within 20 selected villages in Tehran province, which were located between 34° to 36.5° N and 50° to 53° E.

Sampling

The sample size was calculated using the Cochran formula for prevalence studies. Given an estimated prevalence (p) of 30% and a precision (d) of 4.5%, the final sample size was determined to be 800 ticks, ensuring statistical reliability [14]. To ensure representative sampling, a cross-sectional study was conducted, covering both mountainous and plain regions. The selection of livestock was randomized among those showing visible tick infestation, with veterinary supervision ensuring consistency in sample collection across different geographical zones. The chosen method aligns with established epidemiological studies on tick distribution.

The prevalence value (p=0.3) was selected based on prior studies on tick prevalence in similar regions in Iran, indicating an estimated infestation rate of 30%. The margin of error (d=0.045) was determined considering a 95% confidence level, ensuring a balance between precision and the feasibility of sample collection.

Study Area

The study was performed in two separate climatic zones, including 6 mountainous villages and 14 plain villages. After collecting geographical and ecological information, 1623 domestic animals were selected [14]. Using a cross-sectional study design, the distribution of ticks was studied in different study areas from spring to the end of winter of 2019. Ticks were collected using curved forceps from the host body (livestock's earlobes, groin, tail base, and back, and poultry's underarms, groin, and abdomen). To identify the collected ticks' genus and species, valid diagnostic keys were used [15]. The tick species were identified using the diagnostic keys outlined by Jongejan et al [15] and Camicas et al [16], which provide detailed morphological descriptions and illustrations for the identification of both soft and hard ticks. These keys are widely recognized for their accuracy and reliability in the identification of tick species in the Middle East and neighboring regions [16,17]. Based on the distribution of ticks in the study area, the prepared maps, the identification of infested carriers, climate conditions, and host animals of these tick species were analyzed, providing an understanding of the existing situation in the province [16-20]. Two professional stereo microscopes were used to

identify tick species: the Leica S9i and the Zeiss Stemi 508. The Leica S9i has 10× to 60× magnification and includes a 10 megapixel camera, while the Zeiss Stemi 508 offers 8× to 50× magnification with superior optical quality. Both tools are essential for the detailed investigation of tick morphology in entomological research.

Results

Determining the Frequency of Livestock Ticks by Climate Type

In this study, 1623 domestic animals infested with ticks were studied, including chickens, camels, cattle, dogs, pigeons, and sheep. A total of 806 ticks were collected, and 685 (85%) and 121 (15%) of them were detected as hard ticks and soft ticks, respectively. The distribution of collected ticks indicates that out of 806 collected ticks, 361 (44.8%) belonged to the mountainous region, and 445 (55.2%) belonged to the plain region (Table 1). In the mountainous region, the genus *Rhipicephalus*, with 51.2% (185/361), was the most frequent, and the genera *Hyalomma* and *Ornithodoros* were the least frequent. In the plain region, the genus *Hyalomma*, with 66.3% (295/445), was the most frequent, and the genera *Haemaphysalis* and *Boophilus* were the least frequent (Table 1).

Table 1. The number and frequency of ticks caught by genera according to climatic topography, Tehran province, 2019.

Genera	Mountainous region, n (%)	Plain region, n (%)	Total, n (%)
Rhipicephalus (n=307)	185 (60.3)	122 (39.7)	307 (100)
Hyalomma (n=295)	0 (0)	295 (100)	295 (100)
Argas (n=102)	93 (91.2)	9 (8.8)	102 (100)
Haemaphysalis (n=80)	80 (100)	0 (0)	80 (100)
Ornithodoros (n=19)	0 (0)	19 (100)	19 (100)
Rhipicephalus (Boophilus) (n=3)	3 (100)	0 (0)	3 (100)
Total (n=806)	361 (44.8)	445 (55.2)	806 (100)

Argas persicus from the genus *Argas*, *Rhipicephalus bursa* and *Rhipicephalus sanguineus* from the genus *Rhipicephalus*, and all species from the genera *Boophilus* and *Haemaphysalis* were found in the mountainous region of Tehran province. Among the ticks found in the mountainous region, *R sanguineus*, with 48.8% (176/361), was the most frequency. In comparison, *Rhipicephalus (Boophilus) annulatus*, with 0.8% (3/361), was the least frequent (*Hyalomma marginatum*, *Hyalomma asiaticum*, *Hyalomma dromedarii*, *Hyalomma anatolicum*, *Ornithodoros lahorensis*, *Argas reflexus*, and *Hyalomma detritum* were not found in the mountainous region). All *Ornithodoros* species, all *Hyalomma* species, *A reflexus*, and *R sanguineus* were collected from the plain region. Among the tick species found in the plain region, *Hy marginatum*, with 34.3% (152/445), was the most frequent, and *Hy detritum*, with 1.3% (6/445), was the least frequent (*A persicus*, *Haemaphysalis sulcata*, *Haemaphysalis inermis*, *Haemaphysalis erinacei*, *R bursa*, and *R (B) annulatus* were not found in the plain region; Table 2).

The results show that 38.7% (628/1623) and 6.13% (995/1623) of studied animals belonged to the mountainous and plain regions, respectively (Table 3). Of the 230 infested animals, 97 (42.2%) and 133 (57.8%) belonged to the mountainous and plain regions, respectively.

Table 2. The number and frequency of ticks caught by species according to climatic topography, Tehran province, 2019.

Species	Mountainous region, n (%)	Plain region, n (%)	Total, n (%)
Rhipicephalus sanguineus (n=298)	176 (59.1)	122 (40.9)	298 (100)
Hyalomma marginatum (n=152)	0 (0)	152 (100)	152 (100)
Argas persicus (n=93)	93 (100)	0 (0)	93 (100)
Hyalomma anatolicum (n=21)	0 (0)	21 (100)	21 (100)
Hyalomma dromedarii (n=49)	0 (0)	49 (100)	49 (100)
Hyalomma asiaticum (n=67)	0 (0)	67 (100)	67 (100)
Haemaphysalis sulcata (n=47)	47 (100)	0 (0)	47 (100)
Ornithodoros lahorensis (n=19)	0 (0)	19 (100)	19 (100)
Haemaphysalis inermis (n=24)	24 (100)	0 (0)	24 (100)
Haemaphysalis erinacei (n=9)	9 (100)	0 (0)	9 (100)
Argas reflexus (n=9)	0 (0)	9 (100)	9 (100)
Rhipicephalus bursa (n=9)	9 (100)	0 (0)	9 (100)
Rhipicephalus (Boophilus) annulatus (n=3)	3 (100)	0 (0)	3 (100)
Hyalomma detritum (n=6)	0 (0)	6 (100)	6 (100)
Total (n=806)	361 (44.8)	445 (55.2)	806 (100)

Table 3. The number and frequency of animals infested by ticks in different climate regions, Tehran province, 2019.

Climate region	Collected ticks (n=806), n (%)	Infested animals (n=230), n (%)	Studied animals (n=1623), n (%)
Mountainous	361 (44.8)	97 (42.2)	628 (38.7)
Plain	445 (55.2)	133 (57.8)	995 (61.3)

When analyzing the data, it was found that domestic animals had the highest rate of tick infestation in the spring, while the lowest rate of infestation was observed in the winter. It indicates a seasonal variation in tick distribution and infestation rates among domestic animals. In the spring, all genera (except *Boophilus*) were found. The genera *Rhipicephalus* and *Hyalomma* were distributed in the summer. In the autumn, the genus *Hyalomma* was the most abundant, and in the winter, the species *A persicus* (belonging to the family of soft ticks) had a high abundance (Table 4). The frequency of all 6 ticks (4 hard ticks and 2 soft ticks) in different seasons of the year is described below.

Table 4. The number and frequency of ticks caught according to the season (n=806), Tehran province, 2019

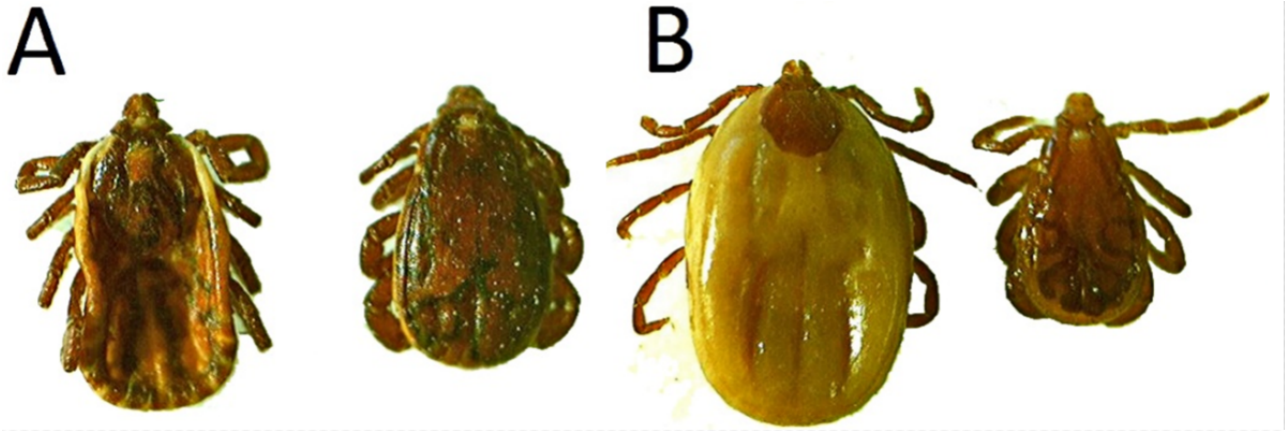
Species	Seasons				
	Spring, n	Summer, n	Autumn, n	Winter, n	Total, n (%)
Rhipicephalus sanguineus	251 (31.1)	23 (2.9)	18 (2.2)	6 (0.7)	298 (37)
Hyalomma marginatum	74 (9.2)	53 (6.6)	14 (1.7)	11 (1.4)	152 (18.8)
Argas persicus	34 (4.2)	0 (0)	41 (5.1)	18 (2.2)	93 (11.5)
Hyalomma asiaticum	33 (4.1)	22 (2.7)	9 (1.1)	3 (0.4)	67 (8.3)
Hyalomma dromedarii	11 (1.4)	13 (1.6)	7 (0.9)	18 (2.2)	49 (6.1)
Haemaphysalis sulcata	28 (3.5)	15 (1.9)	0 (0)	4 (0.5)	47 (5.8)
Hyalomma anatolicum	14 (1.7)	0 (0)	0 (0)	7 (0.9)	21 (2.6)
Ornithodoros lahorensis	12 (1.5)	3 (0.4)	4 (0.5)	0 (0)	19 (2.4)
Haemaphysalis erinacei	9 (1.1)	0 (0)	0 (0)	0 (0)	9 (1.1)
Haemaphysalis inermis	12 (1.5)	0 (0)	12 (1.5)	0 (0)	24 (3)
Argas reflexus	3 (0.4)	0 (0)	6 (0.7)	0 (0)	9 (1.1)
Rhipicephalus bursa	3 (0.4)	0 (0)	0 (0)	6 (0.7)	9 (1.1)
Rhipicephalus (Boophilus) annulatus	0 (0)	3 (0.4)	0 (0)	0 (0)	3 (0.4)
Hyalomma detritum	2 (0.2)	0 (0)	0 (0)	4 (0.5)	6 (0.7)
Genera	13 (N/A[a])	7 (N/A)	8 (N/A)	9 (N/A)	14 (N/A)
Total	486 (60.3)	132 (16.4)	111 (13.8)	77 (9.6)	806 (100)

[a]N/A: not applicable.

Seasonal Activity and Fauna of Rhipicephalus (Hard Ticks)

In this study, 307 ticks of the genus *Rhipicephalus* were caught, which was 38.1% (307/806) of the total sample collected (the highest frequency among the genera). *R sanguineus* of this genus was caught in all seasons, while *R bursa* was only caught in the spring and winter (Figure 1).

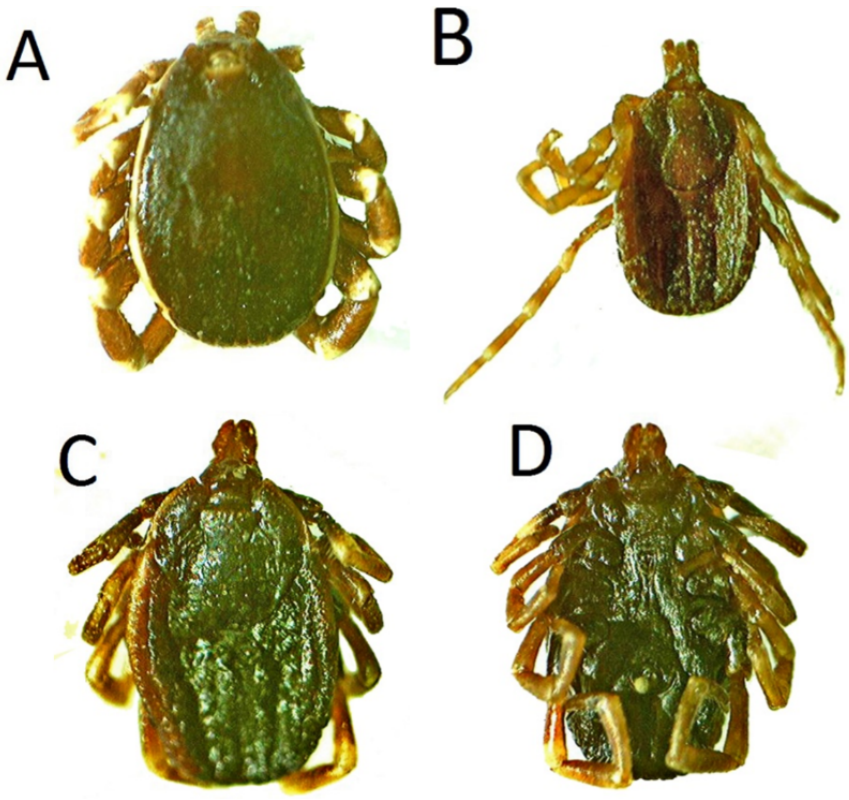
Figure 1. Rear view of (A) *Rhipicephalus sanguineus* (male on the right and female on the left) and (B) *Rhipicephalus bursa* (original; male on the right and female on the left).



Seasonal Activity and Fauna of Hyalomma (Hard Ticks)

The genus *Hyalomma*, with 295 ticks, was the second most abundant genus, accounting for 36.6% (295/806) of the total sample. *Hy marginatum*, *Hy asiaticum*, and *Hy dromedarii* were caught in all seasons, but *Hy anatolicum* and *Hy detritum* were only caught in the spring and winter (Figure 2).

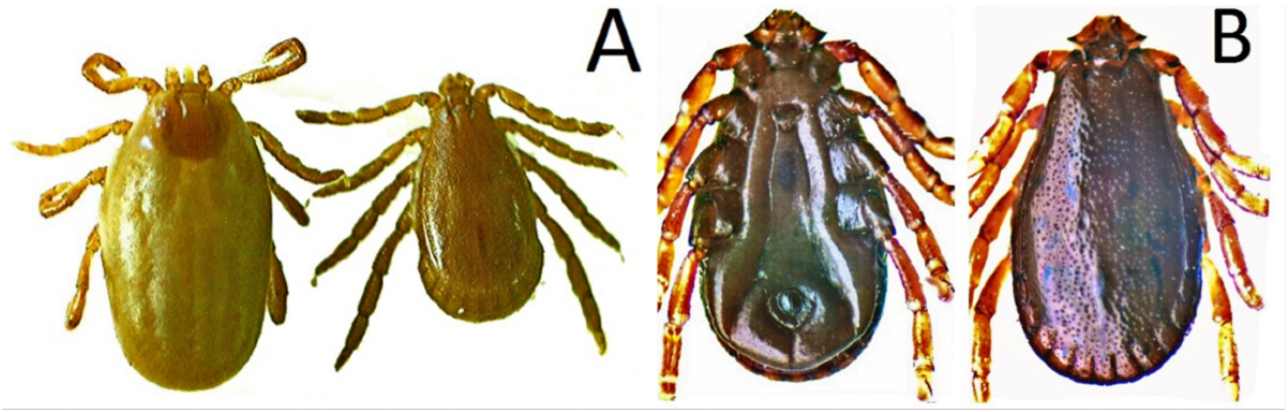
Figure 2. Rear view of (A) male and (B) female *Hyalomma marginatum*; and (C) dorsal view and (D) abdominal view of female *Hyalomma detritum* (original).



Seasonal Activity and Fauna of Haemaphysalis (Hard Ticks)

The genus *Haemaphysalis* was the third genus of the hard tick family detected in this study, and it has 3 species of *Hae sulcata*, *Hae inermis*, and *Hae erinacei*; a total of 80 ticks were caught, with a frequency of 9.9% (80/806). *Hae sulcata* was found in all seasons except for autumn, *Hae inermis* was found in the spring and autumn, and *Hae erinacei* was only caught in the spring (Figure 3).

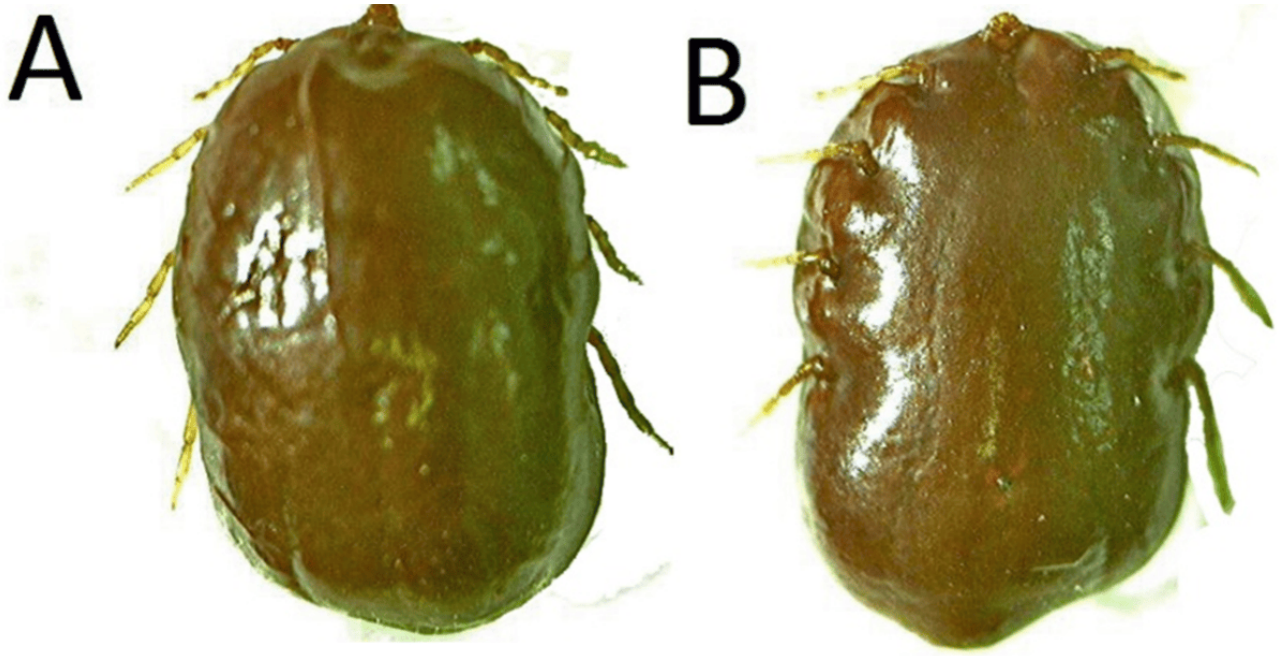
Figure 3. (A) Rear view of *Haemaphysalis sulcata* (male on the right and female on the left) and (B) abdominal view of *Haemaphysalis erinacei* (original; male on the right and female on the left).



Seasonal Activity and Fauna of Rhipicephalus (Boophilus) (Hard Ticks)

In this study, the genera *Boophilus* was only caught in the summer with 1 species, *R (B) annulatus*, with an abundance of 3 ticks (Figure 4).

Figure 4. (A) Rear view and (B) abdominal view of female *Rhipicephalus (Boophilus) annulatus* (engorged; original).

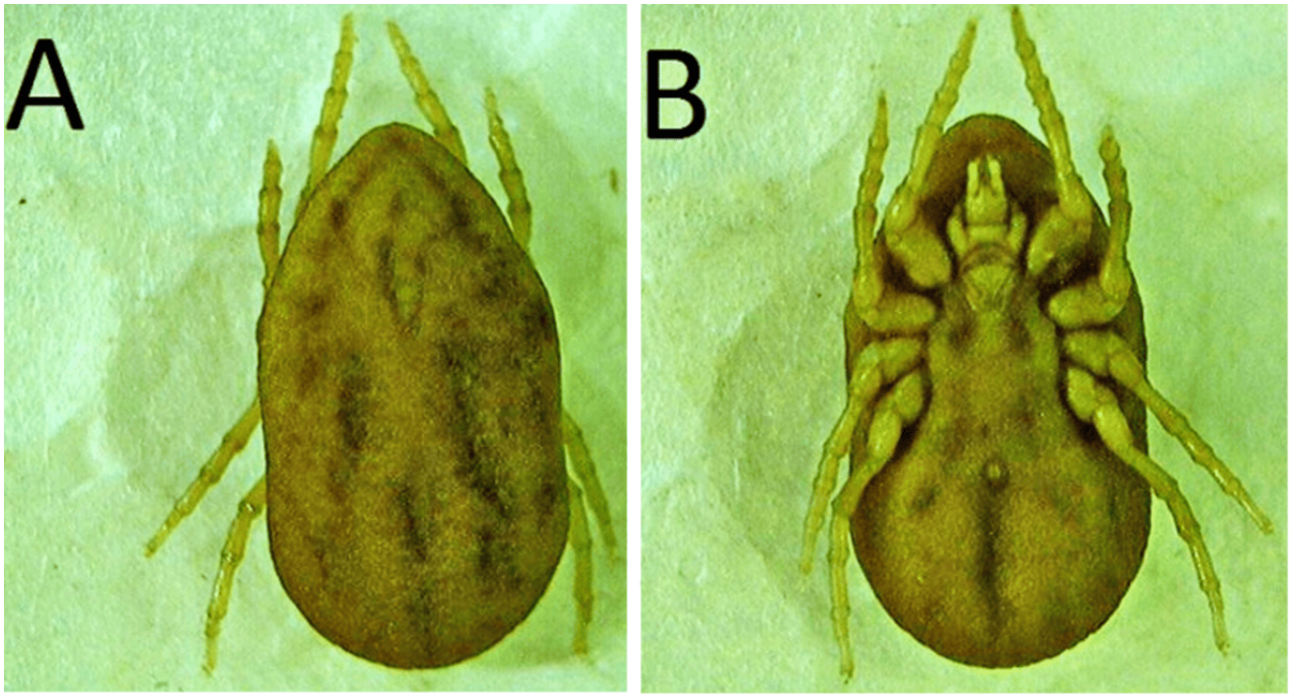


Seasonal Activity and Fauna of Argas and Ornithodoros (Soft Ticks)

In this study, 121 (15% of the total 806) ticks belonging to the soft tick family were caught, which included the genus *Argas* with 2 species, *A persicus* and *A reflexus* (102/121, 84.3%), and the genus *Ornithodoros* with only 1 species, *O lahorensis* (19/121, 15.7%). *A persicus* was caught in all seasons except for the summer, *A reflexus* was caught in the spring and autumn, and *O lahorensis* was caught in all seasons except for the winter (Figures 5 and 6).

Figure 5. (A) Rear view and (B) abdominal view of *Argas persicus* (original).

Figure 6. (A) Rear view and (B) abdominal view of *Ornithodoros lahorensis* (original).



Discussion

Principal Findings

This study is the only codified and comprehensive study on *Ixodidae* and *Argasidae* ticks and their climatic and seasonal activities during 2019 in Tehran province. The *Ixodes* genus was not found in our study because this genus is more distributed in the country's northern provinces, such as Gilan, Mazandaran, and Golestan provinces [21].

A study in Pakistan confirmed the dominance of the tick species of *R (B) annulatus* and *Hy anatolicum*, also prevalent in Tehran province, particularly impacting sheep and cattle. In contrast, a study done in Turkey highlighted that *Ixodes ricinus* is predominantly found in humid regions, which differs from Tehran province's dry climate. Studies from India are also aligned with the findings from Tehran province, showing *R sanguineus* as the most common species. Conversely, Japan reported *Haemaphysalis longicornis* as the most frequent species, influenced by temperate climates. Southern Europe exhibits some similarities with Tehran province, particularly regarding *R sanguineus*; however, it shows higher *I ricinus* prevalence. Northern Europe shows a dominance of *I ricinus* due to temperate climates, differing from Tehran province's dry conditions. In North Africa, tick species like *Hy anatolicum* can be found, which aligns with the findings from Tehran province, while sub-Saharan Africa presents contrasting species like *Amblyomma variegatum*, which thrive in humid climates. Overall, the analysis emphasizes how climate and ecology influence tick species composition and host preferences across regions [22-28].

The observed seasonal trend aligns with the biological cycles of tick species. Hard ticks such as *Rsanguineus* and *Hymarginatum* exhibited peak abundance in the spring and summer due to higher temperatures and increased host activity. Conversely, soft ticks (*Apersicus*) showed resilience during colder months, likely due to their ability to survive in sheltered environments. This seasonal variability highlights the importance of targeted tick control strategies, particularly in warm seasons when the transmission risk of tick-borne diseases is the highest [29-31]. Furthermore, this genus is more distributed in the cold and wet seasons of the year. In our study, different cities in Tehran province did not have high humidity compared to the northern provinces. Therefore, it is assumed that high humidity is a limiting factor in the distribution of this tick in our study area.

In a study in Golestan province, 6 genera and 15 species of ticks, including soft and hard ticks, were reported [32], which is considerably similar to our study conducted in Tehran province. This may be due to the climatic similarities of the two provinces and the proximity and parallelism of research. *A persicus* soft ticks have been caught in Shemiranat city in Tehran province, which has a mountainous climate, with a frequency of 11.5%. *A persicus* is caught in all seasons except for the summer. Its highest frequency was observed in the autumn, which is consistent with studies conducted in the cities of Sanandaj, Boyer-Ahmad, and Bijar in Kurdistan province [33-35].

R sanguineus was the most abundant among the ticks caught in Tehran province. This species has been caught in the cities of Tehran, Islamshahr, Shemiranat, Shahreri, Pakdasht, and Varamin from both plain and mountainous climate regions, which is consistent with other studies carried out in Ghaemshahr, Mazandaran province [36]. In general, the results of this study agree with the studies of other researchers due to the similarity of climate conditions. However, slight differences were observed between the results, which can be attributed to climatic diversity and the susceptibility of different breeds of livestock.

Conclusions

The distribution of collected ticks indicated that out of 806 collected ticks, 44.8% and 55.2% belonged to the mountainous and plain regions, respectively. This study demonstrated significant abundance and diversity of *Ixodidae* and *Argasidae* ticks in livestock in different places of Tehran province.

Acknowledgments

The authors would like to thank the Veterinary Office of Tehran Province and the research vice-chancellor of Shiraz University of Medical Sciences for their cooperation. This study received no grants from commercial, public, or nonprofit entities.

Data Availability

All data obtained from this research are included in the paper's main text.

Authors' Contributions

EA designed the study, collected the ticks, identified tick species, recorded geographic coordinates and area information, wrote the manuscript, and confirmed and submitted the paper.

Conflicts of Interest

None declared.

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Edited by J Ren; submitted 02.12.24; peer-reviewed by J Shen, V Noaman; comments to author 05.02.25; revised version received 11.02.25; accepted 16.02.25; published 31.03.25.

Please cite as:
Abbasi E
Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study
JMIRx Bio 2025;3:e69542
URL: https://bio.jmirx.org/2025/1/e69542
doi:10.2196/69542
PMID:

Population Interaction in the J mon Society via 3D Data of Human Crania: Geometric Morphometric Study

Hisashi Nakao[1], PhD; Akihiro Kaneda[2], PhD; Kohei Tamura[3,4,5], PhD; Koji Noshita[6], PhD; Mayu Yoshida[7], MA; Tomomi Nakagawa[8], PhD

[1]Department of Anthropology and Philosophy, Nanzan University, 18 Yamazato, Showa, Nagoya, Japan
[2]Graduate School of Humanities and Social Sciences, Mie University, Mie, Japan
[3]The Center for Northeast Asian Studies, Tohoku University, Sendai, Japan
[4]Graduate School of Environmental Studies, Tohoku University, Sendai, Japan
[5]Center for Integrated Japanese Studies, Tohoku University, Sendai, Japan
[6]Graduate School of Science, Kyushu University, Fukuoka, Japan
[7]Graduate School of Letters, Tohoku University, Sendai, Japan
[8]Graduate School of Letters, Nagoya University, Nagoya, Japan

Corresponding Author:
Hisashi Nakao, PhD
Department of Anthropology and Philosophy, Nanzan University, 18 Yamazato, Showa, Nagoya, Japan

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Abstract

Background: The Jōmon period (14,000 to 800 cal BC), when people on the Japanese archipelago engaged mainly in hunting and gathering and experienced relatively more severe climate changes, is an important prehistoric period for investigating how people reacted to environmental fluctuations in human evolutionary history. Anthropologists have extensively discussed the population history of the Jōmon period of the Japanese archipelago via their morphological variations. Some have supported the notion of relative morphological uniformity within the Jōmon population, which could be sustained by widespread population interactions, although others have claimed that spatiotemporal morphological differences (especially geographical clines) may exist to some extent.

