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2 **AJUR History and Editorial Board**

3 **Enzymatic and Structural Characterization of Alanine Racemase from *Enterococcus faecium* by Kinetic and Computational Studies**
Arie Van Wieren, Emma Bouchard, & Sudipta Majumdar

13 **Is Play Sexually Dimorphic in the Polygamous Squirrel Monkey?**
Seta Aghabablan, Anita Stone, & Christopher Brown

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Enzymatic and Structural Characterization of Alanine Racemase from *Enterococcus faecium* by Kinetic and Computational Studies

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ABSTRACT
The surge in vancomycin-resistant enterococci (VRE) strains poses a serious threat to public and clinical health. VRE strains are the leading cause of multi-drug resistant enterococcal infections and are commonly acquired from medical devices. Therefore, it is essential to discover new antibacterial targets and drugs for this pathogen. Alanine racemase could be a valuable drug target due to its crucial role in *E. faecium* survival. Alr from *E. faecium* (EF_Alr) was heterologously produced and purified from *E. coli*., and the steady-state kinetic constants were determined at different pH values. Using a coupled reaction with L-alanine dehydrogenase, rate of production of NADH was measured at 340 nm to observe EF_Alr activity in the D- to L-alanine direction. The highest catalytic efficiency, 8.61 ± 0.5 s^{-1} mM^{-1}, was found at pH 9. Additionally, the tentative active site residues, Lys40 and Tyr268, for the alanine racemization reaction were assigned by homology modeling and sequence comparison studies. Using UCSF Chimera, the structure of the EF_Alr homology model was superimposed and compared to the crystal structure of Alr from *E. faecalis*.

KEYWORDS
Alanine Racemase; *Enterococcus faecium*; Vancomycin-Resistant Enterococci; Homology Modeling; pH Optimum; Kinetic Characterization; SWISS-MODEL Server; Steady-State Kinetics

INTRODUCTION

Vancomycin-resistant enterococci (VRE) strains have gained significant attention due to their high prevalence in hospital-acquired infections and resistance to vancomycin, a drug used to treat life-threatening gram-positive bacterial infections unresponsive to other antibiotics.[1] Additionally, these enterococci possess intrinsic resistance to a myriad of other antibiotics and are noted for their ability to acquire resistance to all currently available antibiotics by way of mutation or by genetic transfer through plasmids and transposons.[2] Enterococci can be responsible for multiple types of infections. These infections, notably endocarditis and bacteremia, can be life-threatening and often require specific treatments due to their multidrug-resistant properties.[3] For these reasons, VRE have flourished in the hospital environment. In their recent report, the Centers for Disease Control and Prevention (CDC) categorized VRE as a "serious threat" and reported that VRE infections were responsible for an estimated 54,500 cases of infection in hospitalized patients each year in the United States.[4]

Enterococcus faecium is a Gram-positive VRE bacterium that has emerged in the last two decades as a leading cause of multidrug-resistant enterococcal infections in the United States. Out of a large set of *E. faecium* clinical isolates 36.4%, 77.3%, and 90.1% of strains were found to be resistant to vancomycin, gentamicin, and streptomycin respectively.[5] *E. faecium* infections, as with other VRE infections, have been found to pose serious threats in hospital environments. The National Healthcare Safety Network reported that the majority of device-associated infections (central lines, urinary drainage catheters, and ventilators) were due to either vancomycin- or ampicillin-resistant *E. faecium*.[6] This situation is especially concerning in light of the COVID-19 pandemic, where ventilators and other medical devices were utilized on a large scale to treat immunocompromised patients.[7] As of May 2020, there has been a report of co-infection of COVID-19 and *E. faecium*.[8] Clearly, there is a need to explore novel antibiotic drug targets for this pathogen so that its impact on hospital-acquired infections and public health can be mitigated.

One such antibiotic drug target is alanine racemase (Alr), a pyridoxal 5'-phosphate (PLP)-dependent bacterial enzyme that functions to racemize L-alanine and D-alanine. This enzyme plays a vital role in bacterial cell wall synthesis as D-alanine is one of the principal components in the synthesis of peptidoglycan.[9] Alr has long been considered an appealing target for drug development due to its prevalence in bacteria and its fundamental role in their survival.[10–12] The Alrs from several different pathogens, including another VRE pathogen *E. faecalis*, have been structurally and kinetically characterized.[13–20] However, no work has yet been published for the Alr from *E. faecium* (EF_Alr). In this paper, we report the cloning, expression, purification, and functional characterization of EF_Alr. Additionally, we propose the catalytic residues, Lys40 and Tyr268, for alanine racemization

reaction using sequence comparison and homology modeling studies based on the crystal structure of Alr from *E. faecalis*. We anticipate that our report will pave the way for antibiotic drug development against this infectious human pathogen.