Objective: The aim of this study is to examine the morphological interphase and interregion variations among the Jōmon populations to investigate the population interactions during this period (ie, how widely and continuously they interacted).

Methods: This study used geometric morphometrics of a much larger sample of 3D data of Jōmon human crania than previous studies (n=363 from 97 sites, including 146 females, 215 males, and 2 unknown-sex individuals). The configurations of landmarks were processed with generalized Procrustes analysis and principal component analysis. The principal scores were statistically analyzed with the Steel-Dwass test. We also compared the Jōmon crania with the Yayoi crania in the same way.

Results: Notably, the results of statistical tests on the Jōmon crania show that principal component (PC) 1 did not show any significant differences both in spatial and temporal comparisons. Regional differences did not show geographical clines in PC1. PC2 indicated that statistically significant differences were found in some regions (eg, the Tohoku and Tokai regions: Z=6.375, P<.001; the Kanto and Tokai regions: Z=4.880, P<.001), and a gradual geographical cline was found among the different regions and in phases (eg, the Early and Final phases: Z=3.118, P=.02; the Middle and Final phases: Z=4.233, P<.001). Comparative results between the Jōmon and the Yayoi populations also showed that the Jōmon populations were spatiotemporally less varied than the Yayoi populations and that individual variation within a site was more variable in the Jōmon site than that of the Yayoi site.

Conclusions: This observation is consistent with the possibility that the population interactions of the Jōmon people had been widespread and continuous, which has an important implication for their resilience against severe climate changes at that time. It is possible that the relative stability of the Jōmon society was sustained by their frequent interactions with various populations, as suggested by insights from relevant archeological, ethnographic, and genetic research.

(JMIRx Bio 2025;3:e72432) doi:10.2196/72432

KEYWORDS

Jomon; Japan; crania; cranium; bones; anthropology; archaeology; morphology; morphometrics

Introduction

General Background

On the Japanese archipelago, the Jōmon period (14,000-800 cal BC; Incipient: 14,000-9300 cal BC; Initial: 9300-5200 cal BC; Early: 5200-3400 cal BC; Middle: 3400-2400 cal BC; Late: 2400-1200 cal BC; Final: 1200-800 cal BC [1]; the Incipient period was excluded in this research because no human skeletal remains from this period were found), when people engaged mainly in hunting and gathering, is prehistoric period that is particularly important for researchers investigating how people reacted to environmental fluctuations in human evolutionary history. This period experienced more severe climatic changes, including the Younger Dryas, than did subsequent Yayoi (800 - 250 AD) and Kofun (250 - 600 AD) societies [2-5]. Many have claimed that prehistoric societies undergoing radical environmental fluctuations tended to be accompanied by societal change [6-9]. The Jōmon society may be no exception. Research has suggested that climate change has affected population size in many regions [10,11], which may have influenced other aspects of the Jōmon society.

However, features such as the emergence of social hierarchies, shifts in subsistence patterns, and significant technological developments commonly seen in other periods were less radical in the Jōmon society. Jōmon people predominantly adhered to a hunter-gatherer lifestyle and persisted in using stone tools for over 10 millennia. Although recent findings at the Usu-Moshiri site in Hokkaido found evidence of violent injuries in more than 5 individuals, such occurrences remained relatively rare within the broader context of the Jōmon period. This stands in stark contrast to the following Yayoi period [5,12-16] and can be distinguished from patterns observed in other relevant regions worldwide [17-19]. Although the notion of a “static view” of the Jōmon society has been challenged over the years [2,20], it remains clear that societal changes during this era were relatively less drastic, even in the face of more drastic climatic variations.

One potential strategy for mitigating the impact of external environmental stresses is sustained interaction between different populations. The continuous exchange of genetic material, knowledge, skills, and resources can enhance the resilience and adaptability of populations in the face of environmental disruptions. Many relevant studies of genetic diversity in organisms other than humans support this possibility, including plants, various animal species, and whole ecosystems [21-23]. The specific mechanisms behind the genetic diversity and resilience remain controversial, although it is generally argued that genetic diversity is somehow related to morphological and functional diversity, and that damage to populations due to ecological disturbance could be compensated for by such diversity.

This is also evident in modern hunter-gatherer societies, which exhibit higher genetic diversity, indicative of ongoing interactions and gene flow between groups [24-26]. Moreover, ethnographic research shows that mobile hunter-gatherer communities with lower population densities often seek mates from distant groups [27-29]. Archeological and anthropological research also suggest that higher mobility of prehistoric hunter-gatherers was an important factor for their societal stability [11,30,31].

Previous Research on Jōmon Population Interactions

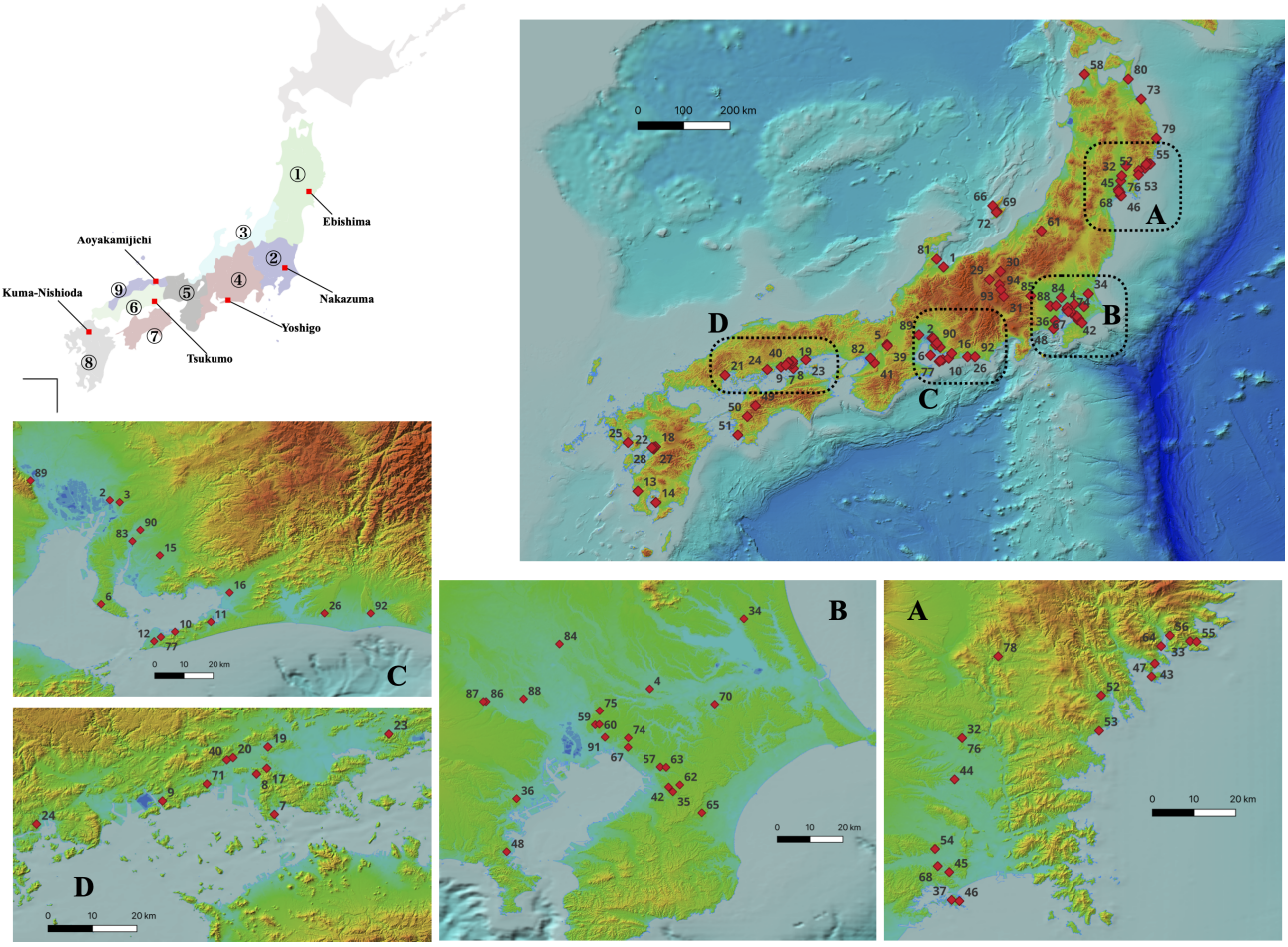
Anthropologists have extensively discussed the population interaction history during the Jōmon period of the Japanese archipelago. Typically, previous research used a traditional biodistance method using distances between specific measuring points[32,33] (see also Figure 1), and statistically significant differences between morphological biodistances among different populations have been regarded as populational and genetic differences. For example, as stated in the next paragraph, if geographical clines and statistically significant differences are found among morphological biodistances of different populations, it is commonly suggested that populations moved in a certain geographical direction and that they did not migrate widely.

Some research on human skeletal remains including crania and limbs has supported the notion of relative morphological uniformity within the Jōmon population, which could be sustained by widespread population interactions, although others have claimed that spatiotemporal differences may exist to some extent [34-42]. Dodo [34,35] argued that statistical differences found in cranial biodistances among Jōmon populations in the Tohoku, Kanto, and western regions were much smaller than among Jōmon populations and more recent populations. On the other hand, it has often been claimed that geographical clines are found from north to south because the Jōmon people were assumed to be influenced by both the southern and northern populations outside the Japanese archipelago. Fukase et al [36] claimed that such geographical clines were found in the length of limbs. Hanihara and Ishida [37] and Kondo [38] argued that geographical clines were also found in cranial biodistances. Ogata [40] and Yamaguchi [41,42] showed that the body size of the Incipient and Early Jōmon people was smaller than that of the other subperiods due to the dietary conditions.

Figure 1. Locations of landmarks. 3D data are from the cranium of the Miyano shell midden, owned by the Ofunato City Museum; locations were selected based on traditional Martin (1928) and Caple and Stephan (2016) locations. Numbers 9, 20, 21, 28, and 29 are not depicted.

Many archeological studies have also discussed population interactions via a widespread distribution and exchange of artifacts such as pottery and lithics over extensive geographic regions during this period [2,3,43-45]. It has often been pointed out that a certain type of pottery was widely distributed across regions. An example is the Ento-kaso type distributed in both the Hokuriku and the northern Tohoku regions of the early phase (see Figure 2 for regions in the Japanese archipelago). Even if the distribution of a type of pottery was regionally restricted, it may have been affected by a distant region. The Funamoto type of the Middle phase was distributed mainly in the Sanyo regions, and this type of pottery strongly influenced the pottery in the Kyushu region during that same phase. In the Late phase, when samples were obtained from the largest number of different regions in this study, it was found that a large quantity of the Horinouchi type I pottery originating from the Kanto region was excavated in the Kinki region; similarly, the Nakatsu type pottery from the Kinki region was transported to the Kanto region and influenced the origin of Shomyoji pottery [45,46]. Such diverse and widespread distribution of pottery suggests that the Jōmon people interacted widely, although it has often been argued that Jōmon society was regionally diverse and distinct, with differences in settlement styles, lifestyles, and types of archeological remains, such as clay figurines. This is why the Jōmon cultural complex has been referred as “Jōmon cultures” [1,47,48].

Figure 2. Regions and site locations investigated in this study. Top left panel: 1. Tohoku, 2. Kanto, 3. Hokuriku, 4. Tokai, 5. Kinki, 6. Sanyo, 7. Shikoku, 8. Kyushu, 9. Sanin. The site locations in the remaining images are labeled: 1. Odake, 2. Tamanoi, 3. Oguruwa, 4. Nakazuma, 5. Awazukotei, 6. Hayashinomine, 7. Azuhashiride, 8. Fukuda, 9. Tsukumo, 10. Ikawazu, 11. Yoshigo, 12. Kawaji, 13. Ichiki, 14. Kunugibaru, 15. Horiuchi, 16. Inariyama, 17. Harasaki, 18. Adaka, 19. Hatori, 20. Satogi, 21. Miyazono, 22. Ono, 23. Ohashi, 24. Ota, 25. Ropponmatsu, 26. Shijimizuka, 27. Sobata, 28. Todoroki, 29. Boji, 30. Yukura Cave, 31. Tochibara Iwakage, 32. Ebishima, 33. Miyano, 34. Wakaumi, 35. Daizennominami, 36. Shosenzuka, 37. Satohama, 38. Satoki, 39. Ishiyama, 40. Suzumimatsu, 41. Kou, 42. Ariyoshiminami, 43. Monzen, 44. Aoshima, 45. Kawakuradi Hibiki, 46. Murohama, 47. Nakasawahama, 48. Shomyoji, 49. Kamikuroiwa Iwakage, 50. Nakatsugawado, 51. Hirajo, 52. Tagara, 53. Maehama, 54. Higashiyogai, 55. Nonomae, 56. Ohora, 57. Kusakari, 58. Sotomeyachi, 59. Akiyamamukaiyama, 60. Nakabyo, 61. Muroya, 62. Takada, 63. Kasori North, 64. Shimofunato, 65. Shimooda, 66. Sadootsuka, 67. Fujisaki, 68. Kaigarazuka, 69. Sannomiya, 70. Soyaaranami, 71. Nakatsu, 72. Fujizuka, 73. Kumanobayashi, 74. Takanekido, 75. Kainohana, 76. Kaitori, 77. Hobi, 78. Kumaana, 79. Isodori-Ebimori, 80. Iyasakadaira, 81. Tomari Cave, 82. Morinomiya, 83. Motokariya, 84. Shinmei, 85. Myoonji, 86. Mizuko, 87. Okkoshi, 88. Enshoji, 89. Hazawa, 90. Imokawa, 91. Kosaku, 92. Nishi, 93. Shimekake, 94. Getsumeisawa. The map is based on the color elevation map published by the Geospatial Information Authority of Japan, with information on the sea area obtained from the Hydrographic and Oceanographic Department, Japan Coast Guard, and it was modified by author HN using QGIS (3.20.3).



The geometric morphometrics of 3D data, the approach used in this research, which explores shape variations of targeted objects typically using the coordination of landmarks, has gained traction in various fields, extending to objects such as lithics [49-51]. Although the morphological variations of the Jōmon people have been explored in previous studies using the traditional biodistance method, this study has the following novelties. The 3D data of Jōmon crania have rarely been

examined using geometric morphometrics, with some exceptions [52-56], and these exceptions focused on smaller samples from restricted regions. Less biased larger samples are important for estimating macroscopic population interactions. Furthermore, when analyzing the resulting data, geometric morphometrics is able to track morphological variation among configurations of each measuring point at once, whereas the traditional biodistance method compares each measured distance independently. These differences could provide some new insights.

The Aim of This Research

This study aims to examine the hypothesis that the Jōmon populations interacted widely and continuously in an anthropological way (ie, through geometric-morphological analysis of the cranial features of the Jōmon people) using a much larger 3D cranial sample collected from broader regions than previous studies. If the populations interacted widely and continuously, there should be fewer morphological differences among different regions and phases (ie, fewer statistically significant differences between each region and phase are found). Moreover, when the Jōmon populations are compared with populations from a different period, the hypothesis would expect that interregion and interphase variations should be lower than in the populations from a different period.

Methods

Landmark Selection for Geometric Morphometric Examinations

We chose 31 representative and/or less complicated landmarks for this geometric morphometric study, which were placed on 3D models using the *geomorph* package in R (version 4.3.2; R Foundation for Statistical Computing; Figure 1). Our research focused on the human cranium, enabling us to use the landmark method, which was used in similar studies [54-58]. Landmark selection was based on those described by Martin [32] and Caple and Stephan [33] (see Figure 1 for the Bookstein type of landmark [33] selected in this study).

3D Data Collection

We collected relatively well-preserved and less skewed 3D cranial data from the Jōmon period in collaboration with local archeological centers, museums, and universities. We used a range of scanning technologies, specifically Creaform HandySCAN BLACK, HandySCAN BLACK Elite, and Einscan HD, in addition to Structure from Motion and Multi-View Stereo techniques based on 2D photographs [59,60]. Previous studies have confirmed the consistency of the models generated by the aforementioned methods for human crania [60,61]. Our dataset comprised 363 Jōmon crania (including 146 females, 215 males, and 2 individuals with unknown sex), with specific information available in Table 1 and Multimedia Appendix 1. These 3D data were collected from various regions spanning the island of Honshu within the Japanese archipelago, encompassing regions from Tohoku to Kyushu (Figure 2). Although buried crania from the prehistoric period tend to be distorted due to soil conditions, we chose to measure crania that are regarded as less skewed and better preserved based on their appearance and relevant excavation information.

Table . Data for the crania examined in this study.

Phases	Initial			Early			Middle			Late			Final			Unknown			Yayoi			Total		
Area	F	M	U	F	M	U	F	M	U	F	M	U	F	M	U	F	M	U	F	M	U	F	M	U
Tohoku	0	0	0	1	1	0	9	14	0	6	14	0	4	4	0	6	5	0	0	0	0	26	38	0
Kanto	0	1	0	3	1	0	10	20	0	14	35	0	1	0	0	0	0	0	0	0	0	28	57	0
Hokkaido	1	1	0	2	6	0	0	2	0	0	1	0	0	0	0	0	0	0	0	0	0	3	10	0
Tokai	3	3	0	0	1	0	0	0	0	4	11	0	42	48	1	0	0	0	0	0	0	49	63	1
Kinki	2	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	4	2	0
Sanin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	13	0	5	13	0
Sanyo	0	1	0	0	1	0	7	10	0	4	3	0	15	16	0	0	1	0	0	0	0	26	32	0
Shikoku	2	2	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	3	3	1
Kyushu	0	0	0	0	1	0	1	1	0	2	6	0	0	0	0	4	2	0	4	18	0	11	28	0
Total	8	8	1	6	11	0	27	48	0	32	71	0	62	68	1	11	9	0	0	0	0	155	246	2

[a]F: female.

[b]M: male.

[c]U: sex unknown.

To provide a basis for comparison, we included cranial data from the Kuma-Nishioda site of the Middle Yayoi period (350 cal BC to AD 30) in the Kyushu region and the Aoyakamijichi site of the Late Yayoi period (AD 30 to 250) in the Sanin region, comprising 22 and 18 crania, respectively. The choice of the Kuma-Nishioda site was motivated by the common assumption that Yayoi people, especially in the northern Kyushu region, might exhibit lower genetic and morphological diversity due to a genetic bottleneck caused by gene flow from migrating Korean populations [55,56,62,63]. The Aoyakamijichi site was selected because it is the only one with a relatively large number of 3D samples outside of the Kyushu region [64]. Since we could not gather good 3D data from the Early Yayoi period, we did not include this period.

Data Processing

The file size of the original 3D data was so large that it took a long time to read the data and locate the landmarks in R; the files ranged from 100 to 600 MB depending on the measurement methods. To address this issue, we reduced the size to 2.5% - 15% of the original using MeshLab [65]. Previous studies have confirmed that this reduction process does not result in a model that is significantly different from the original [59,66,67].

Some prehistoric crania were incomplete, so we attempted to reconstruct lost areas in 3D models. This reconstruction involved mirroring remaining portions along the median line, passing through the nasion, prosthion, and bregma [32,55,56,68,69]. Since this mirroring could include some subjective biases, mirroring was limited to cases with smaller distortions and clearer original positions of remaining parts. In cases where mirroring was not possible, particularly when corresponding parts were entirely lost, we applied the estimate missing function, using the thin-plate spline method within the *geomorph* package (version 4.0.4) of R (version 4.2.1; R Foundation for Statistical Computing) and R studio (2022.07.0 + 548) [70-72].

Sex and age estimation of skeletal remains depended mainly on published excavation reports, where anthropologists estimated the sex and age. If any relevant information was not given in the reports, we made educated guesses by investigating pelves and crania (especially postcranial features and sizes of the zygomatic arch and mastoid process) for sex [73], while the pubic symphyses [74] and cranial sutures were used to estimate age [75,76].

Statistical Analysis

After conducting generalized Procrustes analysis to rotate and adjust the landmark configurations for all Jōmon and Yayoi crania, we performed principal component analysis (PCA) to investigate the spatiotemporal variations of these crania. We did not perform PCA for each phase or region because no region had samples from all phases, and no phase had samples from all regions. Additionally, we also performed the Steel-Dwass test, which is a nonparametric multiple comparison test with an expansion of the Mann-Whitney *U* test, to evaluate the principal component (PC) scores of Jōmon crania based on each phase and region. All statistical analyses were performed using R and R Studio.

We also conducted the PCA and the same statistical tests on all the Jōmon and Yayoi populations to clarify how varied the Jōmon populations were when compared to populations from a different period. Next, we selected cranial data from Jōmon sites with relatively larger sample sizes to compare with the Kuma-Nishioda site. They were compared via PCA by each site. The latter analysis included the Ebishima (from the Tohoku region of the Middle phase), Nakazuma (from the Kanto region of the Late phase), Yoshigo (from the Tokai region of the Final phase), and Tsukumo sites (from the Sanyo region of the Final phase), and their locations are indicated in Figure 2 and Multimedia Appendix 1. Such comparisons of the Jōmon populations from different phases and regions with the Yayoi population could reveal the degree of variation of individual Jōmon populations.

Ethical Considerations

Ethical approval was not required for this study according to the local legislation and institutional requirements because under the Law for the Protection of Cultural Properties in Japan, human skeletal remains are regarded as cultural properties [77]. Curators of all samples are described in Multimedia Appendix 1.

Results

Spatiotemporal Variations Among the Jōmon Populations

The PCA results indicated cumulative contribution rates exceeding 75% up to the 20th PC (Table 2). To streamline the discussion, this study primarily focused on PCs with magnitudes larger than 5% (PC1-PC5). PC1 was positively correlated with narrow facial width, prognathism (protrusion of the jaws), and high and short occipital areas, while PC2 was positively linked to temporal length and negatively related to facial length. PC3 primarily captured temporal length, and PC4 reflected facial height. PC5, primarily representing cranial asymmetry, was not included in the analysis as it was influenced by ground pressure during burial (Figure 3). We also showed deformation patterns of a cranium from the Fujizuka shell midden (ID 341; see Multimedia Appendix 1) according to the morphological change each PC captured (Figure 4).

Table . Contribution rates and cumulative proportion in principal component analysis.

	Contribution rate, %	Cumulative proportion, %
PC1[a]	11.1	11.1
PC2	9.0	20.1
PC3	6.4	26.5
PC4	6.1	32.6
PC5	5.4	38.0
PC6	4.9	42.9
PC7	4.1	47.0
PC8	3.6	50.6
PC9	3.0	53.6
PC10	3.0	56.6
PC11	2.7	59.3
PC12	2.5	61.8
PC13	2.3	64.1
PC14	2.1	66.2
PC15	2.0	68.2
PC16	1.8	70.1
PC17	1.7	71.8
PC18	1.6	73.4
PC19	1.5	74.9
PC20	1.4	76.4

[a]PC: principal component.

Figure 3. Configuration changes captured by each PC. The numbers correspond to the ones provided in Figure 1. PC: principal component.

Figure 4. A deformed human skeletal remain (excavated from the Fujizuka shell midden) according to the morphological change of each PC. PC: principal component.

The PCA results, graphically presented in Figures 5 and 6, suggested that the differences between phases are relatively small, in line with the statistical tests on PCs that indicated statistical differences only in the Early and Final (Z=3.118, P=.02), Middle and Final (Z=4.233, P<.001), Late and Final (Z=4.040, P=.001) in PC2, and Late and Final phase (Z=2.946, P=.038) in PC4 (see Multimedia Appendix 2). Notably, the scatter plots in Figure 5 and the box plots in Figure 6 showed more prominent regional differences, consistent with the statistical tests on PCs showing significant distinctions between various regions. These differences were particularly notable between the Tohoku and Tokai regions (Z=6.375, P<.001), the Tohoku and Sanyo regions (Z=5.852 P<.001), the Kanto and Tokai regions (Z=4.880, P<.001), the Kanto and Sanyo regions (Z=4.180, P<.001), the Hokuriku and Tokai regions (Z=3.773, P=.004), and the Hokuriku and Sanyo regions (Z=3.829, P=.003) in PC2. Additionally, there were significant differences in PC4 between the Kanto and Shikoku regions (Z=3.302, P=.02) and the Shikoku and Kyushu regions (Z=3.088, P=.04). It should be noted that there were no significant differences observed in PC1 and PC3, and the regional differences remained relatively limited. Furthermore, there were few sexual differences (see Multimedia Appendix 3), with some exceptions, specifically PC3 and PC4 in the early phase of the Tokai region and PC4 in the middle phase of the Kanto region, which was supported by statistical tests between the sexes in each PC score (U=15,926, P=.81 in PC1, U=13,925, P=.07 in PC2, U=12,693, P=.002 in PC3, and U=13,695, P=.004 in PC4 comparisons).

Figure 5. Principal component analysis results for Jōmon crania. PC: principal component.

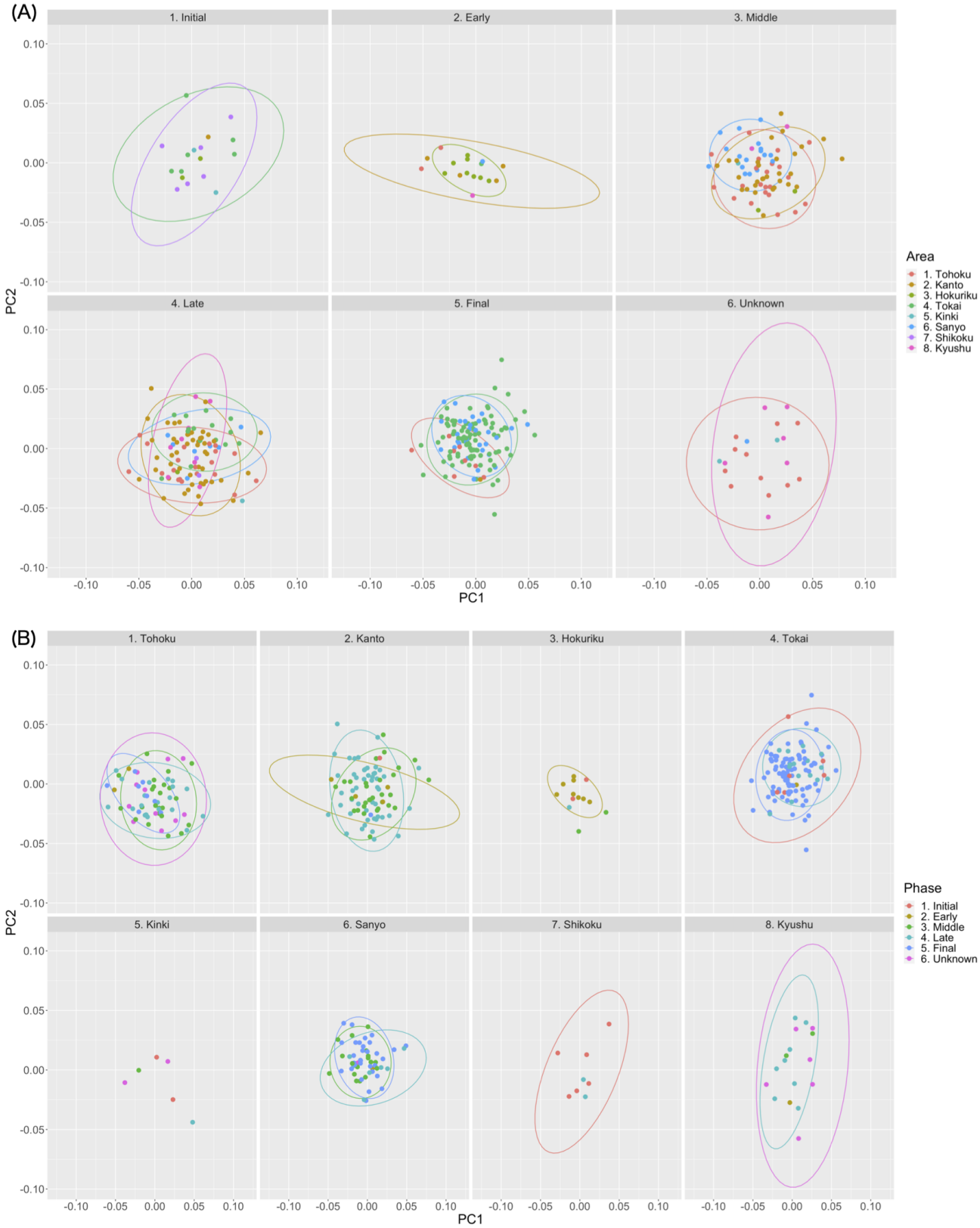


Figure 6. Box plots of PC1 and PC2 by phase and region. PC: principal component. *P<.01, **P<.05.

Comparisons Between the Jōmon Populations and the Yayoi Population

The two comparative results between the Jōmon and Yayoi populations were summarized in Figures 7 and 8 and Multimedia Appendices 2 and 4. In the second site-to-site comparison, to streamline the presentation, these figures showed only PC1 and PC2, as all sites displayed substantial overlap in PC3 and PC4 except the results between the Yoshigo and Kuma-Nishioda sites (see Multimedia Appendix 4).

Figure 7. The comparative results between the Jōmon and Yayoi populations. PC: principal component.

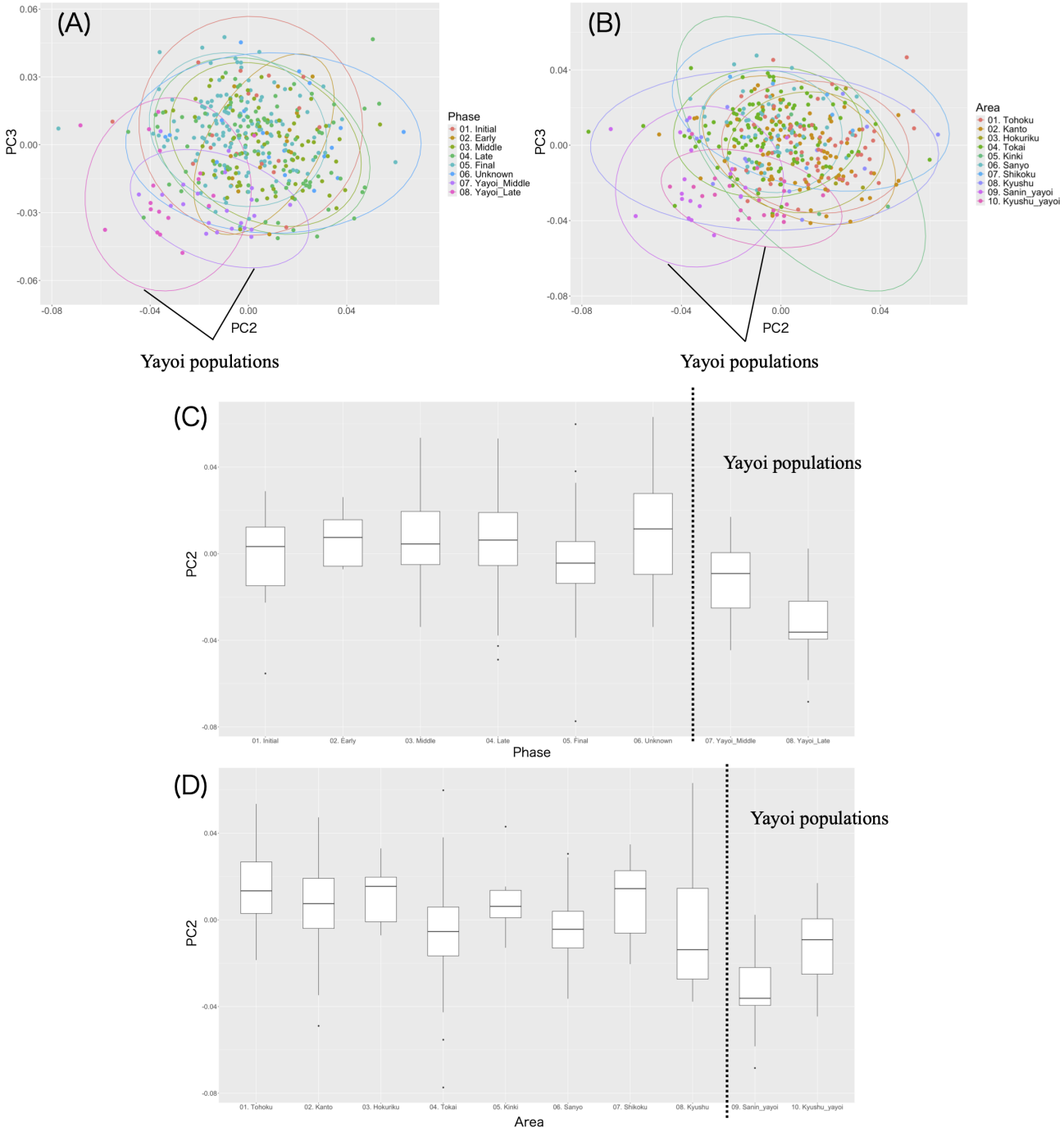
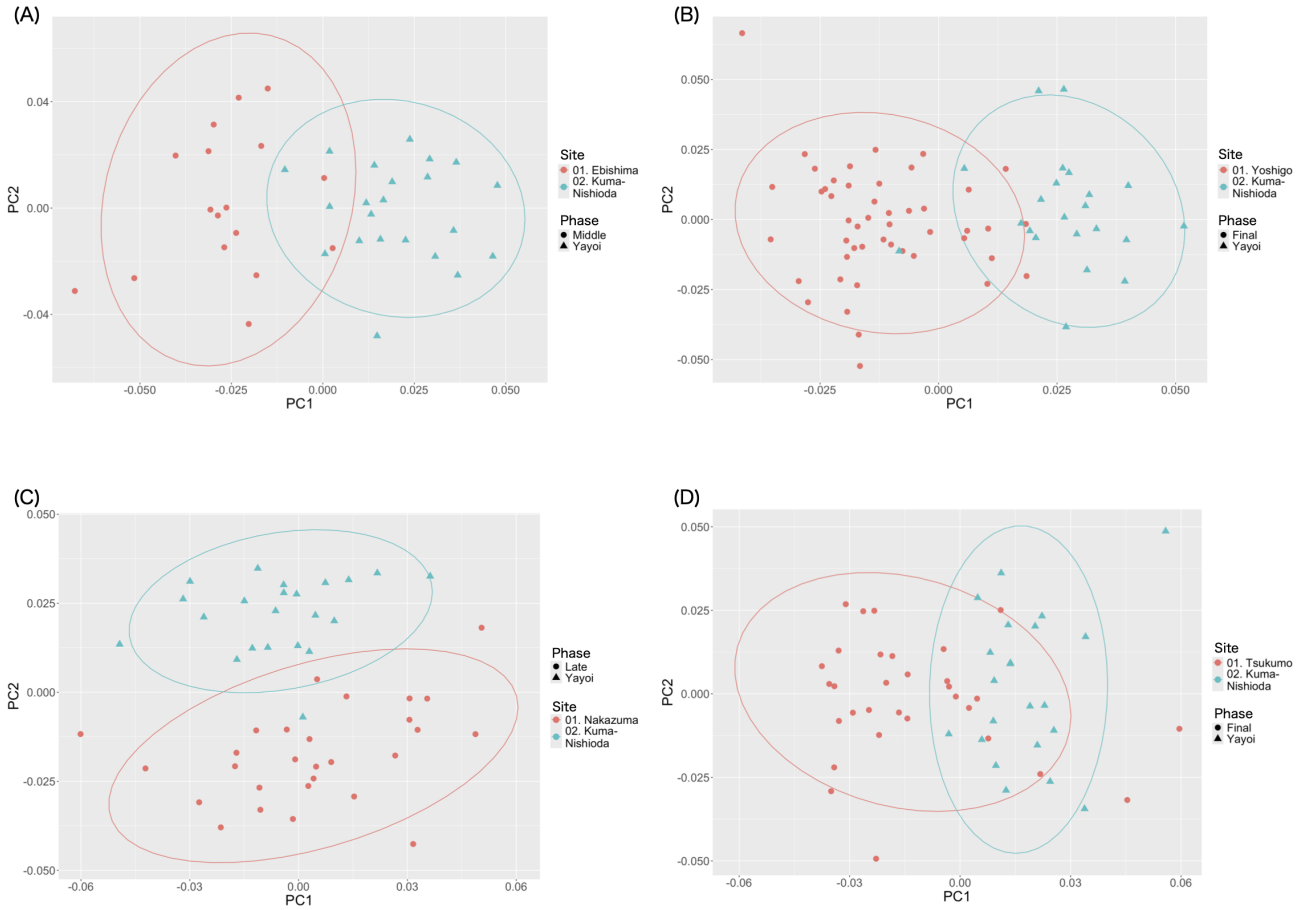


Figure 8. Comparative results between Jōmon sites (Ebishima, Nakazuma, Yoshigo, and Tsukumo) and Yayoi sites (Kuma-Nishioda). Refer to Multimedia Appendix 3 for results between each Jōmon site. PC: principal component.



The statistical results of the first comparison indicated that statistically significant results were found only in PC2 and PC3. PC2 focused on the interphase and interregion differences among all populations, and PC2 and PC3 captured the difference between the Jōmon and Yayoi periods (Multimedia Appendix 2). Figure 7 showed that the Jōmon populations substantially overlapped, while the Yayoi populations were more scattered spatiotemporally in PC2 and PC3, which was supported by the fact that the effect sizes of statistical tests between the Yayoi populations (Z/n=.113 in spatial and temporal comparisons in PC2) were higher than the ones between the Jōmon populations (Z/n<.055 in spatial comparisons and <.029 in temporal comparisons in PC2; Multimedia Appendix 2).

Figure 8 showed that the most pronounced differences of the second comparison were observed in PC1, with one notable exception seen when comparing the Ebishima and Kuma-Nishioda sites, where the differences were more conspicuous in PC2. Overall, the Jōmon individual populations were more varied than the Yayoi population. Figure 9 shows a visual representation of the configurational changes in landmarks for each comparison. The figure was constructed using the plotRefToTarget function of the *geomorph* package in R. Straight bars indicate the degree of variation in each landmark. These overall differences are primarily related to facial height and tooth position, specifically highlighting that individuals from the Kuma-Nishioda site tend to exhibit taller facial features and a more anterior tooth placement.

Figure 9. Landmark configuration changes in comparisons between some Jōmon sites and the Kuma-Nishioda site. Comparisons with the Kuma-Nishioda site for (A) Ebishima (Middle Jōmon), (B) Nakazuma (Late Jōmon), (C) Tsukumo (Final Jōmon), and (D) Yoshigo (Final Jōmon).

Discussion

Principal Findings and Comparison With Previous Research

The results of the PCA presented herein were not straightforward. PC2 showed statistically significant spatiotemporal differences between some regions and phases, which was consistent with previous biometric studies claiming interphase variation [40-42] and geographical clines from north to south [36-38,41], although it is difficult to conclude that their differences are pronounced. This is because PC1 did not show any statistical differences, and a similar study of the Kofun period, examining a larger set of 3D data of human crania using geometric morphometrics, exhibited a geological cline in PC1 [56]. Moreover, as mentioned in the Introduction, given the archeological evidence for the spatiotemporal distinctiveness of the Jōmon material cultures, it would be expected to find statistically significant differences in PC1 as well. Thus, it is possible that the spatiotemporal differences throughout the Jōmon period were more nuanced or relatively small.

Comparisons between the Jōmon and Yayoi populations are consistent with the above interpretations. It is suggested that morphological variations among Jōmon populations are relatively less varied spatiotemporally than in the Yayoi populations, and individual populations are more diverse than the Yayoi population from the Kuma-Nishioda site. It has been widely proposed that Yayoi populations, particularly in the Kyushu region, are descended from migratory groups originating from the Korean peninsula [55,62,63]. Suppose that a genetic bottleneck resulting from this migration contributed to the reduced variation observed in the Yayoi people of northern Kyushu. It suggests that the morphological and genetic diversity among the Jōmon populations were not relatively limited.

Examining population interactions in the Jōmon period could provide important insights into their resilience to environmental fluctuations at the time. As stated in the Introduction, the Jōmon period experienced more severe climate changes than subsequent periods such as the Yayoi and Kofun periods, which sometimes showed a radical societal change. The reason why less evidence of societal changes—such as the occurrence of warfare and emergence of social hierarchies—was found in the Jōmon period may be because the Jōmon populations interacted widely and continuously and frequently exchanged knowledge, skills, and resources against environmental disruptions.

Limitations and Future Directions

First, even if our results suggest that Jōmon people moved or interacted widely, it is still possible that such movements or interactions were not *continuous* but rather *discontinuous* with some drastic environmental changes such as the Kikai-Akahoya eruption and the 4.2 ka event [78-80]. The frequency and degree of interactions naturally depended on the region and phase, as suggested by research on pottery distributions [45]. It should be noted, however, that certain regional interactions must be maintained to sustain homogeneity across different regions. How frequent and continuous interactions could maintain the morphological variations revealed in this study should be explored in future work.

Second, our discussions have focused on PCs with a contribution rate of higher than 5 percentage points, which covers less than 40% of total cumulative proportions. Although the results of PCA on 3D data typically tend to be dispersed across more PCs [55,56,81], we should be careful not to overestimate the present results. The question of how frequently the Jōmon people interacted should be explored from additional aspects, including quantitative investigations of relevant archeological remains and mathematical simulations or modeling of the Jōmon population.

Third, some results from genetic or isotopic research on Jōmon skeletal remains, suggesting that Jōmon people interacted with populations from different regions, are consistent with our interpretation [82,83]. Cooke et al [84] argued that Jōmon people on the mainland of the Japanese archipelago were isolated from other islands, which might have contributed to the morphological homogeneity of Jōmon people. Nevertheless, to strengthen or verify this hypothesis, further genetic data and morphological data from other relevant regions overseas should be collected and combined [53,85,86]. For instance, our sample size is much larger than that in a study by Buck et al [53], which claimed that dietary factors influenced the shape of the Jōmon neurocranium, while our results should also be combined with isotopic dietary data in future work.

Fourth, while the dataset comprising larger sample sizes is acknowledged, potential sampling bias may exist, particularly due to restrictions on obtaining samples, notably in the Kyushu region. However, previous biometric studies have suggested that individuals from the northern Kyushu region do not exhibit significant differences compared to other regions [42]. This contributes to the overall reliability of the findings presented in this study. Our study exclusively focuses on cranial 3D data, although previous investigations have examined other parts of skeletal remains, such as limbs [36,39], warranting a comprehensive examination of their 3D data.

Finally, the selection of landmarks in this study is a critical aspect that warrants consideration. Although the chosen landmarks were selected for their clarity and suitability, the effectiveness of the landmarks in capturing morphologically significant changes may vary depending on various factors such as geographic regions, time periods, age groups, and other contextual factors.

Acknowledgments

We thank for the following cultural property or archeological centers, institutions, museums, and universities (see Multimedia Appendix 1 for detailed information): Iwate Prefectural Museum, Osaki City Board of Education, Ofunato City Museum, Kamikuroiwa Archaeological Museum, Shiura Historical Museum, Tohoku History Museum, Tome City History Museum,

Niigata University of Health and Welfare, Yokohama City Archaeological Center, Hamamatsu City Museum, Matsudo City Board of Education, Anjo City Museum of History, Okayama University of Science, Kasaoka City Board of Education, Kyoto University, Takayama Village History Museum, National Museum of Nature and Science, Sakaki Town Archaeological Center, Sakiyama shell midden Museum, Shiga Prefectural Archaeological Center, Kagoshima Prefecture Archaeological Center, Toride City Archaeological Center, Shirokawa History Museum, Aomori Prefectural Museum, Chiba Prefectural Board of Education, Osaka Metropolitan University, Tahara Municipal Museum, the Tohoku University Museum, Minamichita Town Board of Education, Toyama Prefecture Archaeological Center, Hirajo Public Hall, Nagoya City Archaeological Center, Nagoya City Museum, Kariya City History Museum, Kasukabe City History Museum, Mizuko shell midden Museum, Saitama City Urawa Museum, Kaizu History Museum, Tobinodai Historic Site Park Museum, Iwata City Board of Education, and Kazuhiko Tanaka (Nagano Nishi High School). We also appreciate the following researchers for their highly valuable suggestions, comments, information, and cooperation: Kazuhiro Sakaue (National Museum of Science), Atsushi Fujisawa (The Tohoku University Museum), Takafumi Nara (Niigata University of Medicine and Welfare), Mikiko Abe, Masatake Kai (Osaka Metropolitan University), Naoto Tomioka (Okayama University of Science), Isao Yumura (Aoya Kamijichi Historical Park), and Ai Takeuchi (Nanzan University).

Funding

The present research was supported by JSPS (No. 24H02201 and 20K00019) and Nanzan University Pache Research Subsidy II-B for the 2025 academic year.

Authors' Contributions

All authors designed the research. HN, TN, MY, and KT gathered the 3D data. HN analyzed the data and wrote the original draft. All authors edited the paper and approved the final manuscript.

Conflicts of Interest

None declared.

Multimedia Appendix 1
Metadata of the samples.
[XLSX File, 293 KB - xbio_v3i1e72432_app1.xlsx]

Multimedia Appendix 2
Summary of the statistical tests.
[XLSX File, 38 KB - xbio_v3i1e72432_app2.xlsx]

Multimedia Appendix 3
Plots of sexual differences.
[PDF File, 7731 KB - xbio_v3i1e72432_app3.pdf]

Multimedia Appendix 4
Comparisons of the Jōmon and Yayoi populations.
[PDF File, 2661 KB - xbio_v3i1e72432_app4.pdf]

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Abbreviations

PC: principal component
PCA: principal component analysis

Edited by A Schwartz; submitted 10.02.25; peer-reviewed by D Crampton, O Kondo; revised version received 08.08.25; accepted 24.09.25; published 12.11.25.

Please cite as:
Nakao H, Kaneda A, Tamura K, Noshita K, Yoshida M, Nakagawa T
Population Interaction in the Jōmon Society via 3D Data of Human Crania: Geometric Morphometric Study
JMIRx Bio 2025;3:e72432
URL: https://bio.jmirx.org/2025/1/e72432
doi:10.2196/72432

Peer Review of “Novel Fatigue Profiling Approach Highlights Temporal Dynamics of Human Sperm Motility (Preprint)”

Daniela Saderi[1]; Rosario Rogel-Salazar[2]; Chidi Asuzu[3], MBBS, MMCi; Toba Isaac Olatoye[4]; Mitchell Collier[5]

[1]PREreview, -, Portland, OR, United States

[2]Universidad Autónoma del Estado de México, Toluca, Mexico

[3]Duke University Medical Center, Durham, NC, United States

[4]Kwara State Teaching Service Commission, Ilorin, Nigeria

[5]Temple University, Philadelphia, PA, United States

Related Article:

https://www.biorxiv.org/content/10.1101/2025.04.27.650828v1

(JMIRx Bio 2025;3:e80919) doi:10.2196/80919

KEYWORDS

reproduction; male fertility; sperm motility; motion tracking; computer-assisted analysis; fatigue

This is the peer-review report for the preprint “Novel Fatigue Profiling Approach Highlights Temporal Dynamics of Human Sperm Motility.”

This review is the result of a virtual collaborative live review discussion organized and hosted by PREreview and JMIR Publications on June 26, 2025. The discussion was joined by 14 people: 2 facilitators from the PREreview team, 1 member of the JMIR Publications team, 1 author, and 10 live review participants. The authors of this review have dedicated additional asynchronous time over the course of 2 weeks to help compose this final report using the notes from the live review. We thank all participants who contributed to the discussion and made it possible for us to provide feedback on this preprint.

Summary

This preprint [1] presents a well-structured study that introduces the concept of “sperm fatigue” as a novel framework for assessing motility deterioration in human sperm. The study aims to develop and validate a new profiling method to assess infratrajectory motility decline in human spermatozoa. Using a metric termed the “Fatigue Index” and data from computer-assisted sperm analysis systems, the authors demonstrate that infratrajectory motility decline is both measurable and biologically plausible, with potential links to mitochondrial dysfunction and oxidative stress. This approach aids in identifying sperm with potential subclinical impairments and informs the development of predictive biomarkers for sperm functionality and male fertility evaluation. The methodology is clearly described, and the inclusion of shared code and data exemplifies strong open science practices. Below, we summarize the main points discussed during the live review and offer suggestions for improving the manuscript. Minor enhancements to documentation and accessibility could further support its broader application across disciplines.

List of Major Concerns and Feedback

- Clarify dataset selection and segmentation process: The manuscript lacks sufficient detail regarding the selection and segmentation of video clips from the VISEM dataset. To improve transparency and reproducibility, please provide a clear explanation of how video segments were selected for analysis, including specific criteria such as duration, quality, sample characteristics, or relevance to the study’s aims. Additionally, include a brief description of the origin of the VISEM dataset, emphasizing that the clips used in this study were curated from full-length videos. Please clarify how the original dataset was constructed by its curators and how your study further selected, filtered, or modified these data. Finally, cite the original VISEM publication and relevant documentation to support clarity and reproducibility.
- Missing ethics statement: Although the study likely follows ethical standards, it would be best practice to include a short ethics statement. Since the VISEM dataset is based on human sperm samples, the authors could briefly mention the original ethical approval and cite the VISEM source publication to clarify this point.

List of Minor Concerns and Feedback

- Reproducibility details can be improved: While the authors share code and data, it is not clear which versions of libraries and frameworks were used and how they were applied in the analysis. Please provide a list of all software tools and libraries used, including their versions and sources. For example, “Data analysis was conducted using Python (version number) with the following open-source packages: scikit-learn (version number) for model building, PyMC (version number) for Bayesian inference, matplotlib (version number) for visualization, and pandas (version

number) for data manipulation." Doing this will help validation and reuse, especially in less-resourced settings.
- Figures need better resolution: Some figures (eg, 1A and 1B) appear small or in a low resolution and are difficult to read without zooming in. Increasing the image resolution and adjusting the layout for clarity would improve readability. Also, authors may consider removing box characters from figure legends to make their content more readable and clear.
- Clarify limitations and generalizability: The author mentions limitations, but it would be helpful to more clearly state what the study cannot determine. Also, specifying the populations from which the samples were obtained and discussing whether results might differ in other populations would improve transparency.
- Visual abstract for nonexpert audience: Given the potential interest from a broad audience, including nonexperts, reviewers suggested adding a nontechnical summary of the findings—potentially in the form of a visual abstract. It may also be helpful to reflect on which other disciplinary fields could find this topic relevant.

Concluding Remarks

Overall, live review participants found this to be a well-constructed study.

Acknowledgments

PREreview and JMIR Publications thank the authors of the preprint for posting their work openly for feedback. We also thank all participants of the live review for their time and for engaging in the lively discussion that generated this review.

Conflicts of Interest

None declared.

Reference

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Edited by A Schwartz; submitted 18.07.25; this is a non–peer-reviewed article;accepted 18.07.25; published 30.07.25.

Please cite as:
Saderi D, Rogel-Salazar R, Asuzu C, Olatoye TI, Collier M
Peer Review of "Novel Fatigue Profiling Approach Highlights Temporal Dynamics of Human Sperm Motility (Preprint)"
JMIRx Bio 2025;3:e80919
URL: https://bio.jmirx.org/2025/1/e80919
doi:10.2196/80919

Peer Review of “Machine Learning Ensemble Identifies Distinct Age-Related Response to Spaceflight in Mammary Tissue (Preprint)”

Sylvester Sakilay; Mitchell Collier; Arya Rahgozar[1], PhD; Toba Olatoye[2]; Simon Muhindi Savai; Myron Pulier; Randa Salah Gomaa Mahmoud; Clara Amaka Nkpoikanke Akpan; Sayan Mitra; Julie Moonga

[1]Ottawa Hospital Research Institute, Ottawa, ON, Canada

[2]Kwara State Teaching Service Commission, Ilorin, Nigeria

Related Article:

https://www.biorxiv.org/content/10.1101/2025.02.17.638732v1

(JMIRx Bio 2025;3:e75688) doi:10.2196/75688

KEYWORDS

machine learning; spaceflight; mammary tissue; gene expression; mice; breast cancer; cardiovascular health

This is the peer-review report for the preprint “Machine Learning Ensemble Identifies Distinct Age-Related Response to Spaceflight in Mammary Tissue.”

This review is the result of a virtual collaborative live review discussion organized and hosted by PREreview and JMIR Publications on March 21, 2025. The discussion was joined by 21 people: 3 facilitators from the PREreview team, 1 member of the JMIR Publications team, 1 author, and 16 live review participants, including 3 who agreed to be named: Matthew W Darlison, Luciana Gallo, and Meghal Gandhi. The authors of this review have dedicated additional asynchronous time over the course of 2 weeks to help compose this final report using the notes from the live review. We thank all participants who contributed to the discussion and made it possible for us to provide feedback on this preprint.

Summary

In this study [1], a small existing dataset of mammary gland cell gene transcription in female mice was subjected to various combinations of artificial intelligence binary classification techniques to distinguish young versus old female mice and those exposed or not exposed to a prolonged stay in space. The authors applied machine learning (ML) methods to analyze data on mammary gland gene expression in mice newly returned from a prolonged stay in Earth orbit as compared to controls remaining on the ground in order to identify which genes were affected by the spaceflight experience and how the age of a mouse influenced this response. The underlying theory is that a cell’s “strategy” for adapting to certain stressors may change as a mouse ages, a qualitative change rather than an overall quantitative deterioration of resiliency.

Discovery of key genes involved pinpointing which ones stood out as enabling classification of mice as either young or old and, separately, as having flown in space or not. Because of the small size of the dataset (which had been collected for other research), a conventional random forest approach lacked sufficient power to identify critical genes. Instead, the authors describe trying various ensembles of ML tools until eventually selecting several candidate genes. By associating those genes with metabolic pathways, they then suggest a plausible description of cells of younger mice activating cell structure/cell adhesion–related mechanisms, while older mice activate pathways involved in cortisol synthesis and cardiac muscle contraction.

The application of innovative, computerized techniques (eg, ML and algorithms to better understand gene expression) offers fresh insight into spaceflight in animal models. More specifically, the research sheds new light on molecular pathways implicated in spaceflight-related health risks. This is particularly important in understanding the pathogenesis of a large number of diseases such as cancer that is often characterized by the development of abnormal tissues. However, the study has a few shortfalls as outlined above. Perhaps, a section of the paper should be devoted to limitations of the research. A brief ethical explanation could provide more clarity with the approach of the research. It should be made clear early that the experiment/analysis was done “in silico.” Additionally, the experimentation on mice may overlook biological properties in humans; therefore, arguments should only be extended and scoped on mice.

List of Major Concerns

- The title should be more specific with respect to the source of mammary tissue: identify “mouse mammary gland tissue” in the title or, perhaps, simply “murine mammary tissue.”
- While the methodology is interesting and the findings certainly warrant further study, this should be clearly identified as formative research. There was no preregistration of hypotheses and methods, and the findings (list of key genes and of pathways differing according to age) are just suggestive and not at all robust or convincing.

Accordingly, some detail about the experiences of the mice and physiological values are beside the point, so we suggest it is moved to a "Supplements" section along with more specifics about ML parameters, etc, that could help researchers attempting similar approaches.
- With respect to the OSD-511 dataset, the details of Rodent Research Reference Mission 1 need revision, as it was mentioned that there are 40 female BALB/cAnNTac mice, while the total number of animals used was 43: 21 younger mice and 22 older mice. Moreover, the 8 younger mice that were kept in standard cages were exposed to different conditions from the 7 older mice that were housed in flight hardware.
- In addition, it was mentioned that each group of space-flown mice had corresponding control groups (ground control), but it is not clear which basal controls (10 mice euthanized 1 day post launch) are used to compare which group. This is important to explain the single group called "non-flight" that is mentioned later in the paragraph, and indicate if these latter details from the original experiment are not available to the authors.
- In the Discussion section, or as a separate Limitations section, consider explicitly pointing out that data of experimental mice that were collected just once after 40 days in space and 2 days post return recovery provides only cross-sectional data and does not capture changes in the mice that could be evident while in space or longer after return from space. Also, the description for Figure 1 mentions Figure 1E and F, which are not available in the figure.
- The small sample size should be acknowledged, which means the outcome models may not be able to generalize well on unseen data in downstream tasks.

List of Minor Concerns

- The title could be enhanced to make it clear that this was an experiment based on a model organism (mouse) and not human.
- The reviewers acknowledge the availability of details that enable the reproducibility of the study, such as publicly accessible data sources and detailed description of data handling and analysis procedures. However, the reviewers wondered whether the source code used could be availed for enhancing the reproducibility.
- The total number of mice stated that were used in the study does not correspond with the total number used, based on the breakdown of individual group numbers. Authors need to cross-check the numbers to ensure that they tally with the numbers used.
- Clarify the composition of the control cohort, refer to those mice in a consistent way, and discuss differences that were found to exist between the subsets of controls.
- On page 4, under the Data Transformation section, it is stated that "four filtering methods were performed," but Figure 2B only represents three filters. Kindly clarify if the fourth filtering method was used but not included in the figure or whether there was a mistake in either the figure or the text for the sake of consistency.
- On page 6, the last paragraph, a linear regression model was used to predict the weight of mice at euthanasia, but the significance of this prediction was not discussed. The significance should be discussed for a better understanding of its applicability. Add a brief discussion of the significance of the model, which may include a statistical test validation such as *P* values and/or CIs.
- On page 15, under the Conclusion section, it is also mentioned that "The dysregulation of ECM remodeling, cytoskeletal function, and stress response pathways was observed in radiation-exposed mice," but radiation exposure was not the intervention applied. Revise this statement to accurately reflect the intervention applied in this study (spaceflight) and ensure the conclusion is per the experimental conditions.
- In the Discussion section, some results are repeated instead of being analyzed in depth. Focus more on interpreting the results, compare them with similar studies, and discuss their significance.
- Only accuracy is reported for model performance metrics. Add other metrics, including area under the receiver operating characteristic curve, sensitivity, specificity, and F_1-score, to enhance the assessment of the model's predictive ability.
- Under the algorithms discussion, remove possessive apostrophe from the "1950's."
- It may help to add a statement to make it explicit whether ethics approval was necessary for the study. In addition, it would add value in discussing ethical implications of collecting the dataset used in the manuscript with reference to any discussion in previous publications or from the authors who collected the original data.