METHODS AND PROCEDURES

Chemicals and reagents utilized for this study were purchased from Sigma-Aldrich, Fisher, or Alfa Aesar and were of the highest chemical grade available. Kinetic measurements were made using buffers with 50 mM sodium monobasic/dibasic phosphate, 100 mM NaCl (pH 6.5, 7, 7.5, 8, 8.5) and 50 mM sodium carbonate/bicarbonate, 100 mM NaCl (pH 9, 9.5, 10). Multiskan GO (Fisher Scientific) was used to collect UV-vis Spectrophotometric data. Protein purifications were carried out using BioLogic DuoFlow (Bio-Rad) medium flow chromatography system. The genomic DNA of *E. faecium* TEX16 (ATCC BAA-472) was purchased from American Type Culture Collection (Rockville, MD). L-Alanine dehydrogenase used in this study was produced and purified as described previously.[21]

Sequence alignment

An initial analysis was completed to determine the maximum score and amino acid sequence identity between EF_Alr and the Alr from five other organisms by running a BLAST search.[22] These five isoenzymes were selected from the hitlist due to having published crystal structures and E-values less than $1e^{-50}$. Then, a multiple sequence alignment of these six Alrs was performed using Clustal Omega.[23] The amino acid sequences of these proteins were acquired from Uniprot, a freely accessible resource for protein sequences (Uniprot.org). Protein sources and accession numbers are as follows: *E. faecium,* I3TYG8; *E. faecalis*, Q837J0; *S. pneumoniae*, P0A2W8; *G. stearothermophilus*, P10724; *P. aeruginosa,* Q9HTQ2; *M. tuberculosis*, P9WQA9.

Homology modeling

The sequence of EF_Alr was retrieved from Uniprot (entry: I3TYG8) and was used as a target for homology modeling using the SWISS-MODEL server.[24,25] This tool produced a 3D model for the EF_Alr target sequence by searching a library of experimentally determined protein structures from the Protein Data Bank to identify a suitable template. The template was used to perform a target-template sequence alignment which resulted in the generation of the finished model. The highest quality homology model was selected according to two statistical parameters: Global Model Quality Estimation (GMQE) and Qualitative Model Energy Analysis (QMEAN) Z-score. [26,27] Additionally, the EF_Alr homology model was uploaded to the SAVES server Version 5 to assess the overall stereochemical quality according to PROCHECK.[28]

Comparison of the overall folding of alanine racemases and determination of the active site

The EF_Alr homology model was visualized using UCSF CHIMERA.[29] The MatchMaker function was used to superimpose the EF_Alr homology model and crystal structure of *E. faecalis* Alr.[30] The EF_Alr homology model resulted in a tentative model of the EF_Alr active site containing the PLP cofactor. The FindHBond tool in UCSF CHIMERA was employed to identify putative active site residues involved in catalytic activity and stabilization of the PLP cofactor.

Plasmid construction

The EF_Alr gene was amplified from *E. faecium* genomic DNA with standard PCR methods using primers containing the restriction sites for *Nde*I and *Xho*I restriction enzymes (Integrated DNA technologies) which are listed in **Table 1**.

Primer	Sequence
EF_Alr_NdeI_FP	5'- GGAAGACTAGAAAGAAGGAAAA<u>CATATG</u>GTTGTCGCTTGGCACC -3'
EF_Alr_XhoI_RP	5'- CTCCTCTTTTTACCATAGGA<u>CTCGAG</u>TTATTCTTGATATTCTCTTGG -3'

Table 1. Primer sequences for EF_Alr gene amplification. Restriction sites are underlined.

The PCR mixture (50 µL) was comprised of 1 µL of 100 ng/µL template genomic DNA, 25 µL of Phusion master mix (2X), 1.5 µL DMSO, 2.5 µL of 10 µM forward primer, 2.5 µL of 10 µM reverse primer, 0.5 µL of Phusion High fidelity (2 U/µL) enzyme (Thermo Scientific), and 17 µL ddH_2O. PCR amplification was performed in a T100 Thermal Cycler (Bio-Rad) at the following parameters: 98 °C for 10 s, 65 °C for 20 s, and 72 °C for 30 s, followed by a final extension time of 7 min at 72 °C. Following the manufacturer's protocol, the PCR products were purified by gel extraction (Qiagen). The amplified DNA product was digested with corresponding restriction enzymes (New England Biolabs) before ligation into the pET28b expression vector (Novagen), which was cut by the same enzymes, to form the recombinant plasmid pET28b-EF_Alr.