Concerns With Figures and Tables

- Most figures have poor resolution, which makes them difficult to understand or interpret. It would be helpful to regenerate the figures with better resolution.
- It would be helpful to add details to the captions to include what's represented in each panel and any elements of statistics.
- Creating a table to present the various groups and their characteristics, including ground control, would help improve readability.
- Figure 1 lacks an adequate explanation of each panel, which will clarify what they represent.
- Table 1 is not clear, making it difficult to read. The top and left parts of Figure 7 are cropped, and its possible important information is omitted.
- The legend refers to plots by layout (left/right), duplicating the role of (a)-(d) labels. Also, plot titles are not the most prominent text and are not referenced in the text.
- In Figure 4, the term "accuracy" is used without clarification.
- Abbreviations used in Figures 2 and 3 are not explained.
- The Figure 3 legend does not clearly describe the difference between the left and right diagrams.

- The manuscript refers to Table 1 subsections “e” and “f,” which are not present. Some figures are also unclear and not explanatory enough.
- Figure 5: Fonts are too small to read, and part of the legend is cropped.
- In Figure 1, the caption states that the left plots represent ground mice and the right plots represent space mice, which is not reflected in the figure.
- On page 4, the principal components analysis statement interpreting Figure 1A and D is misleading. The statement suggests that both Figure 1A and D show the principal components analysis for spaceflight, whereas Figure 1A only represents ground mice.
- The text for Figure 1 describes Figure 1E and F, but these panels are not present.

Additional Comments

- Consider revising the title and abstract to identify that the study was conducted with data collected in a model organism or murine model.
- The second page, second sentence of the first paragraph: “Female astronauts in particular have an increased risk of breast cancer due to exposure to galactic cosmic radiation (7).” Please revise the reference, as Kumar et al [2] did not investigate or conclude the mentioned data.
- On the second page, in the last sentence of the first paragraph, “Female astronauts...this increased vulnerability.” Please provide a reference for the mentioned data.
- The second page, second paragraph: “Machine learning (ML) has been leveraged but to a much lesser extent (15).” Please revise the reference Larrañaga et al [3], as ML’s role in bioinformatics has been widely expanded since 2006.
- Page 6, second paragraph: It was mentioned that “The support vector machine was created by Hava Siegelmann and Vladimir Vapnik,” and there is a reference to Cortes and Vapnik [4], while this work [5] was published in 2001.
- Page 11, pathway enrichment analysis: Please identify the abbreviation “KEGG” as “Kyoto Encyclopedia of Genes and Genomes.”
- Page 11, pathway enrichment analysis: Please identify the abbreviation “FDR” as “False Discovery Rate.”

Concluding Remarks

- In the Data Transformation section, groups were introduced for the first time in the manuscript “FLT vs GC and YNG vs OLD”; these categories are defined later, but it would be good to spell out the names the first time they are mentioned. That’s true for any other acronym used.
- The article did not introduce a Limitation section. It is helpful to the reader to emphasize the limitations of the methods.

Acknowledgments

PREreview and JMIR Publications thank the authors of the preprint for posting their work openly for feedback. We also thank all participants of the live review for their time and for engaging in the lively discussion that generated this review.

Conflicts of Interest

None declared.

References

1. Casaletto JA, Zhao T, Yeung J, et al. Machine learning ensemble identifies distinct age-related response to spaceflight in mammary tissue. Bioinformatics. Preprint posted online on Feb 23, 2025. [doi: 10.1101/2025.02.17.638732]
2. Kumar K, Angdisen J, Ma J, Datta K, Fornace AJ, Suman S. Simulated galactic cosmic radiation exposure-induced mammary tumorigenesis in ApcMin/+ mice coincides with activation of ERα-ERRα-SPP1 signaling axis. Cancers (Basel) 2024 Nov 26;16(23):3954. [doi: 10.3390/cancers16233954] [Medline: 39682141]
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Abbreviations

ML: machine learning

Edited by A Schwartz; submitted 08.04.25; this is a non–peer-reviewed article;accepted 08.04.25; published 23.04.25.

Please cite as:
Sakilay S, Collier M, Rahgozar A, Olatoye T, Savai SM, Pulier M, Mahmoud RSG, Akpan CAN, Mitra S, Moonga J
Peer Review of "Machine Learning Ensemble Identifies Distinct Age-Related Response to Spaceflight in Mammary Tissue (Preprint)"
JMIRx Bio 2025;3:e75688
URL: https://bio.jmirx.org/2025/1/e75688
doi:10.2196/75688

Peer-Review Report

Peer Review of “Discovery of Novel Inhibitors of HMG-CoA Reductase Using Bioactive Compounds Isolated From Cochlospermum Species Through Computational Methods (Preprint)”

Shailee Rasania; Sylvester Sakilay; Sayan Mitra; Randa Salah Gomaa Mahmoud[1]; Julie Moonga[2]

[1]Faculty of Human Medicine, Zagazig University, Zagazig, Egypt

[2]King’s College London, London, United Kingdom

Related Article:

Companion article: https://www.biorxiv.org/content/10.1101/2025.01.19.633828v1

(JMIRx Bio 2025;3:e74084) doi:10.2196/74084

KEYWORDS

statins; phytochemicals; Cochlospermum; hypercholesterolemia; molecular docking

This is a peer-review report submitted for the preprint “Discovery of Novel Inhibitors of HMG-CoA Reductase Using Bioactive Compounds Isolated From Cochlospermum Species Through Computational Methods.”

This review is the result of a virtual, collaborative live review discussion organized and hosted by PREreview and JMIR Publications on February 21, 2025. The discussion was joined by 13 people: 3 facilitators from the PREreview Team, 1 member of the JMIR Publications team, 1 author, and 8 live review participants. The authors of this review have dedicated additional asynchronous time over the course of 2 weeks to help compose this final report using the notes from the live review. We thank all participants who contributed to the discussion and made it possible for us to provide feedback on this preprint.

Summary

Cholesterol is an essential component of cellular membranes and a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D. However, elevated low-density lipoprotein cholesterol is a major contributor to atherosclerosis and cardiovascular diseases, which are leading causes of morbidity and mortality worldwide. Inhibiting HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase (HMGR) is a key therapeutic strategy for managing hypercholesterolemia, with statins serving as the most widely used competitive inhibitors; however, their prolonged use is associated with adverse effects. This study aims to identify novel, natural inhibitors of HMGR as potential alternatives to statins.

This study [1] used a molecular docking method to investigate the inhibitory potential of 84 phytochemicals from *Cochlospermum planchonii* and *Cochlospermum tinctorium* against human HMGR. Molecular docking is a purely computational technique used to predict how small molecules bind to proteins. Specifically, the author used a semirigid docking approach, meaning that the structure of the receptor was not allowed to change while the phytochemicals and statins were given some degree of flexibility at the binding pocket. The phytochemicals were screened for their drug-likeness and absorption, distribution, metabolism, excretion, and toxicity properties based on Lipinski’s rule of five, and 32 were docked against the enzyme’s HMG-binding site alongside its native ligand and 6 statins as controls. Docking results identified 10 promising inhibitors of HMGR. These compounds, including 3-O-methylellagic acid, all displayed strong binding affinities and interactions that were either comparable to or exceeding those of the statins used as control ligands.

These findings highlight the therapeutic potential of natural compounds in treating hypercholesterolemia. However, as indicated in the manuscript, further in vitro and in vivo experiments will be needed to establish their efficacy and safe therapeutic use.

Concerns and Feedback

All reviewers found that the study was well written and comprehensive. There were no major concerns regarding the techniques or analyses. A few points were made during the discussion and are highlighted below:

- Reviewers appreciated the depth and thoroughness of the search through the literature of peer-review research. Some reviewers were surprised about the date (1991) of some studies related to the high-performance liquid chromatography–UV analysis of phytochemicals identified in the ethanolic and methanolic extract of *C tinctorium* and

wondered whether there may be more recent studies to also consider.
- To increase the reproducibility of the study, some reviewers wondered if it would be possible to make the data and code used to analyze the data openly available.
- The figures and tables are comprehensive and clearly presented, with well-written descriptions. If feasible, reviewers would suggest ways to visually highlight key compounds listed in tables using colors, bold text, or labels. Furthermore, incorporating chemical structures directly within the relevant tables or as supplementary figures would further enhance the understanding of their molecular characteristics and potential interactions.
- While the author acknowledges the need for in vitro and in vivo validation studies, explicitly addressing potential computational limitations—such as docking inaccuracies, semirigid approach versus more flexible ones, or the absence of dynamic modeling—would further strengthen the discussion.
- Some reviewers suggested adapting the part of the study that identified the compounds through literature review into a systematic review.

Concluding Remarks

We thank the author of the preprint for posting their work openly and for allowing the review of their work openly via live review. We also thank all participants of the live review call for their time and for engaging in the lively discussion that generated this review.

Acknowledgments

PREreview and JMIR Publications thank the authors of the preprint for posting their work openly for feedback. We also thank all participants of the live review call for their time and for engaging in the lively discussion that generated this review.

Conflicts of Interest

None declared.

Reference

1. Olatoye TI. Discovery of novel inhibitors of HMG-CoA reductase using bioactive compounds isolated from Cochlospermum species through computational methods. BioRxiv. Preprint posted online on January 22, 2025 2025. [doi: 10.1101/2025.01.19.633828]

Abbreviations

HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A
HMGR: HMG-CoA reductase

Edited by A Schwartz; submitted 17.03.25; this is a non–peer-reviewed article;accepted 17.03.25; published 24.03.25.

Please cite as:
Rasania S, Sakilay S, Mitra S, Mahmoud RSG, Moonga J
Peer Review of "Discovery of Novel Inhibitors of HMG-CoA Reductase Using Bioactive Compounds Isolated From Cochlospermum Species Through Computational Methods (Preprint)"
JMIRx Bio 2025;3:e74084
URL: https://bio.jmirx.org/2025/1/e74084
doi:10.2196/74084
PMID:

Peer Review of “Population Interaction in the J mon Society via 3D Data of Human Crania: Geometric Morphometric Study”

Osamu Kondo

Nippon Steel Res Inst Corp, Tokyo, Japan

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https://bio.jmirx.org/2025/1/e72432/

(JMIRx Bio 2025;3:e85567) doi:10.2196/85567

KEYWORDS

Jomon; Japan; crania; cranium; bones; anthropology; archaeology; morphology; morphometrics

This is the peer-review report for “Population Interaction in the Jōmon Society via 3D Data of Human Crania: Geometric Morphometric Study.”

Round 1 Review

General Comments

This paper [1] studied 3D data of Jōmon and Yayoi cranial series and analyzed them in terms of temporal and geographical variations. While conventional craniometric studies of Jōmon specimens have been much accumulated, almost no comprehensive ones have analyzed both temporal and regional variation at once. In this sense, the approach of this paper is promising. However, I have major reservations regarding the approaches used to achieve this target; the authors’ present approach is not able to clarify the smallness or wideness of the temporal and/or regional variation among Jōmon crania. Another major reservation is the data, as the 3D morphology of the Jōmon crania sustains unavoidable destruction and deformation during burial processes; thus, most of them were manually repaired. The data used in this study did not mention this bias properly.

Specific Comments

Major Comments

1. One major reservation is in the analytical design; the present one seems suitable for the study target, which aims to clarify the temporal (between Jōmon phases) and geographical (between regions in Japan) variations of the Jōmon crania. The authors concluded that there were pronounced morphological variations within individual populations versus smaller variations between different phases and geographical regions (from the Abstract). Unfortunately, this seems unclear to me. Probably a reason for this could be the lack of comparative targets. Figures 5 and 6 present the temporal and regional variations along principal component (PC) 1 and PC2 and indicate only regional variations along PC2 as being significant, but this seems too weak for readers to reconfirm the conclusion of smaller temporal and regional variations. In my idea, as the authors perform the geometric morphometric and principal component analysis procedures for all the Jōmon and Yayoi individuals, all the comparisons can include all the subdivisions of the Jōmon and Yayoi. Afterward, readers can understand the temporal variation among Jōmon crania and also compare the degree of variation compared to those of the Yayoi, and the same is the case for the regional variation.

2. Another aspect that needs to be considered is the power of expression in the PC scores. The present analysis almost confines the results to PC1 and PC2 scores, which explain only 20% of the total variation (Table 2). When we accept the authors’ idea of the advantage of 3D geometric morphometric analysis being inclusion of whole cranial morphology, selection and only a portion of the PCs may lead to disadvantages. I do not have any ideas for tackling this problem; the authors should care about this.

3. One other reservation is about the Jōmon cranial reconstruction. Most of the fragmentary and heavily reconstructed specimens suffered unavoidable skews and deformation. Because most researchers cannot repair the reconstruction, available options are few, but the authors should mention that they care about this bias. I suggest checking for the position in the plot (eg, outlier position) and the degree of preservation.

Minor Comments

Page 3, lines 3-5. Subdivision of the Jōmon period is usually capitalized (eg, Incipient or Initial Jōmon phases).

Page 7, line 1. “Facies symphysialis” can be rephrased as “pubic symphyses.”

Page 7, line 12. Cranial morphology should be considered as an outcome being affected by many factors; it should not be simplified as “a human trait.”

Page 7, lines 18-21. The purpose of the selection of several Jōmon site data for comparison with the Yayoi site is unclear—do you mean site-to-site comparison?

Page 8, line 15. “We also deform a cranium” is better reworded as “We also show deformation patterns of a cranium...”

Page 8, line 19. “Statistical tests on PCs...no significant differences” test results (Steel-Dwass test?) should be indicated (probably in a table).

Page 8, line 21. Significant regional differences were found only along the PC2, is that right?

Page 37, Figure 7. The legend of the Kuma-Nishioda site should be in the same color.

Page 37, Figure 7. The plot of Kuma-Nishioda and Nakazuma seems wrong; many individuals were overlapped at the same points among those from the two sites. It is unusual.

Page 37, Figure 7. Several individuals fall at the outlier positions.

Page 37, Figure 7 and page 7, lines 23-24. Description of the method of the second comparison is unclear. When and from where do you calculate the PC score data in Figure 7? Did you recalculate them based on the selected pairs? The PC1 and PC2 scores of Kuma-Nishioda sites are different from each case of pairs.

Page 10, lines 1-2. “Remarkably small interphase differences.” I think the authors should indicate the evidence of the “smallness.” Please see the major comment.

Page 10, lines 8-9. “Discrepancy may be due to methodological differences...” The authors’ inference seems to have been made without a reason. Please explain the reasons if you have them. The authors wrote “Geometric morphometrics could examine morphological variation as a whole,...” However, they analyzed only portions (PC1-PC5) of the whole variation. If they insist on the methodological superiority of geometric morphometrics including the whole morphological variation, the authors should use higher-order PC scores.

Round 2 Review

General Comments

I read the Word file as the revised one in this revision, not the PDF file, which seems like an older version. After reading the revised version, I could find some revised points and progressed. However, I still have some reservations about the main conclusions on the wide and continuous interaction among temporal and geographical subpopulations of the Jōmon period.

Specific Comments

Major Comments

1. The most serious reservations are the main conclusions of the widespread and continuous population interactions in the Jōmon population. The related results are seemingly found in two portions; one is the cranial variations within the Jōmon population and the other is the comparison of those of the Jōmon and Yayoi populations.

As for the former, the authors hypothesized that “If the populations interacted widely and continuously, there should be less morphological differences among different regions and phases, ie, fewer statistically significant differences between each region and phase are found” (page 8). In this context, however, the results indicated that statistically significant differences are found in PC2. The hypothesis was thus rejected at least in the results along PC2. The authors stressed in the results that “there are no significant differences observed in PC1 and PC3, and the regional differences remain relatively limited” (page 14). There seems to be no reason for this statement. In addition, the authors also mentioned in the Discussion section that “the boxplots of PCs in Figure 6 by region, with the exception of PC2, do not exhibit such clear clinical patterns” (page 15). However, this result seems to me to indicate that a clear morphological cline can be found (at least along PC2) in the Jōmon cranial series, as with those previously proposed by traditional biometric studies. In sum, the conclusion of the small regional differences seems much less confident to be accepted.

2. Concerning the temporal differences, I feel the same kind of ambiguity about it. The principal component analysis results seem to indicate that the interphase differences are small. This is correct, but it is also without any confidence. A couple of interphase comparisons are actually significant. In the Discussion section, the smallness of the interphase difference was contrasted with those previously reported claims (page 15). This is also without any confidence.

3. In the case of comparisons of Jōmon and Yayoi specimens, the authors hypothesized that “interregion and interphase variations should be lower than the populations from a different period (Yayoi period)” (page 8). In this context, the result in Figure 7 was described as “interregion and interphase variations should be lower than the populations from a different period” (page 14). This description is also without any confidence. The authors should provide several statistical test results to compare the magnitude of interphase and interregion differences among the Jōmon and Yayoi samples.

Minor Comments

4. In the Results section of the Abstract (page 3), “individual populations were more than the Yayoi population” is ambiguous. It seems “more variable than...,” but it still contradicts the preceding expression of “the Jōmon populations were spatiotemporally less various than the Yayoi populations.” When I seek the corresponding lines in the text, they seem to match the site-to-site comparisons in Figure 8. If this is true, it seems to match the line of “the Jōmon individual populations are more various than the Yayoi population (page 14 - 15).” If this is the case, the authors’ expressions seem wrong. As Figure 8 presents the site-to-site comparison, each circle represents the individual variation within each site. Thus, the correct expression seems to be “individual variation within a site was more variable in the Jōmon site than that of the Yayoi site.”

5. Page 4, lines 17 - 19. The following expression is not acceptable: “shift in subsistence patterns and significant technological developments...are conspicuously absent in Jōmon society.”

6. Page 7, lines 3 and 5. “Middle” and “late” phases should be capitalized as Middle and Late.

7. Page 14, line 23. Please check the site of “Ebishima,” which is correct? Is this indicated to Nakazuma?

8. Page 15, line 1. Please describe how to make Figure 9.

9. Page 16, lines 3 - 5. The suggestion of “the morphological and genetic diversity among the Jōmon populations was not relatively limited, but homogeneous across regions and phases” is not understandable.

10. Page 16, lines 10 - 11. The expression is not understandable: “The reason why fewer evidence of such a societal changes were found in the Jōmon period is possibly wide and continuous population interactions.”

Conflicts of Interest

None declared.

Reference

1. Nakao H, Kaneda A, Tamura K, Noshita K, Yoshida M, Nakagawa T. Population interaction in the Jōmon society via 3D data of human crania: geometric morphometric study. JMIRx Bio 2025;3:e72432. [doi: 10.2196/72432]

Abbreviations

PC: principal component

Edited by A Schwartz; submitted 09.10.25; this is a non–peer-reviewed article;accepted 09.10.25; published 12.11.25.

Please cite as:
Kondo O
Peer Review of “Population Interaction in the Jōmon Society via 3D Data of Human Crania: Geometric Morphometric Study”
JMIRx Bio 2025;3:e85567
URL: https://bio.jmirx.org/2025/1/e85567
doi:10.2196/85567

Peer Review of “Population Interaction in the J mon Society via 3D Data of Human Crania: Geometric Morphometric Study”

Denise Amber Crampton

Liverpool John Moores University, Liverpool, United Kingdom

Related Articles:

https://www.biorxiv.org/content/10.1101/2025.02.09.637294v1

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https://bio.jmirx.org/2025/1/e72432/

(JMIRx Bio 2025;3:e85566) doi:10.2196/85566

KEYWORDS

Jomon; Japan; crania; cranium; bones; anthropology; archaeology; morphology; morphometrics

This is the peer-review report for “Population Interaction in the Jōmon Society via 3D Data of Human Crania: Geometric Morphometric Study.”

Round 1 Review

General Comments

The study [1] is well-structured, with a clear methodological framework and comprehensive statistical analyses. The Discussion effectively integrates archeological, genetic, and environmental perspectives to interpret morphological variation. However, the manuscript could benefit from more concise writing, clearer interpretation of statistical results, and better organization of discussion points to avoid redundancy.

Specific Comments

Major Comments

1. The principal component analysis results and statistical tests are well-documented but could be better contextualized. Some *P* values and effect sizes are presented without adequate interpretation of biological significance.I’d suggest the authors expand on how the observed morphological differences relate to adaptive or demographic processes.

2. The paper frequently repeats the idea of widespread and continuous interactions among Jōmon populations. While important, this could be streamlined for clarity. I’d suggest the authors consolidate similar points in the Introduction, Discussion, and Conclusion to reduce redundancy.

3. While the study references past research, it would be helpful to explicitly state how this study differs from previous geometric morphometric studies on Jōmon crania. I’d recommend you include a brief section discussing how the current 3D dataset provides new insights compared to traditional biodistance methods.

4. The Methods mention mirroring and reconstruction of crania but do not address potential biases this may introduce. I’d suggest the inclusion of a statement on limitations associated with missing data and how they were mitigated.

Minor Comments

5. The Abstract is informative but a bit lengthy. Consider summarizing key findings in fewer words.

6. Some terms like “prognathism,” “biodistance,” and “geometric morphometrics” could be briefly defined upon first mention for clarity.

7. Ensure that all figures and tables are referenced in the text where they are discussed. Some figures appear without clear introduction.

8. Check for uniformity in reference formatting, particularly italics in journal names and capitalization.

9. Some long sentences could be split for better readability (eg, in the Discussion section).

10. The Conclusion could briefly outline potential future studies, such as expanding sample sizes or integrating isotopic dietary data.

This study is well-executed and provides valuable insights into Jōmon population interactions using 3D geometric morphometrics. Addressing the clarity of statistical interpretation, reducing repetition, and improving Discussion structure would enhance its impact. I’d also recommend a final round of proofreading for grammar and formatting consistency.

Conflicts of Interest

None declared.

Reference

1. Nakao H, Kaneda A, Tamura K, Noshita K, Yoshida M, Nakagawa T. Population interaction in the Jōmon society via 3D data of human crania: geometric morphometric study. JMIRx Bio 2025;3:e72432. [doi: 10.2196/72432]

Edited by A Schwartz; submitted 09.10.25; this is a non–peer-reviewed article;accepted 09.10.25; published 12.11.25.

Please cite as:
Crampton DA
Peer Review of "Population Interaction in the Jōmon Society via 3D Data of Human Crania: Geometric Morphometric Study"
JMIRx Bio 2025;3:e85566
URL: https://bio.jmirx.org/2025/1/e85566
doi:10.2196/85566

Peer-Review Report

Peer Review of “Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study”

David Wirtshafter[1], BA, MA, PhD

The University of Illinois at Chicago, Chicago, IL, United States

Related Articles:

Companion article: https://www.biorxiv.org/content/10.1101/2024.11.05.622115v1

Companion article: https://bio.jmirx.org/2025/1/e71629/

Companion article: https://bio.jmirx.org/1/e68519/

(JMIRx Bio 2025;3:e71626) doi:10.2196/71626

KEYWORDS

ventral pallidum; nucleus accumbens shell; chemogenetics; sucrose; feeding behavior; food motivation; palatable food; DREADD; designer receptors exclusively activated by designer drugs

This is a peer-review report submitted for the paper “Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study.”

Round 1 Review

General Comments

In this paper [1], the authors present an interesting and well-written paper dealing with the effects of stimulation and inhibition of projections from the ventral pallidum to the nucleus accumbens shell on feeding and food reinforced behaviors. The methods used are cutting edge, and my comments and suggestions are relatively minor.

Specific Comments

Minor Comments

1. In the third paragraph of the Introduction, the sentence beginning with “Parallelly” is very awkward; I am sure there is a way to word this that does not use “parallelly.” Also, the previous sentence could be made clearer as to whether effects on sucrose consumption are found just in female rats.

2. The number of subjects should be listed in the Methods.

3. In the last paragraph of the body of the manuscript, the sentence beginning with “The discrepancies observed across studies of this pathway...” is unfinished, and I am uncertain what the authors intended to say.

4. In discussing the differences between the results observed here and those reported by Vanchez et al [2], is it possible that these may reflect the use of “closed-loop” manipulations linked to the occurrence of licking in the Vanchez et al [2] paper, in contrast to the continuous modulation produced here by the use of the DREADD (designer receptors exclusively activated by designer drugs) technique? Also, in this section, the authors could be a bit clearer as to why the techniques used by Vanchez et al [2] would be expected to label a different subpopulation of cells than was the case in this study.

Conflicts of Interest

None declared.

References

1. Peroutka M, Rivero Covelo I. Effects of ventral pallidum–nucleus accumbens shell neural pathway modulation on sucrose consumption and motivation in female rats: chemogenetic manipulation study. JMIRx Bio 2025;3(1):e68519 [FREE Full text] [doi: 10.2196/68519]

2. Vachez YM, Tooley JR, Abiraman K, Matikainen-Ankney B, Casey E, Earnest T, et al. Ventral arkypallidal neurons inhibit accumbal firing to promote reward consumption. Nat Neurosci 2021 Mar;24(3):379-390 [FREE Full text] [doi: 10.1038/s41593-020-00772-7] [Medline: 33495635]

Abbreviations

DREADD: designer receptors exclusively activated by designer drugs

Edited by O Singh; submitted 22.01.25; this is a non–peer-reviewed article;accepted 22.01.25; published 08.03.25.

Please cite as:
Wirtshafter D
Peer Review of "Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study"
JMIRx Bio 2025;3:e71626
URL: https://bio.jmirx.org/2025/1/e71626
doi:10.2196/71626
PMID:

Peer-Review Report

Peer Review of “Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study”

Jeffrey Grimm

[1]Western Washington University, Bellingham, WA, United States

Related Articles:

Companion article: https://www.biorxiv.org/content/10.1101/2024.11.05.622115v1

Companion article: https://bio.jmirx.org/2025/1/e71629/

Companion article: https://bio.jmirx.org/1/e68519/

(JMIRx Bio 2025;3:e71627) doi:10.2196/71627

KEYWORDS

ventral pallidum; nucleus accumbens shell; chemogenetics; sucrose; feeding behavior; food motivation; palatable food; DREADD; designer receptors exclusively activated by designer drugs

This is a peer-review report submitted for the paper “Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study.”

Round 1 Review

General Comments

The manuscript from Peroutka and Covelo [1] describes the results of chemogenic activation or inhibition of the ventral pallidum–nucleus accumbens shell pathway in adult female rats on sucrose intake (20% sucrose bottle access) versus operant response–provided food pellets delivered on a progressive ratio schedule. The rats were not food restricted. Activation of the pathway decreased sucrose intake while inactivation of the pathway increased sucrose intake. Activation or inactivation did not clearly alter responding for food pellets. The authors provide discussion including an interpretation of the results, such that this pathway is important for sucrose consumption but not motivation for food. This is an interesting study that has some limitations listed below.

Specific Comments

Major Comments

1. Why were only female rats used for this study?

2. What was the approximate age of the rats at the start of the study?

3. The conclusion of the pathway being relevant for sucrose consumption but not food motivation is reasonable, but it would be stronger if the comparisons were made with sucrose consumption versus sucrose motivation and also food consumption versus food motivation.

Minor Comments

4. Are there more objective data from analysis of the immunohistochemistry? What is presented are representative images, but was there any quantification done?

5. The authors discuss cell types but do not specify the likely type of neurons stimulated in this study; is it possible to do so?

Round 2 Review

General Comments

The authors have addressed my concerns from the initial draft.

Conflicts of Interest

None declared.

Reference

1. Peroutka M, Rivero Covelo I. Effects of ventral pallidum–nucleus accumbens shell neural pathway modulation on sucrose consumption and motivation in female rats: chemogenetic manipulation study. JMIRx Bio 2025;3(1):e68519 [FREE Full text] [doi: 10.2196/68519]

Edited by O Singh; submitted 22.01.25; this is a non–peer-reviewed article;accepted 22.01.25; published 08.03.25.

Please cite as:
Grimm J
Peer Review of "Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study"
JMIRx Bio 2025;3:e71627
URL: https://bio.jmirx.org/2025/1/e71627
doi:10.2196/71627
PMID:

Peer-Review Report

Peer Review of "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study"

Jiayi Shen[1]

University of Southern California, Los Angeles, CA, United States

Related Articles:

Companion article: https://preprints.jmir.org/preprint/69542

Companion article: https://bio.jmirx.org/2025/1/e72764/

Companion article: https://bio.jmirx.org/2025/1/e69542/

(JMIRx Bio 2025;3:e72765) doi:10.2196/72765

KEYWORDS

impact of climate; seasonal change; frequency; livestock; ticks; Tehran

This is a peer-review submitted for the paper "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study."