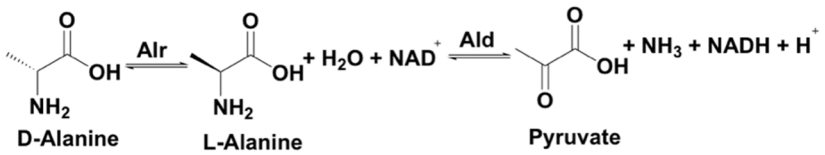
Enzyme expression and purification

Enzyme EF_Alr was produced in *E. coli* BL21 (DE3) cells. A standard bacterial culture for large-scale expression consisted of 2 X 1 L of LB broth medium shaken at 37 °C until the OD_{600} reached 0.6. At this absorbance, expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM, after which the culture was incubated for a period of 48

hours at 20 °C with shaking. The cells were harvested by centrifugation and dissolved in 20 mL cold buffer (20 mM Tris-HCL, 100 mM NaCl, pH 8.0). This was followed by the addition of 20 µL Halt Protease Inhibitor Cocktail (100x). Sonication was employed to lyse the cells and the lysate was cleared by centrifugation. The supernatant was applied to a 5-mL Bio-Scale Mini Nuvia IMAC column (Bio-Rad) and eluted with a linear gradient (100 mL) of 250 mM imidazole buffered with 20 mM Tris-HCl and 100 mM NaCl (pH 8.0). Fractions with pure (>95%) Alr were collected and dialyzed twice against 20 mM Tris-HCl and 100 m*M* NaCl (pH 8.0) and then stored at -80 °C. 10% SDS-PAGE analysis was utilized to verify the purity of the EF_Alr fractions.

Determination of enzyme activity at different pH values

EF_Alr activity was determined at eight different pH values (6.5–10.5) at 30 °C using D-alanine as the substrate. The activity was measured in the D- to L-alanine direction by monitoring the production of NADH (ε_{340} = 6.22 mM^{-1} cm^{-1}) at 340 nm as L-alanine was converted to pyruvate and ammonia by L-alanine dehydrogenase (Ald) (**Scheme 1**). The reaction mixture (250 µL) was comprised of 0.004–16 mM D-alanine (dissolved in buffer), NAD (906 µM), Ald (3.18 µM), and purified EF_Alr (0.201 µM) with an appropriate buffer. Assays corresponding to pH 6.5-8.5 were performed in sodium monobasic/dibasic phosphate buffers while assays corresponding to pH 9-10 were performed in carbonate/bicarbonate buffers. The data were obtained by triplicate kinetic runs at each pH.



Scheme 1. EF_Alr facilitated isomerization of D-alanine to L-alanine followed by NAD^+-dependent oxidation of L-alanine to pyruvate in the presence of alanine dehydrogenase (ALD).

RESULTS

Sequence alignment

Six Alrs from six different bacterial species were aligned based on their amino acid sequences using Clustal Omega (**Figure 1**). The results from the BLAST analysis are reported in **Table 2**. The BLAST results and alignment showed there is high sequence similarity among these Alr proteins. Based on the sequence alignment and homology modeling results, we predict that Lys40 and Tyr268 are most likely involved in the alanine racemization reaction by EF_Alr. The regions near these two proposed active site residues were found to have high homology. Additionally, the homology modeling results, and sequence alignment suggest that conserved residues Tyr44, Arg140, His 170, Ser 208, Arg223, Tyr268, and Tyr357 form hydrogen bonds with the PLP cofactor and play a critical role in its stabilization within the active site.

Alr Isoenzyme	Maximum Score	E-Value	Percent Identity
E. faecalis	486	$2e^{-172}$	63%
S. pneumoniae	380	$1e^{-130}$	52%
G. stearothermophilus	322	$6e^{-108}$	46%
P. aeruginosa	193	$4e^{-58}$	36%
M. tuberculosis	185	$1e^{-54}$	35%

Table 2. Maximum Scores, E-Values, and Percent Identities between EF_Alr and five Alr isoenzymes from BLAST analysis.