Round 1 Review

General Comments

This paper [1] provides a detailed investigation into the distribution and frequency of tick species infecting livestock and poultry in Tehran province, Iran, with a focus on seasonal and climatic variation. The study highlights the significant economic and epidemiological impact of ticks as ectoparasites and pathogen vectors in livestock.

Specific Comments

Major Comments

1. "Material and methods - Sampling": For the tick sampling in this manuscript, what method did you used in this study (ie, how did you decide which tick samples to include and which samples to exclude)? How did you ensure that the sample is representative of the true distribution of ticks in the study area? The distribution and frequency estimates from this sample might not be extended to the whole tick population if the sampling is biased.

2. "Material and methods - Sampling": Could the author provide the rationale or justification of the choice of "*p*" and "*d*" in the sample size calculation?

3. "Discussion": I really like the analysis of seasonal trend presented in Figure 4. Could the author elaborate more on this in the Discussion—the general seasonal trend across all species, the reason why you expect some species to be more abundant in warmer versus colder weather, and the implications from the public health perspective?

Minor Comments

1. Line 41: Spell out "$."

2. Line 44: Remove "(4)"—duplicated reference number.

3. Line 51: Remove "(9)"—duplicated reference number.

4. Line 85, "valid diagnostic keys": Could the author be more specific about the "diagnostic key" being used? Adding a sentence to briefly describe the key would be great.

5. Lines 147-151, "two professional stereo microscopes...in the entomological research": This part should belong to Methods section.

Round 2 Review

Thank you, author, for addressing all my comments and making all necessary changes to the manuscript. I do not have any more comments.

Conflicts of Interest

None declared.

Reference

1. Abbasi E. Assessing the influence of seasonal and climatic variations on livestock tick incidence in Tehran province, Iran: cross-sectional study. JMIRx Bio 2025;3:e69542. [doi: 10.2196/69542]

Edited by J Ren; submitted 17.02.25; this is a non–peer-reviewed article;accepted 17.02.25; published 31.03.25.

Please cite as:
Shen J
Peer Review of "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study"
JMIRx Bio 2025;3:e72765
URL: https://bio.jmirx.org/2025/1/e72765
doi:10.2196/72765
PMID:

Peer-Review Report

Peer Review of "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study"

Vahid Noaman[1]

Razi Vaccine & Serum Research Institute, Karaj, Iran

Related Articles:

Companion article: https://preprints.jmir.org/preprint/69542

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Companion article: https://bio.jmirx.org/2025/1/e69542/

(JMIRx Bio 2025;3:e72766) doi:10.2196/72766

KEYWORDS

impact of climate; seasonal change; frequency; livestock; ticks; Tehran

This is a peer-review submitted for the paper "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study."

Round 1 Review

General Comments

The manuscript [1] presents a comprehensive study on the seasonal and climatic distribution of ticks in Tehran province, Iran. The research is well structured and provides valuable insights into the diversity and abundance of tick species in different climate zones. The study is relevant to the field of veterinary parasitology and has potential implications for tick control strategies in the region. However, there are several areas where the manuscript could be improved in terms of grammar, sentence structure, and adherence to standard academic writing conventions.

Specific Comments

Major Comments

Grammar and Sentence Structure

Overall clarity: The manuscript is generally clear, but there are instances where the sentence structure could be improved for better readability. Some sentences are overly long and could be broken down into shorter, more concise statements.

For example:

- Original: "The presence of ticks on livestock causes localized lesions at the bite site and systemic lesions, leading to death due to anemia and paralysis due to ticks transmitting various diseases such as theileriosis and babesiosis."
- Suggested revision: "The presence of ticks on livestock causes localized lesions at the bite site and systemic effects, which can lead to anemia, paralysis, and even death. Ticks are also vectors for diseases such as theileriosis and babesiosis."

Subject-verb agreement: There are a few instances where the subject-verb agreement is incorrect.

For example:

- Original: "The distribution of collected ticks (in mountain and plain climates) indicated that out of 806 collected ticks, 44.78% and 55.21% belonged to the mountainous and plain regions, respectively."
- Suggested revision: "The distribution of collected ticks (in mountainous and plain climates) indicates that out of 806 collected ticks, 44.78% belonged to mountainous regions, while 55.21% were found in plain regions."

Tense consistency: The manuscript occasionally shifts between past and present tense. It is important to maintain consistency, especially in the Results and Discussion sections.

For example:

- Original: "The study is conducted in two different environments: plains and mountains within 20 selected villages in Tehran Province."
- Suggested revision: "The study was conducted in two different environments: plains and mountains within 20 selected villages in Tehran Province."

Structure and Organization

Abstract: The Abstract is well written and provides a concise summary of the study. However, it could benefit from a brief mention of the key findings related to seasonal variations, as this is a major focus of the study.

Introduction: The Introduction provides a good background on the importance of ticks and their impact on livestock. However, it could be strengthened by including more recent references (post-2020) to highlight the current state of research on tick-borne diseases and climate change.

Methods: The Methods section is detailed and well organized. However, the formula used for sample size calculation is not clearly explained. It would be helpful to provide a brief explanation of the variables used in the formula (eg, p=0.3 and d=0.045).

Results: The Results are presented clearly, with appropriate use of tables and figures. However, some of the tables could be simplified for better readability. For example, Table 4 could be restructured to make it easier to compare seasonal activity across species.

Discussion: The Discussion is thorough and compares the findings with other studies effectively. However, it could be improved by discussing the limitations of the study and suggesting areas for future research.

Similarity and Plagiarism

The manuscript appears to be original, with no significant issues of plagiarism detected. However, it is recommended to run the manuscript through a plagiarism detection tool (eg, Turnitin) to ensure that all sources are properly cited and that there is no unintentional duplication of text.

Adherence to Standard Academic Writing

References: The references are generally appropriate and relevant to the study. However, some references are quite old (eg, references from the 1980s and 1990s). It is recommended to include more recent studies to reflect the current state of knowledge in the field.

Add these references to the manuscript:

1. Noaman V. Identification of hard ticks collected from sheep naturally infected with Anaplasma ovis in Isfahan province, central Iran. Comp Clin Pathol 2012 Feb 21; 21(3):367-369. [doi: 10.1007/s00580-012-1438-1]
2. Noaman V, Abdigoudarzi M, Nabinejad AR. Abundance, diversity and seasonal dynamics of hard ticks infesting cattle in Isfahan province, central Iran. Archives of Razi Institute. 2017 Mar 1;72(1):15-21. [doi: 10.22034/ari.2016.107490]
3. Noaman V, Abdigoudarzi M, Nabinejad AR, Heidari MR, Khalilifard M. (2007). Identification of hard ticks of domestic ruminants in two ecological zones of Isfahan province, Iran. Veterinary Journal (Pajouhesh va Sazandegi). 2008;77:88-95.

Figures and tables: The figures and tables are well presented and support the findings of the study. However, the legends for some figures (eg, Figure 1) could be more descriptive. For example, Figure 1 could include a brief explanation of what the "ratio of caught ticks" represents.

The manuscript presents a valuable contribution to the field of veterinary parasitology, particularly in the context of tick distribution and seasonal activity in Tehran province. With some revisions to improve grammar, sentence structure, and adherence to standard academic writing conventions, the manuscript will be suitable for publication in a reputable journal. The manuscript can be considered for publication in *JMIRx Bio* after major revision.

Round 2 Review

General Comments

The revised manuscript titled "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran" has addressed the previous comments and suggestions effectively. The authors have made the necessary revisions to improve the clarity, structure, and overall quality of the manuscript. Below are my final comments.

Strengths

Improved clarity: The Abstract has been revised to be more concise and now includes key findings related to seasonal variation and the most abundant tick species, enhancing readability and impact.

Focused introduction: The Introduction now more clearly highlights the specific gaps in the literature that this study addresses, particularly in the context of Tehran province.

Streamlined methodology: The Methods section has been clarified, with more details on the randomization process and a more concise description of the study area. The inclusion of supplementary tables for geographical coordinates and ecological information is a welcomed addition.

Organized results: The Results section has been streamlined with the use of subheadings, making it easier to follow. All referenced figures and tables are now included, providing a comprehensive view of the findings.

Enhanced discussion: The Discussion section now more effectively focuses on the implications of the findings for tick control strategies in Tehran province. The comparison with studies from other regions has been made more concise, emphasizing key similarities and differences.

Practical conclusion: The Conclusion has been revised to highlight the practical implications of the findings, particularly the need for seasonal tick control measures in different climate zones.

Consistent references: All references are now formatted consistently according to the journal's guidelines, with complete details provided.

Overall recommendation: The manuscript has been significantly improved and is now suitable for publication. I recommend acceptance of the manuscript in its current form.

Conflicts of Interest

None declared.

Reference

1. Abbasi E. Assessing the influence of seasonal and climatic variations on livestock tick incidence in Tehran province, Iran: cross-sectional study. JMIRx Bio 2025;3:e69542. [doi: 10.2196/69542]

Edited by J Ren; submitted 17.02.25; this is a non–peer-reviewed article;accepted 17.02.25; published 31.03.25.

Please cite as:
Noaman V
Peer Review of "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study"
JMIRx Bio 2025;3:e72766
URL: https://bio.jmirx.org/2025/1/e72766
doi:10.2196/72766
PMID:

Authors' Response to Peer Reviews of "Discovery of Novel Inhibitors of HMG-CoA Reductase Using Bioactive Compounds Isolated From Cochlospermum Species Through Computational Methods: Virtual Screening and Algorithm Validation Study"

Toba Isaac Olatoye[1,2], BSc, MSc

[1]Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Kwara State, Ilorin, Nigeria

[2]Schools and Colleges, Kwara State Teaching Service Commission, Ilorin, Nigeria

Corresponding Author:
Toba Isaac Olatoye, BSc, MSc
Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Kwara State, Ilorin, Nigeria

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https://med.jmirx.org/2025/1/e71675

(JMIRx Bio 2025;3:e78093) doi:10.2196/78093

KEYWORDS

HMGR; statins; hypercholesterolemia; cochlospermum; phytochemicals; molecular docking

This is the authors' response to the peer-review report for "Discovery of Novel Inhibitors of HMG-CoA Reductase Using Bioactive Compounds Isolated From Cochlospermum Species Through Computational Methods: Virtual Screening and Algorithm Validation Study."

My profound appreciation goes to the reviewers [1] for their thoughtful, constructive, and actionable feedback on my manuscript [2]. I'm grateful for the opportunity to revise and improve my work based on the graceful comments provided. Here are the details of my responses to the reviewers' comments and the changes made to the manuscript.

Concerns and Feedback

Reviewers appreciated the depth and thoroughness of the search through the literature of peer-review research. Some reviewers were surprised about the date (1991) of some studies related to the high-performance liquid chromatography–UV analysis of phytochemicals identified in the ethanolic and methanolic extract of Cochlospermum tinctorium and wondered whether there may be more recent studies to also consider.

Response: Thank you for your kind comments and the concern raised. However, just like I mentioned during the live review session, studies related to the isolation and identification of some bioactive compounds from *C tinctorium* are quite old and indigenous with limited data available, but I was still able to pull more recent data from 2012.

To increase the reproducibility of the study, some reviewers wondered if it would be possible to make the data and code used to analyze the data openly available.

Response: The data file and details used for the analysis have now been made available in the manuscript and can be found in Multimedia Appendix 6.

The figures and tables are comprehensive and clearly presented, with well-written descriptions. If feasible, reviewers would suggest ways to visually highlight key compounds listed in tables using colors, bold text, or labels. Furthermore, incorporating chemical structures directly within the relevant tables or as supplementary figures would further enhance the understanding of their molecular characteristics and potential interactions.

Response: The phytochemicals analyzed in this study had already been narrowed down to 10 top-ranked compounds and are contained in Tables 1 and 3 of the manuscript. Also, I have incorporated their chemical structures and a few additional details including those of statins (Table 4) to enhance readers' understanding. For information regarding their molecular characteristics and potential binding interactions, kindly check Multimedia Appendices 2 - 5.

While the author acknowledges the need for in vitro and in vivo validation studies, explicitly addressing potential computational limitations—such as docking inaccuracies, semirigid approach versus more flexible ones, or the absence of dynamic modeling—would further strengthen the discussion.

Response: Of course, computational studies have their own share of limitations especially molecular docking studies, and I'm well aware of certain docking inaccuracies and the advantage that more flexible docking and molecular dynamics simulations algorithms hold over semirigid docking. Nevertheless, a pragmatic approach had already been taken to ascertain the accuracy and validity of the "PyRx AutoDock Vina" algorithm used in this docking study. The details of this submission are in the "Methods" section of the manuscript under the subheading "Molecular Docking Analysis."

Some reviewers suggested adapting the part of the study that identified the compounds through literature review into a systematic review.

Response: This suggestion has been taken into consideration, and a systematic review has now been included as part of the manuscript title. Many thanks to the reviewers for their insightful comments.

Additional revisions have been made to the manuscript in response to the editorial team's valuable feedback, but are not highlighted in my responses above.

I trust that these revisions will address the issues raised by the reviewers and improve the quality of the manuscript.

Artificial intelligence disclosure: The author attests that there was no use of generative artificial intelligence technology in the generation of text, figures, or other informational content of this manuscript.

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Edited by A Schwartz; submitted 26.05.25; this is a non–peer-reviewed article;accepted 26.05.25; published 10.07.25.

Please cite as:
Olatoye TI
Authors' Response to Peer Reviews of "Discovery of Novel Inhibitors of HMG-CoA Reductase Using Bioactive Compounds Isolated From Cochlospermum Species Through Computational Methods: Virtual Screening and Algorithm Validation Study"
JMIRx Bio 2025;3:e78093
URL: https://bio.jmirx.org/2025/1/e78093
doi:10.2196/78093

Authors' Response to Peer Reviews of "Population Interaction in the J mon Society via 3D Data of Human Crania: Geometric Morphometric Study"

Hisashi Nakao[1], PhD; Akihiro Kaneda[2], PhD; Kohei Tamura[3,4,5], PhD; Koji Noshita[6], PhD; Mayu Yoshida[7], MA; Tomomi Nakagawa[8], PhD

[1]Department of Anthropology and Philosophy, Nanzan University, 18 Yamazato, Showa, Nagoya, Japan
[2]Graduate School of Humanities and Social Sciences, Mie University, Mie, Japan
[3]The Center for Northeast Asian Studies, Tohoku University, Sendai, Japan
[4]Graduate School of Environmental Studies, Tohoku University, Sendai, Japan
[5]Center for Integrated Japanese Studies, Tohoku University, Sendai, Japan
[6]Graduate School of Science, Kyushu University, Fukuoka, Japan
[7]Graduate School of Letters, Tohoku University, Sendai, Japan
[8]Graduate School of Letters, Nagoya University, Nagoya, Japan

Corresponding Author:
Hisashi Nakao, PhD
Department of Anthropology and Philosophy, Nanzan University, 18 Yamazato, Showa, Nagoya, Japan

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https://bio.jmirx.org/2025/1/e72432/

(JMIRx Bio 2025;3:e85565) doi:10.2196/85565

KEYWORDS

Jomon; Japan; crania; cranium; bones; anthropology; archaeology; morphology; morphometrics

This is the author's response to peer-review reports for "Population Interaction in the Jōmon Society via 3D Data of Human Crania: Geometric Morphometric Study."

Round 1 Review

Reviewer R [1]

General Comments

The study [2] is well-structured, with a clear methodological framework and comprehensive statistical analyses. The Discussion effectively integrates archeological, genetic, and environmental perspectives to interpret morphological variation. However, the manuscript could benefit from more concise writing, clearer interpretation of statistical results, and better organization of discussion points to avoid redundancy.

Response: We appreciate the supportive and helpful comments. We describe our modifications here.

Specific Comments

Major Comments

1. The principal component analysis results and statistical tests are well-documented but could be better contextualized. Some P values and effect sizes are presented without adequate interpretation of biological significance.

I'd suggest the authors expand on how the observed morphological differences relate to adaptive or demographic processes.

Response: Thank you for the useful comment. We added an explanation from line 22 of page 5 to line 5 of page 6 as follows:

"Typically, previous research employed a traditional biodistance method (eg, Martin 1928 [3]) and statistical differences between morphological biodistances among different populations have been regarded as genetic differences. For example, as stated below, if geographical clines were found in morphological biodistances, it is commonly argued that populations moved in a certain geographical direction."

2. The paper frequently repeats the idea of widespread and continuous interactions among Jōmon populations. While important, this could be streamlined for clarity. I'd suggest the authors consolidate similar points in the Introduction, Discussion, and Conclusion to reduce redundancy.

Response: Thank you for the helpful comment. We tried to reduce the redundancy.

3. While the study references past research, it would be helpful to explicitly state how this study differs from previous geometric morphometric studies on Jōmon crania. I'd recommend you include a brief section discussing how the current 3D dataset provides new insights compared to traditional biodistance methods.

Response: We made a relevant subsection in the Introduction ("Previous Research on the Jōmon Population Interaction") describing how the present research contributes to the previous research.

4. The Methods mention mirroring and reconstruction of crania but do not address potential biases this may introduce. I'd suggest the inclusion of a statement on limitations associated with missing data and how they were mitigated.

Response: Thank you for the important suggestion. We added a statement on limitations and how they were mitigated (lines 5 - 7 of page 11):

"Since this mirroring should include some subjective biases, mirroring was limited to cases with less distorted crania."

Minor Comments

5. The Abstract is informative but a bit lengthy. Consider summarizing key findings in fewer words.

Response: We followed the editorial comment saying, "Although Reviewer R asked for a more concise abstract, this is not in line with journal requirements or expectations, as the abstract can be up to 450 words and should have sufficient information to understand the study as a whole without needing to refer to the main text."

6. Some terms like "prognathism," "biodistance," and "geometric morphometrics" could be briefly defined upon first mention for clarity.

Response: We added a brief explanation of these terms as follows:

- prognathism (protrusion of the jaws) (line 22 of page 12).
- biodistance method using distances between specific measuring points (line 23 of page 5).
- The geometric morphometrics of 3D data, which the present research employed and explores shape variations of targeted objects, typically using coordination of landmarks, has gained traction in various fields, extending to objects such as lithics (lines 15 - 17 of page 7).

7. Ensure that all figures and tables are referenced in the text where they are discussed. Some figures appear without clear introduction.

Response: We ensured the points above were addressed.

8. Check for uniformity in reference formatting, particularly italics in journal names and capitalization.

Response: We checked and modified the reference formatting.

9. Some long sentences could be split for better readability (eg, in the Discussion section).

Response: We tried to split the sentences, especially in the Discussion section.

10. The Conclusion could briefly outline potential future studies, such as expanding sample sizes or integrating isotopic dietary data.

Response: We deleted the Conclusion section following editorial guidelines. We made a subsection in the Discussion and stated the future directions and limitations.

This study is well-executed and provides valuable insights into Jōmon population interactions using 3D geometric morphometrics. Addressing the clarity of statistical interpretation, reducing repetition, and improving Discussion structure would enhance its impact. I'd also recommend a final round of proofreading for grammar and formatting consistency.

Response: Thank you again for the helpful comment. We appreciate it.

Reviewer AE [4]

General Comments

This paper studied 3D data of Jōmon and Yayoi cranial series and analyzed them in terms of temporal and geographical variations. While conventional craniometric studies of Jōmon specimens have been much accumulated, almost no comprehensive ones have analyzed both temporal and regional variation at once. In this sense, the approach of this paper is promising. However, I have major reservations regarding the approaches used to achieve this target; the authors' present approach is not able to clarify the smallness or wideness of the temporal and/or regional variation among Jōmon crania. Another major reservation is the data, as the 3D morphology of the Jōmon crania sustains unavoidable destruction and deformation during burial processes; thus, most of them were manually repaired. The data used in this study did not mention this bias properly.

Response: We appreciate the highly important comments and suggestions. We followed your suggestion, added a comparative analysis between the Jōmon and Yayoi populations, and mentioned some limitations of the present research. Details of the additional analysis and modifications are described below.

Specific Comments

Major Comments

One major reservation is in the analytical design; the present one seems suitable for the study target, which aims to clarify the temporal (between Jōmon phases) and geographical (between regions in Japan) variations of the Jōmon crania. The authors concluded that there were pronounced morphological variations within individual populations versus smaller variations between different phases and geographical regions

(from the Abstract). Unfortunately, this seems unclear to me. Probably a reason for this could be the lack of comparative targets. Figures 5 and 6 present the temporal and regional variations along principal component (PC) 1 and PC2 and indicate only regional variations along PC2 as being significant, but this seems too weak for readers to reconfirm the conclusion of smaller temporal and regional variations. In my idea, as the authors perform the geometric morphometric and principal component analysis procedures for all the Jōmon and Yayoi individuals, all the comparisons can include all the subdivisions of the Jōmon and Yayoi. Afterward, readers can understand the temporal variation among Jōmon crania and also compare the degree of variation compared to those of the Yayoi, and the same is the case for the regional variation.

Response: We added some Yayoi populations (especially from the Aoyakamijichi site in the Late Yayoi period of the Sanin region) and conducted the geometric morphometric and principal component analysis for all the Jōmon populations and Yayoi populations. We could not include the Yayoi population from the Early period and from regions other than the Kyushu and Sanin regions because the excavated Yayoi crania are highly concentrated in the Kyushu region and the Aoyakamijichi site is the only exception with more accurate information and 3D data of more than 5 well-preserved individuals excavated. Moreover, many of the cranial samples from the period are collected in the Kyushu University Museum and Nagasaki University and they do not permit anyone to access the 3D data of the collection. We tried to gather and include as many relatively large and well-preserved samples from the Yayoi population as possible.

The results are summarized in Figure 7 and Multimedia Appendix 3. Figure 7 in particular suggests that variations among the Yayoi population are larger than in the Jōmon populations.

Another aspect that needs to be considered is the power of expression in the PC scores. The present analysis almost confines the results to PC1 and PC2 scores, which explain only 20% of the total variation (Table 2). When we accept the authors' idea of the advantage of 3D geometric morphometric analysis being inclusion of whole cranial morphology, selection and only a portion of the PCs may lead to disadvantages. I do not have any ideas for tackling this problem; the authors should care about this.

Response: We appreciate this highly useful comment. As the editor also gave us a similar point, we mentioned a limitation of and caution regarding the present results in the Discussion section (from line 22 of page 16 to line 3 of page 17) as follows:

"Second, our discussions have focused on PCs with higher than 5 percentages of contribution rate, which covers less than 40 percentages of total cumulative proportions. Although the results of principal component analysis on 3D data typically tend to be dispersed in more PCs [5-7], we should be careful not to overestimate the present results. The question of how frequently the Jōmon people interacted should be explored from more aspects, including quantitative investigations of relevant archeological remains and mathematical simulations or modeling of the Jōmon population."

One other reservation is about the Jōmon cranial reconstruction. Most of the fragmentary and heavily reconstructed specimens suffered unavoidable skews and deformation. Because most researchers cannot repair the reconstruction, available options are few, but the authors should mention that they care about this bias. I suggest checking for the position in the plot (eg, outlier position) and the degree of preservation.

Response: Thank you for the important comment. We rechecked and recalculated all the data and modified some of the plots. Also, we added an explanation for the selection of samples in lines 12 - 15 of page 9 as follows: "Although buried crania from the prehistoric period tend to be distorted due to soil conditions, we chose to measure crania that are regarded as less skewed and well-preserved based on their appearance and excavation information."

Minor Comments

Page 3, lines 3-5. Subdivision of the Jōmon period is usually capitalized (eg, Incipient or Initial Jōmon phases).

Response: We capitalized the subdivisions.

Page 7, line 1. "Facies symphysialis" can be rephrased as "pubic symphyses."

Response: We modified the expression.

Page 7, line 12. Cranial morphology should be considered as an outcome being affected by many factors; it should not be simplified as "a human trait."

Response: We changed "human trait" to "human cranium."

Page 7, lines 18-21. The purpose of the selection of several Jōmon site data for comparison with the Yayoi site is unclear—do you mean site-to-site comparison?

Response: Sorry for the confusion. As the reviewer said, we meant site-to-site comparison here. We conducted the analysis to explore the degree of variation in the individual Jōmon populations. As the reviewer suggested, we added another comparative analysis with some Yayoi populations to explore the interphase and interregion differences among the Jōmon populations (see also the subsection "Comparisons Between the Jōmon Populations and the Yayoi Population" in the Results section). We added an explanation in the Methods section (lines 13 - 15 of page 12) as follows: "Such comparisons of the Jōmon populations from different phases and regions with the Yayoi population could reveal the degree of variation in the individual Jōmon populations."

Page 8, line 15. "We also deform a cranium" is better reworded as "We also show deformation patterns of a cranium..."

Response: We reworded the expression as stated in the comment.

Page 8, line 19. "Statistical tests on PCs...no significant differences" test results (Steel-Dwass test?) should be indicated (probably in a table).

Response: We added a supplementary table (Multimedia Appendix 3: Summary of the statistical tests) summarizing the statistical results.

Page 8, line 21. Significant regional differences were found only along the PC2, is that right?

Response: Fewer significant regional differences were also found along the PC4 and mentioned in the text. We reanalyzed and recalculated all of the data, and regional differences were not found in PC1, PC3, and PC5.

Page 37, Figure 7. The legend of the Kuma-Nishioda site should be in the same color.

Response: We modified the Figure following the comment.

Page 37, Figure 7. The plot of Kuma-Nishioda and Nakazuma seems wrong; many individuals were overlapped at the same points among those from the two sites. It is unusual.

Response: We highly appreciate the important comment. We reanalyzed and recalculated all of the data. Some parts of the results shown in Figure 7 were wrong and were modified. Also, as the statistical differences in the PC2 of the temporal comparisons were missed, we added some explanations as follows (lines 7 to 13 of page 13):

"The PCA results, graphically presented in Figures 5 and 6, suggest that the differences between phases are relatively small, in line with the statistical tests on PCs that indicate statistical differences only in the Early and Final (Z=3.118, *P*=.022), Middle and Final (Z=4.233, *P*<.001), and Late and Final phase (Z=4.040, *P*=.001) in PC2, and the Late and Final phase (Z=2.946, *P*=.038) in PC4 (see also Multimedia Appendix 2)."

Page 37, Figure 7 and page 7, lines 23-24. Description of the method of the second comparison is unclear. When and from where do you calculate the PC score data in Figure 7? Did you recalculate them based on the selected pairs? The PC1 and PC2 scores of Kuma-Nishioda sites are different from each case of pairs.

Response: We recalculated the data by each comparison. We added more details in the Methods section as follows (in lines 8 - 9 of page 12):

"Especially, we selected cranial data from Jōmon sites with relatively larger sample sizes to compare the Kuma-Nishioda site. They were compared via PCA [principal component analysis] by each site."

Page 10, lines 1-2. "Remarkably small interphase differences." I think the authors should indicate the evidence of the "smallness." Please see the major comment.

Response: Thank you for the important comment. As the reviewer suggested, we added data from the Aoyakamijichi site of the Late Yayoi period in the Sanin region and conducted an additional analysis between the Jōmon and the Yayoi populations (from the Aoyakamijichi site and the Kuma-Nishioda site of the Middle Yayoi period in the Kyushu region). The results are summarized in Figure 7 and Multimedia Appendix 3 (Summary of the statistical tests), suggesting that variations between the Jōmon populations were relatively smaller than the Yayoi populations, and that individual populations in the Jōmon period were more various than in the Yayoi period.

Page 10, lines 8-9. "Discrepancy may be due to methodological differences..." The authors' inference seems to have been made without a reason. Please explain the reasons if you have them. The authors wrote "Geometric morphometrics could examine morphological variation as a whole,..." However, they analyzed only portions (PC1-PC5) of the whole variation. If they insist on the methodological superiority of geometric morphometrics including the whole morphological variation, the authors should use higher-order PC scores.

Response: Sorry for the confusion. We did not intend to claim that geometric morphometrics is superior to the traditional biodistance method in that geometric morphometrics could capture all of the variations. What we would like to say is that the traditional biodistance method focuses on each biodistance between specific measuring points, while geometric morphometrics explores the overall variations between each measuring point. In order to avoid such confusion, we deleted the relevant sentence in the Discussion section and modified the explanations in the Introduction as follows (line 23 of page 7 to line 3 of page 8):

"Furthermore, when analyzing the resulting data, geometric morphometrics is able to track morphological variation among configurations of each measuring point at once, whereas the traditional biodistance method compares each measured distance independently. These differences would provide some new insights."

Round 2 Review

Reviewer AE [4]

General Comments

I read the Word file as the revised one in this revision, not the PDF file, which seems like an older version. After reading the revised version, I could find some revised points and progressed. However, I still have some reservations about the main conclusions on the wide and continuous interaction among temporal and geographical subpopulations of the Jōmon period.

Response: We appreciate for the reviewer's careful reading and important comments and suggestions. We mentioned all the reviewer's concerns and described our modifications in the below response.

Specific Comments

Major Comments

1. The most serious reservations are the main conclusions of the widespread and continuous population interactions in the Jōmon population. The related results are seemingly found in two portions; one is the cranial variations within the Jōmon population and the other is the comparison of those of the Jōmon and Yayoi populations.

As for the former, the authors hypothesized that "If the populations interacted widely and continuously, there should be less morphological differences among different regions and phases, ie, fewer statistically significant differences between each region and phase are found" (page 8). In this context, however, the results indicated that statistically significant

differences are found in PC2. The hypothesis was thus rejected at least in the results along PC2. The authors stressed in the results that "there are no significant differences observed in PC1 and PC3, and the regional differences remain relatively limited" (page 14). There seems to be no reason for this statement. In addition, the authors also mentioned in the Discussion section that "the boxplots of PCs in Figure 6 by region, with the exception of PC2, do not exhibit such clear clinical patterns" (page 15). However, this result seems to me to indicate that a clear morphological cline can be found (at least along PC2) in the Jōmon cranial series, as with those previously proposed by traditional biometric studies. In sum, the conclusion of the small regional differences seems much less confident to be accepted.

2. Concerning the temporal differences, I feel the same kind of ambiguity about it. The principal component analysis results seem to indicate that the interphase differences are small. This is correct, but it is also without any confidence. A couple of interphase comparisons are actually significant. In the Discussion section, the smallness of the interphase difference was contrasted with those previously reported claims (page 15). This is also without any confidence.

Response: Thank you for the important comments. Comment 1 and 2 are related, so we responded to them together here. As the reviewer pointed out, we admit that PC2 indicated spatiotemporal variations with some statistical differences, which is consistent with some previous research. On the other hand, PC1 and other PCs did not. Since the results were mixed, we argued that even though spatiotemporal differences were found, it is possible that they were relatively small. We have modified the Discussion to clarify our intentions as follows:

"The results of the PCA [principal component analysis] presented above were not straightforward. PC2 showed statistically significant spatiotemporal differences between some regions and phases, which was consistent with previous biometric studies claiming interphase variation [8-10] and geographical clines from north to south [9,11-13], although it is difficult to conclude that their differences are pronounced. This is because PC1 did not show any statistical differences, and a similar study of the Kofun period, examining a larger set of 3D data of human crania by geometric morphometrics, exhibited a geological cline in PC1 [6]. Moreover, as mentioned in the Introduction, given the archeological evidence for the spatiotemporal distinctiveness of the Jōmon material cultures, it would also be expected to find statistically significant differences in PC1. Thus, it is possible that the spatiotemporal differences in the Jōmon period were more nuanced or relatively small."

3. In the case of comparisons of Jōmon and Yayoi specimens, the authors hypothesized that "interregion and interphase variations should be lower than the populations from a different period (Yayoi period)" (page 8). In this context, the result in Figure 7 was described as "interregion and interphase variations should be lower than the populations from a different period" (page 14). This description is also without any confidence. The authors should provide several statistical test results to compare the magnitude of interphase and interregion differences among the Jōmon and Yayoi samples.

Response: We added effect sizes between the Yayoi populations and between the Jōmon populations as follows:

Figure 7 showed that the Jōmon populations substantially overlapped, while the Yayoi populations were more scattered spatiotemporally in PC2 and PC3, which was supported by the fact that the effect sizes of statistical tests between the Yayoi populations (Z/n=.113 in spatial and temporal comparisons in PC2) were higher than the ones between the Jōmon populations (Z/n<.055 in spatial and Z/n<.029 in temporal comparisons in PC2) (see Multimedia Appendix 2).

Minor Comments

4. In the Results section of the Abstract (page 3), "individual populations were more than the Yayoi population" is ambiguous. It seems "more variable than...," but it still contradicts the preceding expression of "the Jōmon populations were spatiotemporally less various than the Yayoi populations." When I seek the corresponding lines in the text, they seem to match the site-to-site comparisons in Figure 8. If this is true, it seems to match the line of "the Jōmon individual populations are more various than the Yayoi population (page 14 - 15)." If this is the case, the authors' expressions seem wrong. As Figure 8 presents the site-to-site comparison, each circle represents the individual variation within each site. Thus, the correct expression seems to be "individual variation within a site was more variable in the Jōmon site than that of the Yayoi site."

Response: Thank you for the important suggestion. We modified the description as the reviewer suggested.

5. *Page 4, lines 17 - 19. The following expression is not acceptable: "shift in subsistence patterns and significant technological developments...are conspicuously absent in Jōmon society."*

Response: We modified the expression as follows:

"However, features such as the emergence of social hierarchies, shifts in subsistence patterns, and significant technological developments commonly seen in other periods were less radical in Jōmon society."

6. *Page 7, lines 3 and 5. "Middle" and "late" phases should be capitalized as Middle and Late.*

Response: We capitalized them.

7. *Page 14, line 23. Please check the site of "Ebishima," which is correct? Is this indicated to Nakazuma?*

Response: We think "Ebishima" is correct, which is shown in the upper left of Figure 8.

8. *Page 15, line 1. Please describe how to make Figure 9.*

Response: We added explanations as follows:

"Figure 9 shows a visual representation of the configurational changes in landmarks for each comparison. The figure was constructed by the *plotRefToTarget* function in the *geomorph*

package of R. Straight bars indicated the degree of variation in each landmark."

9. *Page 16, lines 3 - 5. The suggestion of "the morphological and genetic diversity among the Jōmon populations was not relatively limited, but homogeneous across regions and phases" is not understandable.*

Response: Sorry for the vague description. We deleted the latter description to make the description more straightforward as follows:

"It suggests that the morphological and genetic diversity among the Jōmon populations were not relatively limited."

10. *Page 16, lines 10 - 11. The expression is not understandable: "The reason why fewer evidence of such a societal changes were found in the Jōmon period is possibly wide and continuous population interactions."*

Response: We have clarified the description as follows:

"The reason why less evidence of societal changes such as the occurrence of warfare and emergence of social hierarchies was found in the Jōmon period is possibly because the Jōmon populations interacted widely and continuously and frequently exchanged knowledge, skills, and resources against environmental disruptions."

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Abbreviations

PC: principal component

Edited by A Schwartz; submitted 09.10.25; this is a non–peer-reviewed article;accepted 09.10.25; published 12.11.25.

Please cite as:
Nakao H, Kaneda A, Tamura K, Noshita K, Yoshida M, Nakagawa T
Authors' Response to Peer Reviews of "Population Interaction in the Jōmon Society via 3D Data of Human Crania: Geometric Morphometric Study"
JMIRx Bio 2025;3:e85565
URL: https://bio.jmirx.org/2025/1/e85565
doi:10.2196/85565

Authors' Response to Peer Reviews

Authors' Response to Peer Reviews of "Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study"

Markie Peroutka[1]; Ignacio Rivero Covelo[1], PhD

Department of Psychology, Professional Counseling, and Neuroscience, University of Wisconsin–Parkside, Kenosha, WI, United States

Corresponding Author:
Ignacio Rivero Covelo, PhD
Department of Psychology, Professional Counseling, and Neuroscience
University of Wisconsin–Parkside
900 Wood Rd
Kenosha, WI, 53144
United States
Phone: 1 8478680045
Email: riveroco@uwp.edu

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Companion article: https://bio.jmirx.org/2025/1/e68519/

(JMIRx Bio 2025;3:e71629) doi:10.2196/71629

KEYWORDS

ventral pallidum; nucleus accumbens shell; chemogenetics; sucrose; feeding behavior; food motivation; palatable food; DREADD; designer receptors exclusively activated by designer drugs

This is the authors' response to peer-review reports for "Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study."

Round 1 Review

Reviewer C [1]

General Comments

In this paper [2], the authors present an interesting and well-written paper dealing with the effects of stimulation and inhibition of projections from the ventral pallidum to the nucleus accumbens shell on feeding and food reinforced behaviors. The methods used are cutting edge, and my comments and suggestions are relatively minor.

Minor Comments

1. In the third paragraph of the Introduction, the sentence beginning with "Parallelly" is very awkward; I am sure there is a way to word this that does not use "parallelly." Also, the previous sentence could be made clearer as to whether effects on sucrose consumption are found just in female rats.

Response: The paragraph has been reworded for clarity and to minimize its possible awkwardness. Moreover, we believe the current phrasing emphasizes that the results were observed only in female rats.

2. The number of subjects should be listed in the Methods.

Response: In the original manuscript, the number of subjects was listed in the Methods section under the subsection "Immunohistochemistry." The authors recognize that this is an

unorthodox location for that kind of information, and now, the number of subjects can be found in the "Subjects" subsection.

> *3. In the last paragraph of the body of the manuscript, the sentence beginning with "The discrepancies observed across studies of this pathway..." is unfinished, and I am uncertain what the authors intended to say.*

Response: The offending sentence has been removed from the paragraph. The authors would like to thank the reviewer for the careful reading of the manuscript.

> *4. In discussing the differences between the results observed here and those reported by Vanchez et al [3], is it possible that these may reflect the use of "closed-loop" manipulations linked to the occurrence of licking in the Vanchez et al [3] paper, in contrast to the continuous modulation produced here by the use of the DREADD (designer receptors exclusively activated by designer drugs) technique? Also, in this section, the authors could be a bit clearer as to why the techniques used by Vanchez et al [3] would be expected to label a different subpopulation of cells than was the case in this study.*

Response: This paragraph has been expanded in an attempt to address Reviewer C's comments. The authors believe that the current version of the manuscript offers a more nuanced discussion of our findings and those of Vachez et al [3].

Reviewer Q [4]

General Comments

> *The manuscript from Peroutka and Covelo [1] describes the results of chemogenic activation or inhibition of the ventral pallidum–nucleus accumbens shell pathway in adult female rats on sucrose intake (20% sucrose bottle access) versus operant response–provided food pellets delivered on a progressive ratio schedule. The rats were not food restricted. Activation of the pathway decreased sucrose intake while inactivation of the pathway increased sucrose intake. Activation or inactivation did not clearly alter responding for food pellets. The authors provide discussion including an interpretation of the results, such that this pathway is important for sucrose consumption but not motivation for food. This is an interesting study that has some limitations listed below.*

Specific Comments

Major Comments

> *1. Why were only female rats used for this study?*

Response: Historically, much of behavioral neuroscience research has focused primarily on males, leading to a lack of understanding of female brain function. While this study could have been conducted in male rats, we decided to use female rats to generate more information about the female rat brain. The authors acknowledge that future studies should consider studying male rats to observe if sex is a relevant variable in the observed behaviors.

> *2. What was the approximate age of the rats at the start of the study?*

Response: The age of the rats at the start of the study has been added to the Methods section.

> *3. The conclusion of the pathway being relevant for sucrose consumption but not food motivation is reasonable, but it would be stronger if the comparisons were made with sucrose consumption versus sucrose motivation and also food consumption versus food motivation.*

Response: This study only uses sucrose as a reward, either in the form of sucrose pellets in the case of the progressive ration task, or 20% sucrose solution in the case of the free-access task. The authors recognize that the use of the term "food" throughout the manuscript might have contributed to some confusion as to the nature of the reward used. In this version, we have minimized the generic use of the word "food" and specified that sucrose was used all along. The authors still believe that the chemogenetic manipulations described in the manuscript affected sucrose consumption but not the motivation to work for food.

Minor Comments

> *4. Are there more objective data from analysis of the immunohistochemistry? What is presented are representative images, but was there any quantification done?*

Response: As described in the Methods, immunohistochemistry was studied qualitatively to assess DREADD (designer receptors exclusively activated by designer drugs) expression in the relevant brain areas. The authors consider this analysis to be sufficient to support the conclusions presented in the manuscript. Future studies could be conducted to assess if the number of DREADD-expressing neurons affects the behavioral outcomes observed, although such studies would require a significantly higher number of animals than those used here.

> *5. The authors discuss cell types but do not specify the likely type of neurons stimulated in this study; is it possible to do so?*

Response: The question of the nature of the cells expressing DREADD is interesting and worth studying in the future. Unfortunately, at this time, it is not logistically possible for the authors to conduct such studies.

Round 2 Review

Reviewer Q [4]

General Comments

> *The authors have addressed my concerns from the initial draft.*

References

1. Wirtshafter D. Peer review of "Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study". JMIRx Bio 2025;3(1):e71626 [FREE Full text] [doi: 10.2196/71626]
2. Peroutka M, Rivero Covelo I. Effects of ventral pallidum–nucleus accumbens shell neural pathway modulation on sucrose consumption and motivation in female rats: chemogenetic manipulation study. JMIRx Bio 2025;3(1):e68519 [FREE Full text] [doi: 10.2196/68519]
3. Vachez YM, Tooley JR, Abiraman K, Matikainen-Ankney B, Casey E, Earnest T, et al. Ventral arkypallidal neurons inhibit accumbal firing to promote reward consumption. Nat Neurosci 2021 Mar;24(3):379-390 [FREE Full text] [doi: 10.1038/s41593-020-00772-7] [Medline: 33495635]
4. Grimm J. Peer review of "Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study". JMIRx Bio 2025;3(1):e71627 [FREE Full text] [doi: 10.2196/71627]

Abbreviations

DREADD: designer receptors exclusively activated by designer drugs

Edited by O Singh; submitted 22.01.25; this is a non–peer-reviewed article;accepted 22.01.25; published 08.03.25.

Please cite as:
Peroutka M, Rivero Covelo I
Authors' Response to Peer Reviews of "Effects of Ventral Pallidum–Nucleus Accumbens Shell Neural Pathway Modulation on Sucrose Consumption and Motivation in Female Rats: Chemogenetic Manipulation Study"
JMIRx Bio 2025;3:e71629
URL: https://bio.jmirx.org/2025/1/e71629
doi:10.2196/71629
PMID:

Author's Response to Peer Reviews

Author's Response to Peer Reviews of "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study"

Ebrahim Abbasi[1], PhD

Department of Medical Entomology and Vector Control, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran

Corresponding Author:
Ebrahim Abbasi, PhD
Department of Medical Entomology and Vector Control
School of Health
Shiraz University of Medical Sciences
Zand Street
JGHF+XFG
Shiraz, 3761833650
Iran
Phone: 98 9124338389
Fax: 98 9124338389
Email: abbasie.ebrahim@gmail.com

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Companion article: https://bio.jmirx.org/2025/1/e69542/

(JMIRx Bio 2025;3:e72764) doi:10.2196/72764

KEYWORDS

impact of climate; seasonal change; frequency; livestock; ticks; Tehran

Author's response to peer reviews for "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study."

Round 1 Review

Reviewer W [1]

General Comments

> *This paper provides a detailed investigation into the distribution and frequency of tick species infecting livestock and poultry in Tehran province, Iran, with a focus on seasonal and climatic variation. The study highlights the significant economic and epidemiological impact of ticks as ectoparasites and pathogen vectors in livestock.*

Response: We sincerely appreciate the time and effort you have taken to review our manuscript [2]. Your feedback is valuable, and we have addressed all of your comments as outlined below.

Specific Comments

Major Comments

> *1. "Material and methods - Sampling": For the tick sampling in this manuscript, what method did you used in this study (ie, how did you decide which tick samples to include and which samples to exclude)? How did you ensure that the sample is representative of the true distribution of ticks in the study area? The distribution and frequency estimates from this sample might not be extended to the whole tick population if the sampling is biased.*

Response: In this study, tick samples were collected from 1623 livestock animals (including chickens, camels, cows, pigeons, dogs, and sheep) infected with ticks. The sampling was carried

out using a cross-sectional study design in two different climate regions: mountainous and plain. Livestock were randomly selected based on visible tick infestation, ensuring that the sample represented the true distribution of ticks in the study area, particularly with the help of local veterinary authorities.

Text to be added to the Sampling section of the Methods: "To ensure representative sampling, a cross-sectional study was conducted, covering both mountainous and plain regions. Selection of livestock was randomized among those showing visible tick infestation, with veterinary supervision ensuring consistency in sample collection across different geographical zones. The chosen method aligns with established epidemiological studies on tick distribution."

2. "Material and methods - Sampling": Could the author provide the rationale or justification of the choice of "p" and "d" in the sample size calculation?

Response: The parameter p=0.3 was selected based on previous studies on tick prevalence in similar regions of Iran, where an approximate prevalence rate of 30% was observed. The margin of error (d=0.045) was chosen to ensure a 95% confidence level while maintaining a practical sample size for data collection.

Text to be added to the manuscript: "The parameter *P* (0.3) was selected based on prior studies on tick prevalence in similar regions in Iran, indicating an estimated infestation rate of 30%. The margin of error (d = 0.045) was determined considering a 95% confidence level, ensuring a balance between precision and feasibility of sample collection."

3. "Discussion": I really like the analysis of seasonal trend presented in Table 4 and Figure 4. Could the author elaborate more on this in the Discussion—the general seasonal trend across all species, the reason why you expect some species to be more abundant in warmer versus colder weather, and the implications from the public health perspective?

Response: The analysis revealed that hard ticks like *Rhipicephalus sanguineus* and *Hyalomma marginatum* were more abundant during the spring and summer, likely due to higher temperatures and increased activity of their hosts. In contrast, soft ticks such as *Argas persicus* were more prevalent in the autumn and winter, as they can survive in sheltered environments and colder conditions.

Text to be added to the Discussion section: "The observed seasonal trend aligns with the biological cycles of tick species. Hard ticks such as *Rhipicephalus sanguineus* and *Hyalomma marginatum* exhibited peak abundance in spring and summer due to higher temperatures and increased host activity. Conversely, soft ticks (*Argas persicus*) showed resilience during colder months, likely due to their ability to survive in sheltered environments. This seasonal variability highlights the importance of targeted tick control strategies, particularly in warm seasons when transmission risk of tick-borne diseases is highest."

Minor Comments

1. Line 41: Spell out "$."

2. Line 44: Remove "(4)"—duplicated reference number.

3. Line 51: Remove "(9)"—duplicated reference number.

4. Line 85, "valid diagnostic keys": Could the author be more specific about the "diagnostic key" being used? Adding a sentence to briefly describe the key would be great.

Response: To clarify the "valid diagnostic key" used for tick identification, we will specify the exact key used for species identification and provide a brief description of its methodology.

Text to be added to the manuscript (Line 85 of Methods): "The tick species were identified using the diagnostic keys outlined by Jongejan et al. (1987) [3] and Camicas et al. (1998) [4], which provide detailed morphological descriptions and illustrations for the identification of both soft and hard ticks. These keys are widely recognized for their accuracy and reliability in the identification of tick species in the Middle East and neighboring regions."

5. Lines 147-151, "two professional stereo microscopes...in the entomological research": This part should belong to Methods section.

Reviewer AX [5]

General Comments

The manuscript [1] presents a comprehensive study on the seasonal and climatic distribution of ticks in Tehran province, Iran. The research is well structured and provides valuable insights into the diversity and abundance of tick species in different climate zones. The study is relevant to the field of veterinary parasitology and has potential implications for tick control strategies in the region. However, there are several areas where the manuscript could be improved in terms of grammar, sentence structure, and adherence to standard academic writing conventions.

Specific Comments

Major Comments

Grammar and Sentence Structure

Overall clarity: The manuscript is generally clear, but there are instances where the sentence structure could be improved for better readability. Some sentences are overly long and could be broken down into shorter, more concise statements.

For example:

- *Original: "The presence of ticks on livestock causes localized lesions at the bite site and systemic lesions, leading to death due to anemia and paralysis due to ticks transmitting various diseases such as theileriosis and babesiosis."*
- *Suggested revision: "The presence of ticks on livestock causes localized lesions at the bite site and systemic effects, which can lead to anemia, paralysis, and even death. Ticks*

are also vectors for diseases such as theileriosis and babesiosis."

Subject-verb agreement: There are a few instances where the subject-verb agreement is incorrect.

For example:

- *Original: "The distribution of collected ticks (in mountain and plain climates) indicated that out of 806 collected ticks, 44.78% and 55.21% belonged to the mountainous and plain regions, respectively."*
- *Suggested revision: "The distribution of collected ticks (in mountainous and plain climates) indicates that out of 806 collected ticks, 44.78% belonged to mountainous regions, while 55.21% were found in plain regions."*

Tense consistency: The manuscript occasionally shifts between past and present tense. It is important to maintain consistency, especially in the Results and Discussion sections.

For example:

- *Original: "The study is conducted in two different environments: plains and mountains within 20 selected villages in Tehran Province."*
- *Suggested revision: "The study was conducted in two different environments: plains and mountains within 20 selected villages in Tehran Province."*

Response: We agree, and several sentences will be broken down for better clarity and conciseness. Below is an example of a revised sentence:

- Original: "The presence of ticks on livestock causes localized lesions at the bite site and systemic lesions, leading to death due to anemia and paralysis due to ticks transmitting various diseases such as theileriosis and babesiosis."
- Revised: "Ticks on livestock cause localized bite-site lesions and systemic effects. They can lead to anemia, paralysis, and even death by transmitting diseases like theileriosis and babesiosis."

Structure and Organization

Abstract: The Abstract is well written and provides a concise summary of the study. However, it could benefit from a brief mention of the key findings related to seasonal variations, as this is a major focus of the study.

Response: Yes, the Abstract will be revised to include more specific quantitative data, such as sample size, species abundance, and seasonal variations.

Revised Abstract (Results section): "Results showed that out of 806 collected ticks, 44.78% were found in mountainous regions and 55.21% in plain regions. The most abundant species was *Rhipicephalus sanguineus* (36.97%), while *Rhipicephalus (Boophilus) annulatus* was the least common (0.37%). Seasonal variation indicated peak infestation in spring (60.3%) and lowest in winter (9.5%)."

Introduction: The Introduction provides a good background on the importance of ticks and their impact on livestock. However, it could be strengthened by including more recent references (post-2020) to highlight the current state of research on tick-borne diseases and climate change.

Methods: The Methods section is detailed and well organized. However, the formula used for sample size calculation is not clearly explained. It would be helpful to provide a brief explanation of the variables used in the formula (eg, p=0.3 and d=0.045).

Response: A brief explanation of the sample size calculation formula will be added to the Methods section for clarity.

Text to be added to the Sample Size Calculation section of the Methods: "The sample size was calculated using Cochran's formula for prevalence studies. Given an estimated prevalence (*p*) of 30% and a precision (*d*) of 4.5%, the final sample size was determined to be 800 ticks, ensuring statistical reliability."

Results: The Results are presented clearly, with appropriate use of tables and figures. However, some of the tables could be simplified for better readability. For example, Table 4 could be restructured to make it easier to compare seasonal activity across species.

Discussion: The Discussion is thorough and compares the findings with other studies effectively. However, it could be improved by discussing the limitations of the study and suggesting areas for future research.

Similarity and Plagiarism

The manuscript appears to be original, with no significant issues of plagiarism detected. However, it is recommended to run the manuscript through a plagiarism detection tool (eg, Turnitin) to ensure that all sources are properly cited and that there is no unintentional duplication of text.

Adherence to Standard Academic Writing

References: The references are generally appropriate and relevant to the study. However, some references are quite old (eg, references from the 1980s and 1990s). It is recommended to include more recent studies to reflect the current state of knowledge in the field.

Add these references to the manuscript:

1. *Noaman V. Identification of hard ticks collected from sheep naturally infected with Anaplasma ovis in Isfahan province, central Iran. Comp Clin Pathol 2012 Feb 21; 21(3):367-369. [doi: 10.1007/s00580-012-1438-1]*
2. *Noaman V, Abdigoudarzi M, Nabinejad AR. Abundance, diversity and seasonal dynamics of hard ticks infesting cattle in Isfahan province, central Iran. Archives of Razi Institute. 2017 Mar 1;72(1):15-21. [doi: 10.22034/ari.2016.107490]*
3. *Noaman V, Abdigoudarzi M, Nabinejad AR, Heidari MR, Khalilifard M. (2007). Identification of hard ticks of domestic ruminants in two ecological zones of Isfahan province, Iran. Veterinary Journal (Pajouhesh va Sazandegi). 2008;77:88-95.*

Response: The following recent references will be added to the manuscript:

1. Noaman V. Identification of hard ticks collected from sheep naturally infected with Anaplasma ovis in Isfahan province, central Iran. Comp Clin Pathol 2012 Feb 21; 21(3):367-369. [doi: 10.1007/s00580-012-1438-1]
2. Noaman V, Abdigoudarzi M, Nabinejad AR. Abundance, diversity and seasonal dynamics of hard ticks infesting cattle in Isfahan province, central Iran. Archives of Razi Institute. 2017 Mar 1;72(1):15-21. [doi: 10.22034/ari.2016.107490]
3. Noaman V, Abdigoudarzi M, Nabinejad AR, Heidari MR, Khalilifard M. (2007). Identification of hard ticks of domestic ruminants in two ecological zones of Isfahan province, Iran. Veterinary Journal (Pajouhesh va Sazandegi). 2008;77:88-95.

Text to be added to the Discussion section: "Our findings align with previous studies on tick diversity in central Iran (Noaman et al., 2012; Noaman et al., 2017), confirming seasonal variations in tick populations. These studies further support the need for region-specific tick control strategies."

> *Figures and tables: The figures and tables are well presented and support the findings of the study. However, the legends for some figures (eg, Figure 1>) could be more descriptive. For example, Figure 1 could include a brief explanation of what the "ratio of caught ticks" represents.*
>
> *The manuscript presents a valuable contribution to the field of veterinary parasitology, particularly in the context of tick distribution and seasonal activity in Tehran province. With some revisions to improve grammar, sentence structure, and adherence to standard academic writing conventions, the manuscript will be suitable for publication in a reputable journal. The manuscript can be considered for publication in JMIRx Bio after major revision.*

Response: We trust that these revisions adequately address the reviewer's concerns. Please let us know if any further modifications are required. We look forward to your feedback on the revised manuscript.

Round 2 Review

Reviewer W

> *Thank you, author, for addressing all my comments and making all necessary changes to the manuscript. I do not have any more comments.*

Reviewer AX

General Comments

> *The revised manuscript titled "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran" has addressed the previous comments and suggestions effectively. The authors have made the necessary revisions to improve the clarity, structure, and overall quality of the manuscript. Below are my final comments.*

Strengths

> *Improved clarity: The Abstract has been revised to be more concise and now includes key findings related to seasonal variation and the most abundant tick species, enhancing readability and impact.*
>
> *Focused introduction: The Introduction now more clearly highlights the specific gaps in the literature that this study addresses, particularly in the context of Tehran province.*
>
> *Streamlined methodology: The Methods section has been clarified, with more details on the randomization process and a more concise description of the study area. The inclusion of supplementary tables for geographical coordinates and ecological information is a welcomed addition.*
>
> *Organized results: The Results section has been streamlined with the use of subheadings, making it easier to follow. All referenced figures and tables are now included, providing a comprehensive view of the findings.*
>
> *Enhanced discussion: The Discussion section now more effectively focuses on the implications of the findings for tick control strategies in Tehran province. The comparison with studies from other regions has been made more concise, emphasizing key similarities and differences.*
>
> *Practical conclusion: The Conclusion has been revised to highlight the practical implications of the findings, particularly the need for seasonal tick control measures in different climate zones.*
>
> *Consistent references: All references are now formatted consistently according to the journal's guidelines, with complete details provided.*
>
> *Overall recommendation: The manuscript has been significantly improved and is now suitable for publication. I recommend acceptance of the manuscript in its current form.*

Conflicts of Interest

None declared.

References

1. Shen J. Peer review of "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study". JMIRx Bio 2025;3:e72765. [doi: 10.2196/72765]

2. Abbasi E. Assessing the influence of seasonal and climatic variations on livestock tick incidence in Tehran province, Iran: cross-sectional study. JMIRx Bio 2025;3:e69542. [doi: 10.2196/69542]
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5. Noaman V. Peer review of "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study". JMIRx Bio 2025;3:e72764. [doi: 10.2196/72766]

Edited by J Ren; submitted 17.02.25; this is a non–peer-reviewed article;accepted 17.02.25; published 31.03.25.

Please cite as:
Abbasi E
Author's Response to Peer Reviews of "Assessing the Influence of Seasonal and Climatic Variations on Livestock Tick Incidence in Tehran Province, Iran: Cross-Sectional Study"
JMIRx Bio 2025;3:e72764
URL: https://bio.jmirx.org/2025/1/e72764
doi:10.2196/72764
PMID:

Discovery of Novel Inhibitors of HMG-CoA Reductase Using Bioactive Compounds Isolated From Cochlospermum Species Through Computational Methods: Virtual Screening and Algorithm Validation Study

Toba Isaac Olatoye[1,2], BSc, MSc

[1]Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Kwara State, Ilorin, Nigeria

[2]Schools and Colleges, Kwara State Teaching Service Commission, Ilorin, Nigeria

Corresponding Author:
Toba Isaac Olatoye, BSc, MSc
Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Kwara State, Ilorin, Nigeria

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Abstract

Background: Cholesterol biosynthesis is a critical pathway in cellular metabolism, with 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMGR) catalyzing its committed step. HMGR inhibition has been widely explored as a therapeutic target for managing hypercholesterolemia, and statins are the most commonly used competitive inhibitors. However, the search for novel, natural HMGR inhibitors remains a vital area of research, due to the adverse effects associated with long-term statin use. *Cochlospermum planchonii* and *Cochlospermum tinctorium* are West African medicinal plants traditionally used to treat metabolic disorders, including dyslipidemia. Despite their usefulness, the specific bioactive compounds responsible for these effects are currently poorly characterized, justifying further investigations.