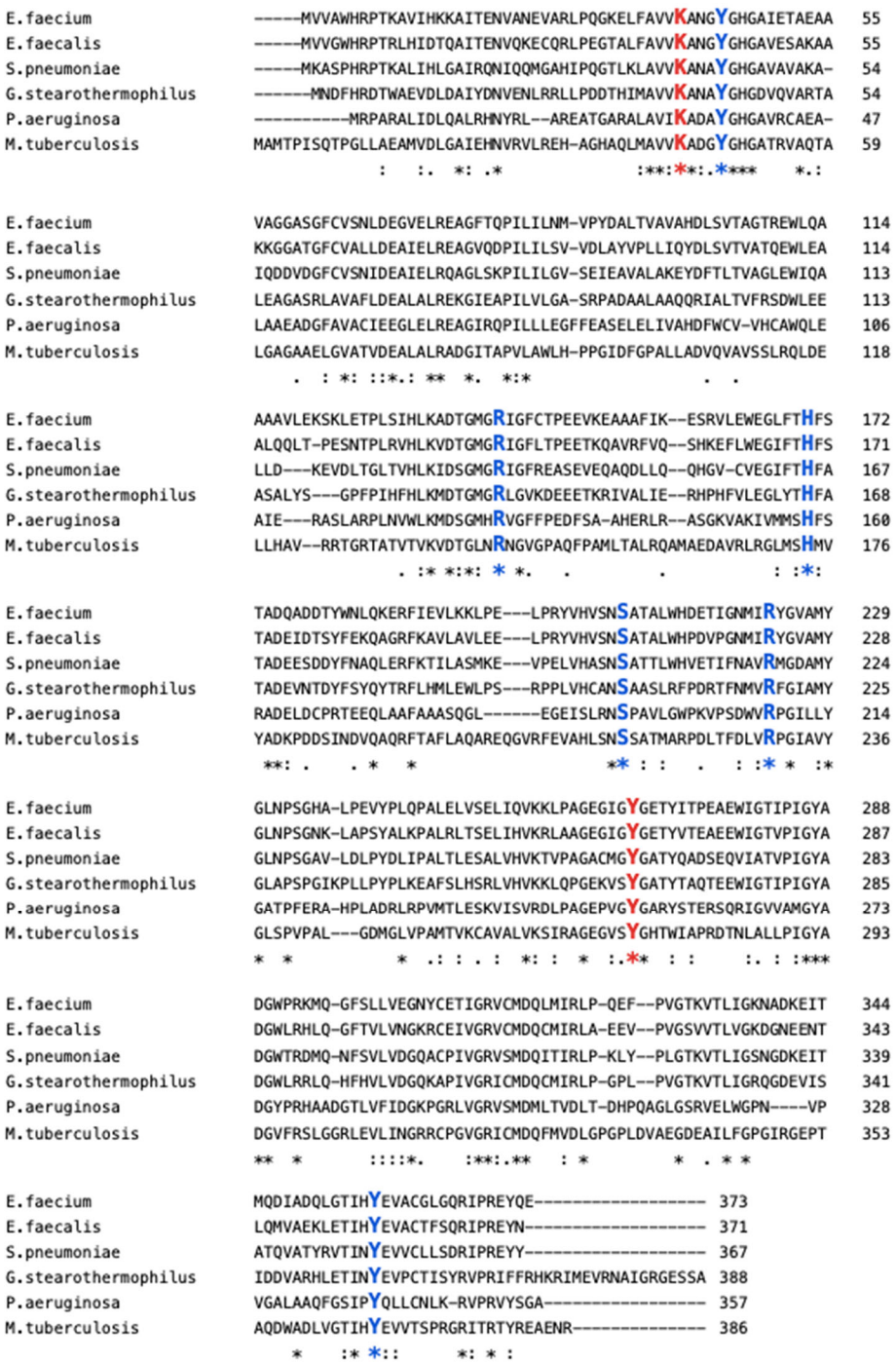


Figure 1. Multiple sequence alignment of alanine racemase from six different bacterial species. Proposed catalytic residues are shown in red. Proposed residues that form Hydrogen bonds with PLP with an interatomic distance less than 3 Å are shown in blue. Positions with a single, fully conserved residue marked by (*). (:) is used to mark positions with conservation between groups of strongly similar properties (approximately equivalent to scoring >0.5 in the Gonnet PAM 250 matrix). Sources and protein accession numbers: *E. faecium,* I3TYG8; *E. faecalis*, Q837J0; *S. pneumoniae*, P0A2W8; *G. stearothermophilus*, P10724; *P. aeruginosa,* Q9HTQ2; *M. tuberculosis*, P9WQA9.