Objective: This study investigates the potential of phytochemicals from *Cochlospermum planchonii* and *Cochlospermum tinctorium* as natural inhibitors of human HMGR using molecular docking techniques.

Methods: A total of 84 phytochemicals from 2 species of *Cochlospermum* as reported in literature, were evaluated as potential inhibitors of HMGR. Using DataWarrior software, their drug-likeness and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties were screened in accordance with Lipinski's Rule of Five. The 32 compounds that met the criteria were docked on PyRx against the HMG-binding site of HMGR, alongside atorvastatin (native ligand) and 6 known statins, which served as control ligands.

Results: Docking analysis of their two best binding modes showed that 10 (31.3%) out of the 32 screened phytochemicals demonstrated strong binding affinities and interactions with the HMG-binding pocket (residues 682 - 694) of HMGR, with binding energy (ΔG) scores ranging from −4.6 to −6.0 kcal/mol, comparable to or exceeding those of statins (−4.6 to −5.7 kcal/mol). Their docking scores (−13.272 to −32.103) also compared favorably with those of statins (−25.939 to −36.584). Interestingly, 3-O-methylellagic acid (ID_13915428) demonstrated the strongest interaction, forming 26 binding interactions with the HMG-binding pocket residues, more than any compound, including statins. One-way ANOVA of the mean and SEM of the binding affinity scores for the phytochemicals and statins (9 replicates each) indicated a statistically significant difference at $P<.05$ (total sample size n=153; actual $P=.0001$).

Conclusions: This study is the first to virtually screen and identify specific bioactive compounds isolated from *Cochlospermum planchonii* and *Cochlospermum tinctorium* with potential cholesterol-lowering effects in humans. The findings not only support the traditional use of these plants in West Africa to manage dyslipidemia and other ailments, but also present the phytochemicals as promising drug candidates for further optimization as natural inhibitors of HMGR. However, while this study provides valuable computational insights into the molecular interactions of the compounds with HMGR, further advanced computational, in vitro, and in vivo studies are still necessary to validate their inhibitory potential and therapeutic applications.

(JMIRx Bio 2025;3:e71675) doi:10.2196/71675

KEYWORDS

HMGR; statins; hypercholesterolemia; cochlospermum; phytochemicals; molecular docking; 3-hydroxy-3-methylglutaryl coenzyme-A reductase

Introduction

Cholesterol is a vital component of cellular membranes and serves as a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D. However, elevated levels of cholesterol, especially low-density lipoprotein (LDL) cholesterol, are strongly associated with the development of atherosclerosis and cardiovascular diseases (CVDs), which are among the leading causes of morbidity and mortality worldwide [1]. Although lifestyle changes in individuals such as exercise, healthy diets, and drug therapies particularly statins, have been touted as effective in the prevention and management of hypercholesterolemia including its attendant cardiovascular complications [1,2]. Nevertheless, the challenge is yet far from over, as these conditions still remain major global concerns, especially in high-income countries like the United States, where about 48% of adults are currently affected [3].

HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase (HMGR), the rate-limiting enzyme in the mevalonate pathway, catalyzes the 4-electron reductive deacylation of HMG-CoA to mevalonate, a crucial precursor of cholesterol biosynthesis in human [4]. Statins, a class of synthetic drugs with inhibition constant (Ki) values in the nanomolar range, are competitive inhibitors of HMGR widely used to lower serum cholesterol levels in human [5]. These drugs occupy the catalytic portion of the enzyme where the substrate, HMG-CoA, binds, thus blocking its access to the active site (Figure 1). Near the carboxyl terminus of human HMGR, several catalytically relevant amino acid residues representing the HMG-binding pocket are disordered in the enzyme-statin complex. If these residues were not flexible, they would sterically hinder the binding of statins [5]. All statins have an HMG-like moiety, with rigid, hydrophobic groups that are covalently linked to them and may be present in inactive lactone form. In vivo, these drugs are enzymatically hydrolyzed to their active hydroxy-acid forms [6]. In addition to lowering cholesterol, statins seem to have other functions, including the nitric oxide–mediated promotion of the growth of new blood vessels [7], stimulation of bone formation [8], protection against oxidative modification of LDL, and anti-inflammatory effects with a reduction in C-reactive protein levels [9]. Nevertheless, the use of statins is often limited by their side effects such as myopathy, liver and kidney dysfunction, and an increased risk of diabetes [10-12]. These limitations have necessitated the search for alternative cholesterol-lowering agents, especially those from natural sources, which may offer safer and more effective therapeutic needs.

Figure 1. (A) Active site of human 3-hydroxy-3-methylglutaryl coenzyme-A reductase in complex with 3-hydroxyl-3-methylglutaric acid (HMG), coenzyme A (CoA), and nicotinamide adenine dinucleotide phosphate ($NADP^+$). (B) Binding of rosuvastatin to 3-hydroxy-3-methylglutaryl coenzyme-A reductase [5]. A: Ala; C: Cys; D: Asp; E: Glu; F: Phe; G: Gly; H: His; I: Ile; K: Lys; L: Leu; M: Met; N: Asn; P: Pro; Q: Gln; R: Arg; S: Ser; T: Thr; V: Val; W: Trp; Y: Tyr. Adapted from Istvan and Deisenhofer [5], with permission from International Union of Crystallography.

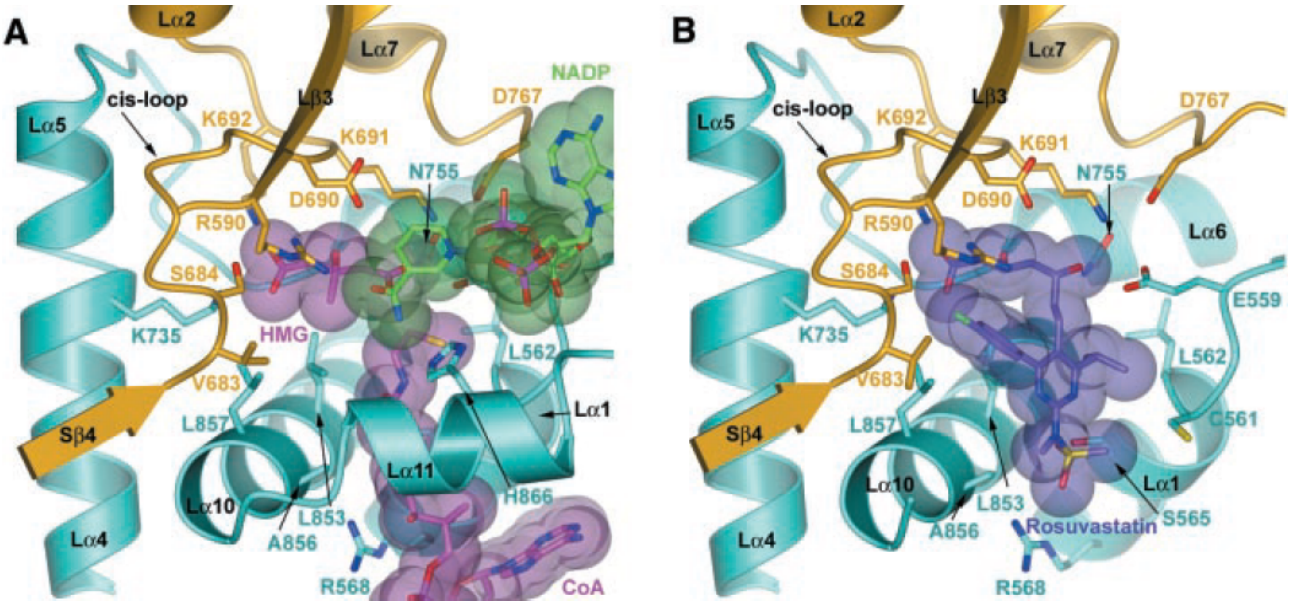
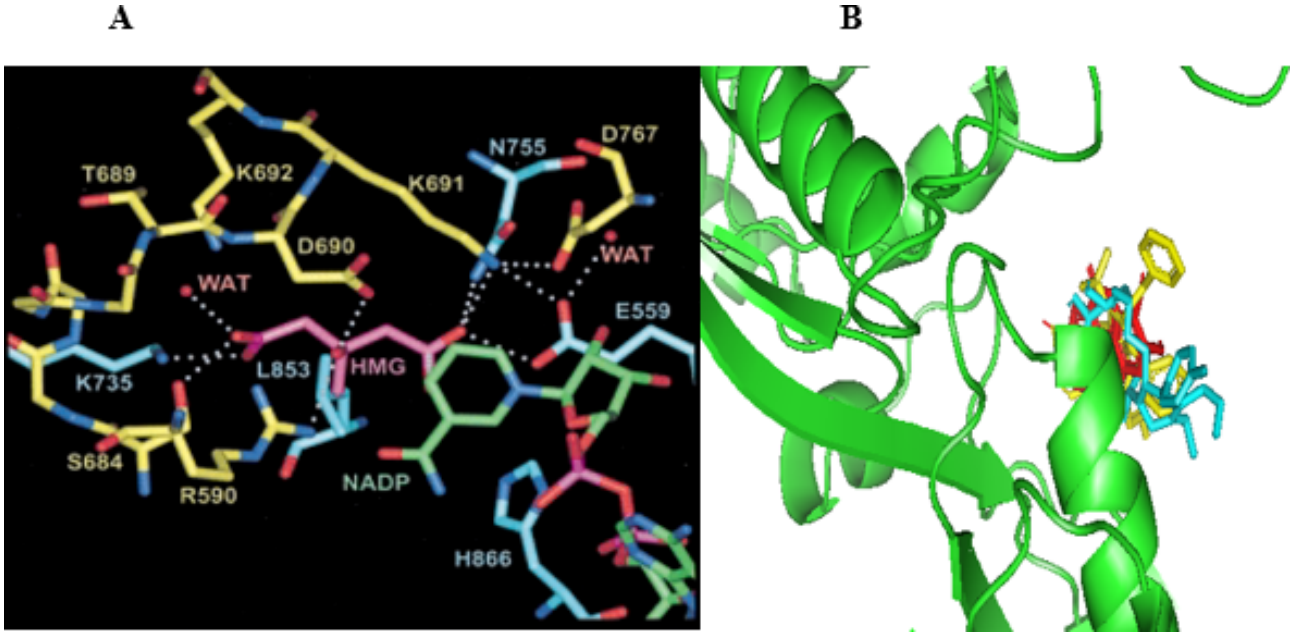


Figure 2. (A) Single-letter abbreviations of residues involved in HMG-binding based on the crystal structure of human 3-hydroxy-3-methylglutaryl coenzyme-A reductase [4]. (B) 3D representation showing the binding modes of cocrystallized atorvastatin (yellow), cerivastatin (cyan), and 3-O-methylellagic acid (red), at the HMG-binding site of human 3-hydroxy-3-methylglutaryl coenzyme-A reductase. A: Ala; C: Cys; D: Asp; E: Glu; F: Phe; G: Gly; H: His; I: Ile; K: Lys; L: Leu; M: Met; N: Asn; P: Pro; Q: Gln; R: Arg; S: Ser; T: Thr; V: Val; W: Trp; Y: Tyr. Adapted from Ensouf [13] and Istvan et al [4], with permission from The American Association for the Advancement of Science.



The 3D crystal structure of human HMGR (Protein Data Bank [PDB] ID: 1HWK) is a tetramer (subunits A: PRO442–HIS861; B: SER463–GLY860; C: LEU462–GLY860; D: SER463–GLY860) that contains the catalytic domains of HMGR in complex with 4 atorvastatin molecules at the interface of 2 adjacent monomers [5,14]. Structurally, these domains are divided into three subdomains: an "N-domain" (residues 460 - 527) connecting the catalytic portion of the enzyme to the membrane domain; a large "L-domain" (residues 528 - 590 and 694 - 861); and a small "S-domain" (residues 592 - 682). In the monomer, the amino acid residues of the L- and S-domains form the 2 active sites: (1) the HMG-binding pocket characterized by a narrow cis-loop (residues 682 - 694) and formed between the S- and L-domains; and (2) the nicotinamide adenine dinucleotide phosphate (NADPH)-binding site (containing residues 592 - 682 of the S-domain), which is also inserted into the L-domain (Figures 1 and 2) [4,5]. As all statins share HMG-like moieties, which enable them to compete with HMG-CoA by sterically preventing its binding at the cis-loop, then it is imperative to computationally explore this mode of binding and mechanism of inhibition, in order to determine whether the phytochemicals of interest (sharing similar polar side groups as the HMG-like moieties of statins but with much less hydrophobic rings), will exhibit similar binding interactions at this narrow binding site.

Cochlospermum planchonii (*C planchonii*) and *Cochlospermum tinctorium* (*C tinctorium*), 2 species of *Cochlospermum*, are plants extensively used in West African herbal medicine to manage several ailments [15-19]. They are known for their rich phytochemical constituents such as tannins, saponins, carotenoids, triterpenoids, flavonoids, and other polyphenolic compounds, which exhibit various pharmacological activities including antimalarial, antidiabetic, antioxidant, anti-inflammatory, antimicrobial, and enzyme-inhibitory properties [16,20-27]. Other studies have also demonstrated the antihyperlipidemic and cholesterol-lowering potential of their extracts (root, rhizomes, and leaf) [17,28], suggesting they contain bioactive compounds capable of managing lipid disorders. As far as the literature is concerned, no specific compounds isolated from *C planchonii* and *C tinctorium* have been directly studied or linked as potential inhibitors of HMGR. However, their phytochemicals, mostly reported to possess antioxidant, antimicrobial, anti-inflammatory, and enzyme-inhibitory activities, are thought to be significant contributors to the plants' lipid-lowering ability [16,17,28,29].

The aim of this study is to explore the efficacy of these phytochemicals as potential inhibitors of human HMGR and as promising alternatives to statins, using molecular docking tools. Molecular docking is a computational technique used to predict the preferred orientation of a small molecule (ligand) when it binds to a target protein (enzyme), allowing researchers to assess the binding mode and affinity, as well as the chemical interactions between the ligand and the enzyme in a complex [30]. Therefore, adopting this approach helps in evaluating the mechanism of phytochemical interactions with HMGR, their mode of binding and affinity, their fitness at the active site, as well as the stability of the HMGR-phytochemical complexes formed, in a context that is relevant to HMGR inhibition.

Methods

Phytochemical Selection

The selection of phytochemicals for this study was guided by a comprehensive review of existing literature. A Google Scholar search was conducted to identify peer-reviewed articles reporting on the phytochemical constituents and pharmacological activities of various extracts from *C planchonii* and *C tinctorium*. This search was conducted using key terms including

"phytochemicals from *C. planchonii*," "phytochemicals from *C. tinctorium*," "HPLC analysis of *C. planchonii* and *C. tinctorium*," "GC-MS analysis of *C. planchonii* and *C. tinctorium*," and "bioactive compounds of *C. planchonii* and *C. tinctorium*." Articles were included if they (1) reported the use of high-performance liquid chromatography (HPLC) or gas chromatography mass-spectrometry (GC-MS) techniques in the phytochemical profiling of *C planchonii* and *C tinctorium*; (2) provided compounds with identifiable names; and (3) described pharmacological activities relevant to hypercholesterolemia or metabolic disorders. In total, 16 articles were evaluated, of which 4 met the inclusion criteria and provided the sufficient details used in the identification of the compounds [15-17,31]. A total of 84 phytochemicals were compiled, with 32 from *C planchonii* and 52 from *C tinctorium* (Tables S1-S3 in Multimedia Appendix 1). The selected compounds were included for this computational study based on the following criteria: (1) reported bioactivities, (2) structural integrity, (3) acceptable molecular weight for molecular docking, and (4) availability of their 2D structure data in the PubChem database. Their 2D structures were retrieved in structure data file (SDF) format from PubChem database on August 5, 2024 [32], and subsequently concatenated using Open Babel software [33], before being used for virtual screening.

Virtual Screening

DataWarrior is excellent for managing and screening large libraries of compounds based on their chemical properties [34,35]. The software was used to narrow down the large pool of 84 potential drug candidates, ensuring that only the most promising ones make it to the docking step. This approach helps save computational resources and time by focusing on most viable candidates. The phytocompounds were subjected to virtual screening to determine their drug-likeness and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties, in accordance with the Lipinski's Rule of Five. According to the Rule of Five, compounds are considered likely to be well absorbed into the systemic circulation when they possess octanol-water partition coefficient (CLogP) value ≤5, molecular weight ≤500, the number of hydrogen bond donors ≤5, the number of hydrogen bond acceptors ≤10, and the topological polar surface area ≤160 Å [36,37]. These properties were calculated for the concatenated compounds after importing them on DataWarrior using the "calculate compound properties from chemical structure" tab of the software. The 32 compounds meeting the criteria were selected and saved in SDF format. Other parameters screened for were mutagenicity, carcinogenicity, reproductive effectiveness, ligand efficiency, drug-likeness, and irritancy (Table 1). These parameters allow screening out compounds that do not meet the physicochemical criteria for drug-like behavior. The 2D structures of statins were also downloaded from PubChem database [32] and subjected to the same screening to serve as reference (Table 2).

Table . Drug-likeness and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of top-ranked phytochemicals of *Cochlospermum planchonii* and *Cochlospermum tinctorium*.

Serial number	Pubchem ID	Compound name	MW[a] (g/mol)	HA[b]	HD[c]	ClogP[d]	TPSA[e]	DL[f]	LE[g]	RE[h]	Mutagenic	Tumorigenic	Irritant
1	73641	Arjunolic acid	488.71	5	4	4.286	97.99	–5.555	0.162	None	None	None	None
2	12305768	Alphitolic acid	472.71	4	3	5.519	77.76	–21.780	0.077	None	None	None	None
3	5281855	Ellagic acid	302.19	8	4	1.277	133.52	–1.598	0.142	None	None	None	None
4	72277	Epigallocatechin	306.26	7	6	1.163	130.61	0.315	0.258	None	None	None	None
5	13915428	3-O-methylellagic acid	316.22	8	3	1.553	122.52	–1.390	0.111	None	None	None	None
6	5280417	3,7-di-O-methylquercetin	330.29	7	3	2.194	105.45	–0.105	0.130	None	None	None	None
7	44446550	3,4'-O-dimethylquercetin	332.31	7	3	1.662	105.45	0.503	0.077	None	None	None	None
8	9064	Catechin	290.27	6	5	1.509	110.38	0.315	0.329	None	None	None	None
9	535203	3-(Azepan-1-yl)-1,2-benzothiazole 1,1-dioxide	264.35	4	0	2.478	58.12	–1.176	0.249	None	None	None	None
10	10120274	(2E,4E,6E,8E,10E,12E)-13-[(1S)-1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl]-2,6,11-trimethyltrideca-2,4,6,8,10,12-hexaenoic acid	396.53	4	2	6.038	74.60	0.101	0.047	None	None	None	None

[a]MW: molecular weight.

[b]HA: hydrogen acceptor.

[c]HD: hydrogen donor.

[d]ClogP: Octanol-water partition coefficient.

[e]TPSA: topological polar surface area.

[f]DL: drug-likeness.

[g]LE: ligand efficiency.

[h]RE: reproductive effectiveness.

Table . Drug-likeness and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of statins.

Serial number	Pubchem ID	Compound name	MW[a] (g/mol)	HA[b]	HD[c]	ClogP[d]	TPSA[e]	DL[f]	LE[g]	RE[h]	Mutagenic	Tumorigenic	Irritant
1	Protein Data Bank ID: 117/obj01	Atorvastatin (cocrystallized control)	558.65	7	4	5.622	111.79	4.451	—[i]	High	None	None	None
2	60823	Atorvastatin	558.65	7	4	5.622	111.79	4.451	0.141	High	None	None	None
3	64715	Mevastatin (Compactin)	390.52	5	1	3.626	72.83	0.578	0.205	None	None	None	None
4	446155	Fluvastatin	411.47	5	3	3.978	82.69	1.804	0.153	High	None	None	None
5	446156	Cerivastatin	459.56	6	3	4.320	99.88	–0.283	0.139	None	None	None	None
6	446157	Rosuvastatin	481.54	9	3	2.100	149.3	3.454	0.139	None	None	None	None
7	54454	Simvastatin	418.57	5	1	4.461	72.83	0.668	0.195	None	None	None	None

[a]MW: molecular weight.

[b]HA: hydrogen acceptor.

[c]HD: hydrogen donor.

[d]ClogP: Octanol-water partition coefficient.

[e]TPSA: topological polar surface area.

[f]DL: drug-likeness.

[g]LE: ligand efficiency.

[h]RE: reproductive effectiveness.

[i]Not applicable.

As shown in Tables 1 and 2, the drug-likeness score is a crucial parameter used in determining whether a compound is likely to be an effective drug. A positive score indicates that a compound possesses structural features similar to known drugs, while a negative score suggests that such compound has structural features that are less common in known drugs. Good drug-like compounds usually have scores greater than zero [35,36]. Ligand efficiency (LE) is a metric used to evaluate the binding efficiency of compounds relative to their size. A higher LE score indicates that a compound achieves its binding affinity with fewer atoms making it more efficient, while a lower LE score suggests that a compound relies on a larger structure to achieve its binding, which might be less desirable [38]. When screening for toxic compounds, those that may bind to unintended off-target sites, which could lead to adverse effects such as genetic mutations, cancer development, or cause irritation to tissues like skin, eyes, or mucous membrane, were eliminated. Reproductive effectiveness parameter was used to predict the potential impact of a compound on reproductive health, including infertility and harm to fetal development [35].

Drug Target Preparation

The 3D crystal structure of human HMGR (PDB ID: 1HWK) complexed with atorvastatin (PDB ID: 117) was retrieved in PDB format August 5, 2024, from PDB [14]. The drug target was prepared by removing redundant subunits (B, C, D), Adenosine diphosphate, heteroatoms, and water molecules using PyMOL visualization tool [39]. The unique ligand atorvastatin (obj01/117), which served as one of the control ligands was extracted from the catalytic subunit A, in addition to the 6 other statins downloaded from PubChem. Both target and ligand were saved in PDB and SDF formats, respectively. Using PyMOL allows one to visualize and predict the grid co-ordinates around the HMG-binding pocket, while Discovery Studio visualizer [40] helps in identifying and characterizing the residues at the binding site.

Molecular Docking Analysis

PyRx virtual screening tool [41] was used for the molecular docking. The prepared drug target HMGR was loaded on PyRx in PDB format, hydrogen atoms were added to ensure the protein is correctly protonated and made as macromolecule, after which the screened phytochemicals were imported in SDF format. These compounds were subjected to energy minimization using the optimization algorithm tool of PyRx, and the required force field was set at “ghemical,” adjusting the positions of atoms in the phytochemicals in order to reduce their overall energy and steric clashes, thus attaining stable conformations. The

compounds were converted to PDBQT format for compatibility with the docking algorithm Autodock Vina. Docking was performed specifically at the HMG-binding pocket (residues 682 - 694) of the protein. The 3D docking grid box which encloses this region, where the compounds will bind was centered at co-ordinates (X: 22.2175, Y: –3.5559, Z: 5.8150) with grid box dimensions of 21.0454 × 28.2041 × 28.7731 Å, along the same axes, respectively. This type of docking is semirigid, where the structure of receptor (HMGR) remains rigid while the phytochemicals and statins have some degree of flexibility at the binding pocket. In the molecular docking, the PyRx AutoDock Vina Wizard exhaustive search docking function was used because of its balance between computational efficiency and accuracy. To ensure the feasibility of the study protocol and accuracy of the docking algorithm, 6 statins (atorvastatin, mevastatin/compactin, fluvastatin, cerivastatin, rosuvastatin, and simvastatin) were downloaded from PubChem database in addition to the native ligand (cocrystallized obj01/117 extracted from the drug target 1HWK). Before docking the phytochemicals, each statin was redocked into the HMG-binding site. The resulting poses, binding interactions, and binding energies were compared with those in the literature, especially the original crystallographic data (PDB IDs: 1HWK, 1HWI, 1HWL, 1HWJ, 1HW8, and 1HW9) [14] reported by Istvan and Deisenhofer [5]. The consistency between the docked results and published experimental data validated the efficiency and accuracy of the Autodock Vina docking algorithm. After this validation, the docking of the 32 hit (screened) phytochemicals was performed. Their results were exported as PDBQT files and visualized using PyMOL and Discovery Studio to evaluate the best poses (binding modes), hydrogen bonding, hydrophobic interactions, and molecular fit within the binding pocket. Their binding energy scores were saved in excel format for statistical analysis. The docking process was repeated for all 84 phytochemicals without screening, to determine whether potential inhibitors, which might have been previously screened out, could be identified as drug candidates. To generate the docking scores for the compounds and statins, another round of docking was performed using the "Dock structure into protein cavity" tab on DataWarrior.

The docking score and binding energy score are two key metrics used in this molecular docking study (Tables 3 and 4). The docking score was a value generated by DataWarrior software to represent the quality of the ligand's fit into the receptor's binding site and is derived using a scoring function based on factors such as hydrogen bonding, van der Waal's, hydrophobic, and electrostatic interactions [35]. The docking score was mainly used to rank the different compounds in terms of how well they bind to the HMG-binding site of HMGR and to compare the quality and fitness of their binding with those of statins. Higher docking scores (more negative) generally indicate a better fit between the compounds and HMGR, and vice versa. However, the docking score is not an absolute energy value.

Table . Molecular docking results of the top-ranked phytochemicals' binding at 3-hydroxy-3-methylglutaric acid–binding pocket of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Protein Data Bank ID: 1HWK).

Serial number[a]	PubChem ID	Compound name	Molecular structure (2D)	Binding energy ΔG (kcal/mol)	Ki[b] (μM)	RMSD[c]	Docking score	HMG binding pocket residues	Other amino acid residues
1	73641	Arjunolic acid	MF[d]: $C_{30}H_{48}O_5$	–6.0	39.7	0.0	–20.320	LYS691, LYS692, ASN686, VAL683	ASN658
2	12305768	Alphitolic acid	MF: $C_{30}H_{48}O_4$	–5.5	92.4	0.0	–19.805	LYS691, LYS692, VAL683	GLU665, ASN658
3	5281855	Ellagic acid	MF: $C_{14}H_6O_8$	–5.1	181.7	0.0	–20.728	SER684, ARG590, LYS692, ASP690	—[e]
4	72277	Epigallocatechin	MF: $C_{15}H_{14}O_7$	–5.1	181.7	0.0	–26.477	VAL683, ARG590, ASP690, SER684, ASN686, LYS692	SER661, GLU665
5[f]	13915428	3-O-methyl ellagic acid	MF: $C_{15}H_8O_8$	–5.0	215.2	0.0	–21.684	VAL683, ARG590, SER684, ASN686, ASP690, LYS692, LYS691	—
6[g]	5280417	3,7-Di-O-methylquercetin	MF: $C_{17}H_{14}O_7$	–5.0	215.2	0.0	–32.103	LYS691, ARG590, SER684, ASN686	MET655, MET657
7[h]	44446550	3,4'-O-Dimethylquercetin	MF: $C_{17}H_{16}O_7$	–4.9	254.8	0.0	–29.303	LYS692, ASP690, LYS691, ARG590	ASN658, SER661, GLU665
8	9064	Catechin	MF: $C_{15}H_{14}O_6$	–4.9	254.8	0.0	–26.213	LYS692, ASP690, VAL683, SER684, ASN686, ARG590	ASN658, SER661, GLU665
9	535203	3-(Azepan-1-yl)-1,2-benzothiazole-1,1-dioxide	MF: $C_{13}H_{16}N_2O_2S$	–4.7	357.3	0.0	–13.272	LYS692, SER684, ARG590, ASP690, LYS691, VAL683	MET657

Serial number[a]	PubChem ID	Compound name	Molecular structure (2D)	Binding energy ΔG (kcal/mol)	Ki[b] (μM)	RMSD[c]	Docking score	HMG binding pocket residues	Other amino acid residues
10	101202074	Cochloxanthin (4E,6E,8E,10E,12E)-13-[(1S)-1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl]–2,6,11-trimethyltrideca-2,4,6,8,10,12-hexaenoic acid	 **MF: $C_{25}H_{32}O_4$**	–4.6	422.6	0.0	–25.083	VAL683, ASP690, LYS691, LYS692	GLU665

[a]The interaction of the ligands with the catalytic residues of 3-hydroxy-3-methylglutaryl coenzyme-A reductase, as presented in the table, are curated from their two best poses (1 and 2), while their binding energy scores are derived from binding pose 1.

[b]Ki: inhibition constant.

[c]RMSD: root mean square deviation.

[d]MF: molecular formula.

[e]Not applicable.

[f]Compound 5: C-3→O-CH_3.

[g]Compound 6: C-3→O-CH_3, C-7→O-CH_3.

[h]Compound 7: C-3→O-CH_3, C-4→O-CH_3.

Table . Molecular docking results of statins' binding at 3-hydroxy-3-methylglutaric acid–binding pocket of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Protein Data Bank ID: 1HWK).

Serial number[a]	PubChem ID	Compound name	Molecular structure (2D)	Binding energy ΔG (kcal/mol)	Ki[b] (μM)	RMSD[c]	Docking score	HMG binding pocket residues	Other amino acid residues
1	Protein Data Bank ID: 117/Obj01	Atorvastatin (cocrystallized control)	 **MF:[d] $C_{33}H_{35}FN_2O_5$**	–5.3	129.6	0.0	–31.329	LYS692, ASP690, ARG590, SER684, VAL683	SER661, LYS662, ASN658
2	60823	Atorvastatin	 **MF: $C_{33}H_{35}FN_2O_5$**	–5.1	181.7	0.0	–33.050	LYS691, ARG590, LYS692, ASN686, SER684, VAL683	ALA769, ASN658, SER661
3	64715	Mevastatin (Compactin)	 **MF: $C_{23}H_{34}O_5$**	–4.9	254.8	0.0	–27.147	ASP690, LYS691, SER684, ARG590, VAL683, LYS692	SER661
4	446155	Fluvastatin	 **MF: $C_{24}H_{26}FNO_4$**	–5.3	129.6	0.0	–33.559	LYS691, ASP690, ARG590, ALA682, VAL683, SER684	MET657
5	446156	Cerivastatin	 **MF: $C_{26}H_{34}FNO_5$**	–4.6	422.6	0.0	–36.584	LYS692, ARG590, SER684, ASP690, LYS691, VAL683	GLU665, MET657, ASN658
6	446157	Rosuvastatin	 **MF: $C_{22}H_{28}FN_3O_6S$**	–4.9	254.8	0.0	–31.207	ARG590, SER684, VAL683, LYS692	SER661, ASN658
7	54454	Simvastatin	 **MF: $C_{25}H_{38}O_5$**	–5.7	66.0	0.0	–25.939	ASP690, ARG590, SER684, LYS691, VAL683	—[e]

[a]The interaction of the statins with the catalytic residues of 3-hydroxy-3-methylglutaryl coenzyme-A reductase, as presented in the table, are curated from their two best poses (1 and 2), while their binding energy scores are derived from binding pose 1.

[b]Ki: inhibition constant.

[c]RMSD: root mean square deviation.

[d]MF: molecular formula.

[e]Not applicable.

In contrast, the binding energy score, represented as the free energy of binding (ΔG) and measured in kcal/mol, refers to the binding affinity and strength of the interaction between a ligand and its target. It was generated by the AutoDock Vina algorithm in PyRx. The binding energy score predicts how strongly a compound will bind to the HMGR under physiological conditions, with higher (more negative) values indicating a stronger binding and more thermodynamically favorable formation of complexes [42]. Unlike the docking score, the binding energy score was directly correlated with inhibition constant (Ki) value using the formula {Ki=eΔG/RT}, where R is molar gas constant (1.987 cal/mol/K), and T is standard temperature in Kelvin (298K). Therefore, the selection of compounds was focused on those with stronger interactions and more effective binding energies rather than on the ones with good docking scores, in addition to using their drug-likeness and ADMET properties.

Statistical Analysis

All binding affinity scores for test compounds and control ligands were expressed as the means of 9 determinations each representing 9 different binding modes and SE of the mean. Statistical evaluation of data was performed using one-way ANOVA on Graphpad Prism (version 8.0; Graphpad Software Inc.). Significance levels were tested at $P<.05$.

Ethical Considerations

This study did not require ethical approval because it involved only computational analyses and did not include any human participants, identifiable personal data, or animal experiments, in accordance with institutional and international guidelines.

Results

To investigate the mechanism of binding and inhibition of the bioactive compounds isolated from *C planchonii* and *C tinctorium* on human HMGR activity, statins and each compound were docked against the HMG-binding pocket of the enzyme. The docking study results revealed that 10 lead compounds, each at 9 different binding poses, exhibited strong binding affinities, with binding energy (ΔG) scores ranging from –4.6 to –6.0 kcal/mol (Figure 3; Table 3). These phytochemicals also interacted well with the relevant amino acid residues at the HMG-binding pocket of the enzyme (Figure 4 and Figures S1-S5 in Multimedia Appendix 2) when compared with the interactions of statins (Figure S6 in Multimedia Appendix 3). Their ΔG scores were comparable to or exceeded those of the control ligands (–4.6 to –5.7 kcal/mol; Table 4). Their docking scores (–13.272 to –32.103) also compared favorably with those of statins (–25.939 to –36.584). One of the lead compounds, 3-O-methylellagic acid (ID_13915428) demonstrated stronger and more substantial binding interactions with the HMG-binding pocket residues of the drug target than any compound, including statins, in addition to exhibiting high binding energy (Table S4 in Multimedia Appendix 4).

Figure 3. Binding potential of statins (red) and top-ranked phytochemicals (blue) at 3-hydroxy-3-methylglutaric acid–binding pocket of human 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Protein Data Bank ID: 1HWK).

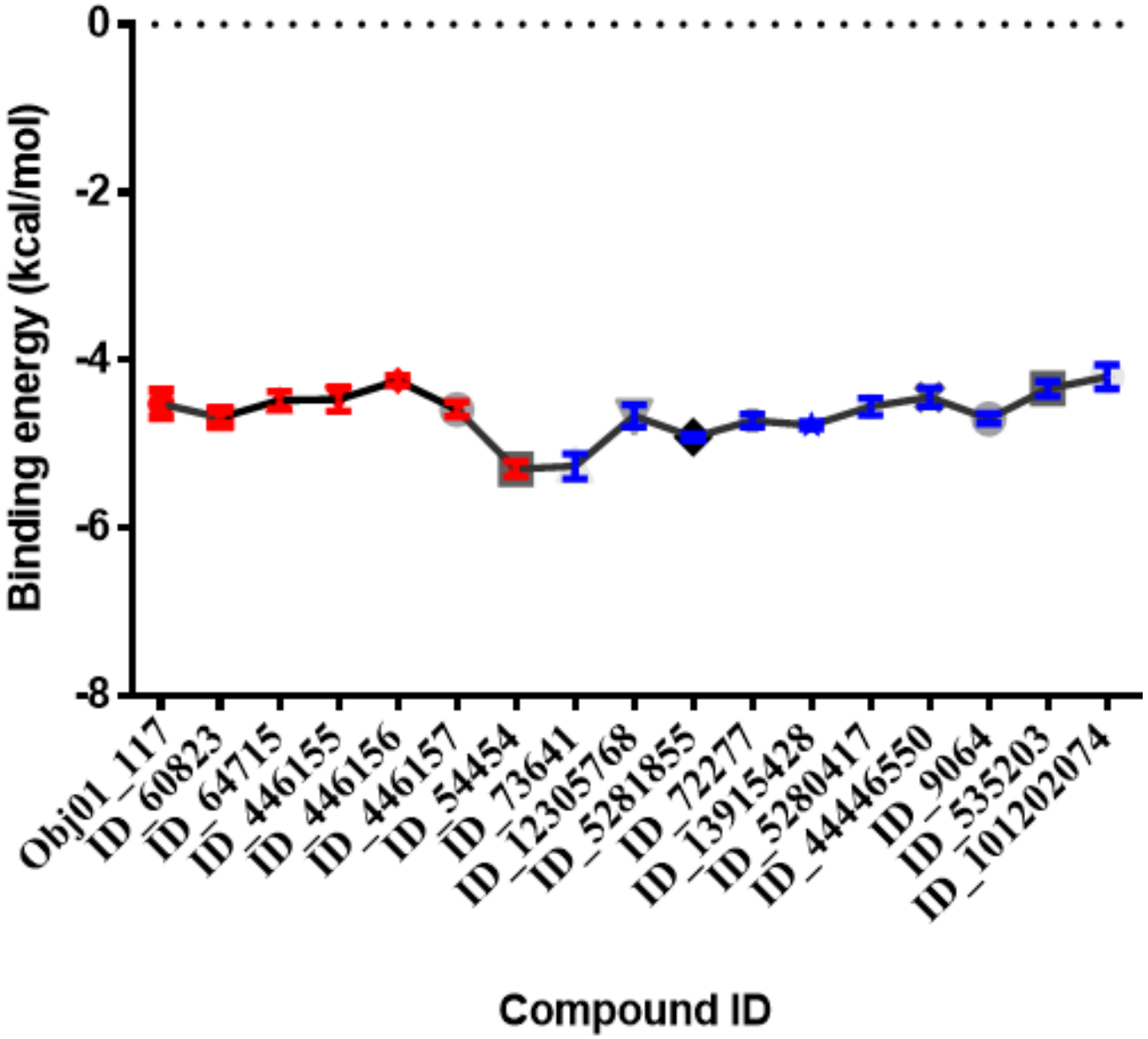
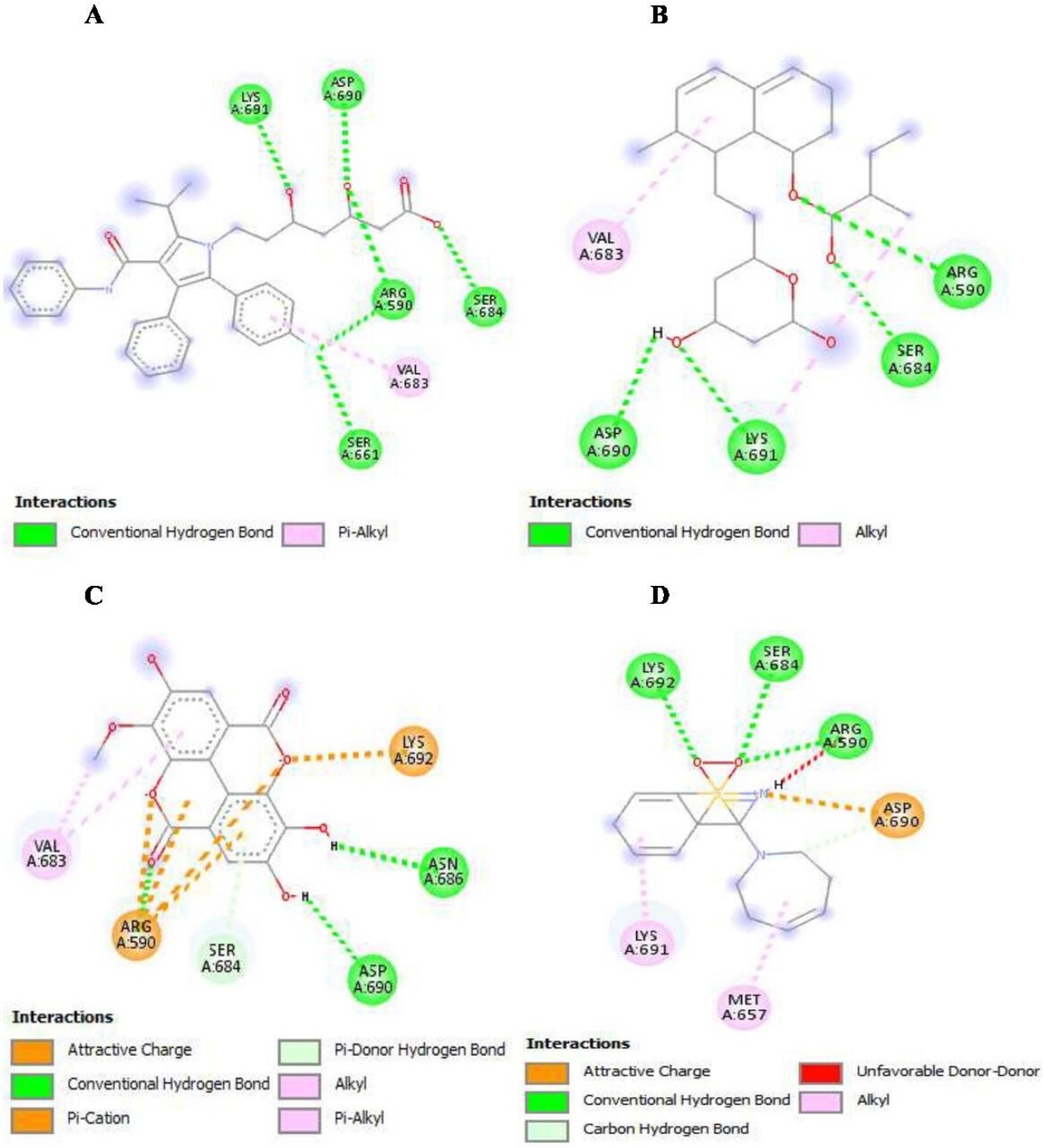


Figure 4. 2D representation showing the binding interactions of (A) Protein Data Bank ID_117/objt01 from literature, (B) ID_64715 pose 1,(C) ID_13915428 pose 1, and (D) ID_535203 pose 1, with 3-hydroxy-3-methylglutaric acid–binding pocket residues of human 3-hydroxy-3-methylglutaryl-coenzyme A reductase.



Discussion

Principal Findings

The molecular docking analysis of the two best binding modes of the 10 top-ranked phytochemicals demonstrated their cholesterol-lowering potential, as they clearly showed strong biochemical interactions and high binding affinities with the relevant amino acid residues that constitute the HMG-binding pocket (residues 682 - 694) of human HMGR (Tables S4-S13 in , Multimedia Appendix 4; Figures 3 and 4), comparable to or better than statins (Tables S14-S20 in Multimedia Appendix 5). This suggests that the phytochemicals could hinder the binding of the substrate HMG-CoA through competitive inhibition, in a similar binding mechanism as statins.

As shown in Table 3, the 10 top-ranked phytochemicals identified in this study, in no particular order, comprise 2 hydrolysable tannins (ellagitannins): ellagic acid (ID_5281855) and 3-O-methylellagic acid (ID_13915428); 4 flavonoids: catechin (ID_9064), epigallocatechin (ID_72277), 3,7-Di-O-methylquercetin (ID_5280417), and 3,4'-O-Dimethylquercetin (ID_44446550); 2 triterpenoid saponins: arjunolic acid (ID_73641) and alphitolic acid

(ID_12305768); 1 carotenoid: cochloxanthin (ID_101202074); and 1 benzothiazole derivative, 3-(Azepan-1-yl)–1,2-benzothiazole-1,1-dioxide (ID_535203).

Comparison to Prior Work

Several polar interactions with the cis-loop residues (Arg^{590}, Ser^{684}, Asn^{686}, Asp^{690}, Lys^{691}, Lys^{692}) of HMGR, are formed by the hydroxyl (-OH) groups of the aromatic rings, carbonyl groups (C=O), and lactone ring oxygen atoms of the ellagitannins. Their bulky rings also establish several electrostatic and hydrophobic contacts with residues Val^{683}, Arg^{590}, Ser^{684}, Asp^{690}, Lys^{691}, and Lys^{692} (Multimedia Appendices 2 and 4). No interactions of these polyphenols were observed with other residues within the HMGR-binding site. Among all the compounds, including statins, 3-O-methylellagic acid (ID_13915428) exhibits the greatest number (26) of binding interactions, indicating that this polyphenolic compound could be a viable drug candidate for HMGR inhibition. A recent in vivo and in vitro study by Lee et al [43] demonstrated that ellagic acid inhibits HMGR by activating AMP-activated protein kinase (AMPK), leading to the phosphorylation and subsequent inactivation of the enzyme. This study, which included rats fed with a high-cholesterol diet, revealed that the administration of ellagic acid (4 mg/kg/d, orally) resulted in significant reductions in serum total cholesterol, LDL-C, and triglyceride levels. Ellagic acid was also found to downregulate the gene expression of sterol regulatory element-binding protein-2 (SREBP-2) and its target protein HMGR, thereby reducing cholesterol biosynthesis in the liver [43]. In addition to its roles in cholesterol metabolism, ellagic acid and its derivatives also exhibit antioxidant and anti-inflammatory properties, which contribute to their protective effects against cardiovascular diseases [44].

The 4 flavonoids identified, belonging to the catechin and quercetin classes of polyphenols (ID_9064, ID_72277, ID_5280417, and ID_44446550), demonstrated their potential to mimic the binding of statins by forming polar hydrogen bonds with cis-loop residues (Arg^{590}, Ser^{684}, Asn^{686}, Asp^{690}, Lys^{691}, and Lys^{692}) and other residues (Asn^{658} and Glu^{665}). They also formed several electrostatic and nonpolar hydrophobic interactions with Val^{683} and other residues, including Met^{655}, Met^{657}, and Ser^{661}, at the HMGR-active site. This capability is attributed to their basic flavan-ring structure with multiple polar -OH, C=O, pyran ring oxygen atoms, and methoxy ($-OCH_3$) groups (Multimedia Appendices 2 and 4). An in vitro experiment showed that catechin isolates from *Uncaria gambir*, an Indonesian plant, exhibit strong inhibitory activities against HMGR with 97.46% efficacy, compared to 85.74% for simvastatin, a performance suggesting it could stand out as a promising therapy for hypercholesterolemia treatment [45]. Surprisingly, epigallocatechin gallate has been shown to potently and reversibly inhibit HMGR in vitro by competing with its cofactor NADPH and binding at the cofactor site instead of the HMG-binding pocket [46]. However, this present study suggests that epigallocatechin gallate may possess both capabilities. Quercetin dihydrate and gallate supplements have also been reported to lower plasma and hepatic cholesterol levels in rats fed with a cholesterol-rich diet. The results of the study concluded that quercetin dihydrate significantly reduced hepatic HMGR activity compared to normal control groups [47]. Furthermore, other several studies have elucidated the ability of quercetin to drastically reduce HMGR activity, inhibit fatty acid and triacylglycerol synthesis in hepatocytes, and alleviate endothelial dysfunction associated with age-related cardiovascular diseases [48-50].

Alphitolic acid and arjunolic acid are the two pentacyclic triterpenoids examined in this study. They generally exhibited fewer binding interactions with the HMG-binding site of HMGR, possibly due to their bulky and less polar triterpene core structure. However, the -OH and carboxylic (-COOH) groups present at both ends of their side chains formed polar hydrogen interactions with relevant residues such as Asp^{690}, Lys^{691}, Lys^{692}, Asn^{658}, and Glu^{665}. In addition, their pentacyclic rings engaged in non-polar hydrophobic interactions with important residues including Val^{683} and Lys^{691} (Multimedia Appendices 2 and 4). Direct studies on the inhibition of HMGR by alphitolic acid and arjunolic acid are currently lacking. However, several studies have shown that other structurally related triterpenoids possess direct inhibitory effects on HMGR. For example, 3α,26-dihydroxytirucalla-7,24-dien-21-oic acid (ARM-2) and 3β-hydroxylanosta-9,24-dien-21-oic acid (RA-5), isolated from *Protorhus longifolia*, demonstrated potent HMGR inhibition with IC_{50} values lower than lovastatin and simvastatin [51]. Similarly, Shen et al [52] reported that the doses of 25 and 125 µg/mL of oleanolic acid, a pentacyclic triterpenoid found in *Cassia mimosoides*, showed inhibitory effects on HMGR that were comparable to those of standard pravastatin groups. A patent report by Wöhrle et al [53] also identified several polyhydroxylated pentacyclic triterpenes as novel HMGR inhibitors, highlighting the therapeutic potential of this class of compounds. Moreover, arjunolic acid has been reported to protect against atorvastatin-induced oxidative stress and apoptosis in renal and hepatic tissues [54]. Its role in activating AMPK and suppressing neuroinflammation in animal models further suggests it may exert an indirect regulatory effect on HMGR inhibition [55].

Cochloxanthin is a carotenoid pigment found in certain plants, including *Cochlospermum* species. This compound showed polar hydrogen interactions between its polar side chain (-OH and -COOH groups) and a few HMG-binding residues, such as Asp^{690}, Lys^{692}, and others Glu^{665}. Additionally, hydrophobic bonds were formed between the carbon atoms of its long polyene chain and relevant residues, including Val^{683} and Lys^{691} (Multimedia Appendices 2 and 4). These relatively few binding interactions likely occurred due to the compound's linear long-chain skeleton, which may not fit properly into the narrow HMG-binding pocket of the enzyme. Metibemu et al [56] in their in-silico study, investigated several carotenoids isolated from *Spondias mombin* and suggested that these compounds possess strong HMGR inhibitory effects, along with antilipidemic and anticancer properties, but there was no direct link established with cochloxanthin. Similarly, in vitro studies by Alvi et al [57] reported that lycopene, a red carotenoid predominantly found in tomatoes, demonstrated significant

inhibitory effects on HMGR, with an IC_{50} value of 36 ng/mL, which surpassed that of pravastatin (IC_{50}=42 ng/mL). Their molecular docking analyses also revealed that lycopene binds effectively to the hydrophobic portion of the HMGR active site, showing a competitive inhibition [57]. In addition, Moreno et al [58] in their own study involving rat liver tissues showed that the administration of 70 mg/kg β-carotene (a precursor of vitamin A) led to a 50% reduction in hepatic HMGR mRNA expression. The authors suggested the role of β-carotene in modulating cholesterol biosynthesis at post-transcriptional level [58].

3-(Azepan-1-yl)-1,2-benzothiazole-1,1-dioxide is a heterocyclic sulfonamide derivative with a benzothiazole scaffold and an azepane ring structure, isolated from *C tinctorium*. Interestingly, this compound revealed promising polar interactions between the sulfonyl functional group (SO_2) of its benzothiazole ring and nitrogen atom with HMG-binding residues including Arg^{590}, Ser^{684}, Asp^{690}, and Lys^{692}. Additionally, its benzene and azepane rings formed several catalytically important hydrophobic contacts with residues Val^{683}, Lys^{691}, Asp^{690}, and Met^{657} (Multimedia Appendices 2 and 4). These interactions suggest the compound may serve as a novel, natural inhibitor of human HMGR. Currently, there is no information available on the effect of this compound on HMGR activities. Nevertheless, a molecular docking study by Ikpa and Tochukwu [59] demonstrated that this compound exhibited higher antiulcer potential than omeprazole by binding strongly to the H^+/K^+-ATPase receptor, a key drug target for proton pump inhibitors. The authors suggested that the compound may have superior gastric proton pump inhibitory potential compared to omeprazole, justifying its traditional use for relieving ulcer in patients.

Strengths and Limitations

This study has several strengths. Firstly, it is the first computational study to virtually screen and identify specific bioactive compounds isolated from two indigenous *Cochlospermum* species as potential inhibitors of human HMGR, through a structured and comprehensive literature review. Secondly, the study integrates several open-source and cost-effective software applications known for their high accuracy and reproducibility, such as PyMOL, PyRx, Open Babel, DataWarrior, and Discovery Studio, in the phytochemical screening and molecular docking analysis. This approach enhances the strength of the findings without the need for immediate wet-lab resources in the discovery of potential drug candidates, thus saving time and cost in the early stages of drug discovery. Lastly, the use of a validated human HMGR structure, with docking focusing on its HMG-binding pocket (cis-loop), a critical region responsible for its catalytic activity, ensures the biological relevance of the docking results. In addition, the inclusion of known statin inhibitors and the native ligand as controls provides a robust benchmark for comparing and assessing the inhibitory potential of the phytochemicals of interest.

However, there are some limitations. Due to resource constraints, this study did not include molecular dynamics simulation (MDS), a computational technique that could have provided additional insights into the dynamic behavior, conformational flexibility, and stability of the HMGR-phytochemical complexes over specific time. Also, the docking approach used was semirigid, where the crystal structure of HMGR is kept rigid and only the statins and phytochemicals have conformational flexibility. This method may not fully account for induced-fit effects, which could potentially lead to an underestimation of the compounds binding affinities and specificities, or a misinterpretation of their binding interactions.

In order to address these limitations, a pragmatic alternative was taken. The accuracy of the PyRx Autodock Vina docking algorithm was validated by cross-checking its docked statin results against the previously reported wet-lab experimental data of statins from the literature, before docking the phytochemicals. The consistency between the docking results and validated data from the literature supports the efficiency, reliability, and accuracy of the computational tools utilized in this study.

Future Directions

Although the literature review approach adopted in this study was crucial in the identification of bioactive compounds isolated from *C planchonii* and *C tinctorium*, however, it did not meet the full PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) criteria for a systematic review, due to limitations in the coverage of database, the lack of a second reviewer, and absence of a registered protocol. Therefore, a more extensive systematic review of phytochemicals from all species of *cochlospermum* is recommended for future studies, as this would help in the identification of potential drug candidates for HMGR inhibition that were not evaluated in this study.

Building on this study's findings, future work should include MDS to better capture the dynamic behavior, stability, and conformational changes of the HMGR-phytochemical complexes over time. These simulations can help in validating the docking results and reveal the compounds potential to maintain stable interactions with HMGR under physiological conditions. Furthermore, introducing other computational techniques such as quantitative structure-activity relationship (QSAR) modeling and free energy calculations (eg, MM/PBSA or MM/GBSA) would also improve the predictive accuracy of the binding affinities of the phytochemicals.

To complement these computational techniques, the 10 top-ranked phytochemicals identified in this study should be subjected to in vitro enzymatic assays and cell-based experiments in order to evaluate their actual inhibitory effects on HMGR activity. These efforts should also be followed by in vivo pharmacokinetic and toxicological studies which are necessary to determine the safety profile and therapeutic viability of these natural compounds. In the end, these combined computational and experimental approaches will be essential in translating the results of this study into meaningful advances in drug discovery.

Conclusions

This study has identified several bioactive compounds isolated from *C planchonii* and *C tinctorium* with potential to inhibit the activity of HMGR. The molecular docking results showed that compounds such as ellagic acid and its derivative, flavonoids, triterpenoids, carotenoids, and a benzothiazole derivative, exhibited significant biochemical interactions with the cis-loop residues of the enzyme, in addition to their high binding affinities. This demonstrates the ability of these phytochemicals of interest to potentially serve as natural and safer alternatives for hypercholesterolemia treatment, addressing the limitations posed by synthetic statins.

The findings are also consistent with previous studies that support the cholesterol-lowering and cardioprotective effects of these compounds, either directly or indirectly, through mechanisms such as AMPK activation, HMGR downregulation, and antioxidant properties. Although this study provides valuable computational insights into the molecular interactions of the compounds with HMGR, further advanced computational, in vitro, and in vivo studies are still necessary to validate their inhibitory potential and therapeutic applications.

Acknowledgments

This study would not have been possible without the grace of God. The author has no funding to declare.

Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files in Multimedia Appendix 6.

Authors' Contributions

TIO: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing.

Conflicts of Interest

None declared.

Multimedia Appendix 1
Phytochemicals identified from *Cochlospermum planchonii* and *Cochlospermum tinctorium*.
[PDF File, 299 KB - xbio_v3i1e71675_app1.pdf]

Multimedia Appendix 2
The best two binding interactions of top-ranked phytochemicals with 3-hydroxy-3-methylglutaric acid–binding pocket residues of human 3-hydroxy-3-methylglutaryl coenzyme-A reductase.
[PDF File, 506 KB - xbio_v3i1e71675_app2.pdf]

Multimedia Appendix 3
The best two binding interactions of statins with 3-hydroxy-3-methylglutaric acid–binding pocket residues of human 3-hydroxy-3-methylglutaryl coenzyme-A reductase.
[PDF File, 417 KB - xbio_v3i1e71675_app3.pdf]

Multimedia Appendix 4
Interaction profiles of the top-ranked phytochemicals at 3-hydroxy-3-methylglutaric acid–binding site of human 3-hydroxy-3-methylglutaryl coenzyme-A reductase.
[PDF File, 183 KB - xbio_v3i1e71675_app4.pdf]

Multimedia Appendix 5
Interaction profiles of statins at 3-hydroxy-3-methylglutaric acid–binding site of human 3-hydroxy-3-methylglutaryl coenzyme-A reductase.
[PDF File, 157 KB - xbio_v3i1e71675_app5.pdf]

Multimedia Appendix 6
Manuscript raw data files and analysis.
[RAR File, 22492 KB - xbio_v3i1e71675_app6.rar]

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Abbreviations

ADMET: absorption, distribution, metabolism, excretion, and toxicity
AMPK: AMP-activated protein kinase
CVD: cardiovascular disease
GC-MS: gas chromatography-mass spectrometry
HMG: 3-hydroxy-3-methylglutaric acid
HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme-A
HMGR: 3-hydroxy-3-methylglutaryl coenzyme-A reductase
HPLC: high-performance liquid chromatography
LDL-C: low-density lipoprotein cholesterol
LE: ligand efficiency
MDS: molecular dynamics simulation
MM/GBSA: molecular mechanics generalized Born surface area
MM/PBSA: molecular mechanics Poisson-Boltzmann surface area
NADPH: nicotinamide adenine dinucleotide phosphate
PDB: Protein Data Bank
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses
SDF: structure data file

Edited by A Schwartz; submitted 23.01.25; peer-reviewed by S Rasania, S Sakilay, S Mitra, R Salah, G Mahmoud, J Moonga; revised version received 18.05.25; accepted 23.05.25; published 10.07.25.

Please cite as:
Olatoye TI
Discovery of Novel Inhibitors of HMG-CoA Reductase Using Bioactive Compounds Isolated From Cochlospermum Species Through Computational Methods: Virtual Screening and Algorithm Validation Study
JMIRx Bio 2025;3:e71675
URL: https://bio.jmirx.org/2025/1/e71675
doi:10.2196/71675

Publisher:
JMIR Publications
130 Queens Quay East.
Toronto, ON, M5A 3Y5
Phone: (+1) 416-583-2040
Email: support@jmir.org