Homology modeling

An alignment and homology model for EF_Alr was generated using the SWISS-MODEL server (**Figure 2, Table 3**). The *E. faecalis* Alr crystal structure was selected as a suitable template protein structure for the target because it was the only VRE Alr crystal structure available and exhibited high sequence identity (63.34%) and high coverage (99% of target EF_Alr aligned to the template).[13] The GMQE and QMEAN scoring functions were used as the preliminary methods to distinguish between suitable

and poor models. The high alignment values and GMQE and QMEAN4 values suggested a statistically acceptable model was generated for EF_Alr (**Table 3**).

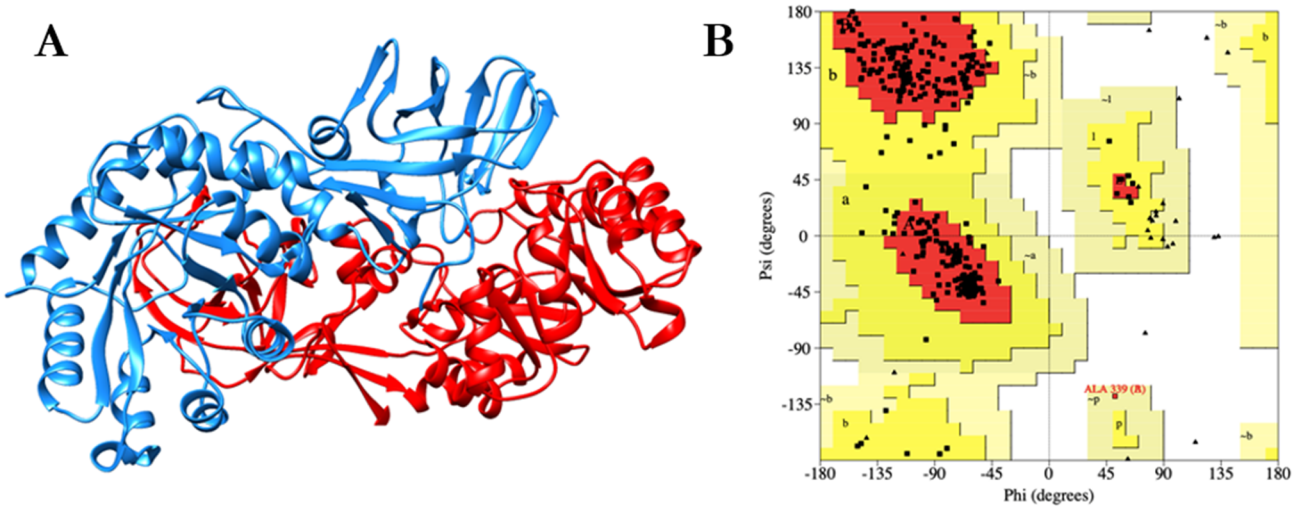


Figure 2. EF_Alr homology model and corresponding Ramachandran plot. (A) EF_Alr homology model produced with SWISS-MODEL that had suitable sequence coverage with the *E. faecalis* Alr template protein. EF_Alr is a homodimer. Individual chains are represented in blue and red. (B) Ramachandran plot for EF_Alr homology model obtained by PROCHECK, exhibiting dihedral angles Psi and Phi of amino acid residues. The most favored zones are represented by red; additional allowed zones by yellow; generously allowed zones by beige; and white areas are disallowed zones.

Template protein	Template PDB code	Sequence Identity	GMQE	QMEAN4	Most favored zones	Additional allowed zones	Generously allowed zones	Disallowed zones
E. faecalis Alr [13]	3E6E	63.34%	0.83	-0.80	90.5%	9.1%	0.3%	0.0%

Table 3. Summary of *E. faecium* Alr homology model and statistical validation.

Additionally, the Ramachandran plot for the model provided further confirmation of its acceptability. The results, shown in **Figure 2** and **Table 3**, indicated that 90.5% of residues in the homology model were within the most favored zones (red), 9.1% of residues in the homology model were inside the additional allowed zones (yellow), 0.3% of residues in the homology model were within the generously allowed zones (beige), and 0.0% of residues in the homology model were within the disallowed zones (white). The results of this analysis revealed the high reliability of the overall stereochemical properties of the generated EF_Alr homology model.

Comparison of the overall folding of alanine racemases and determination of the active site

Using the MatchMaker tool in UCSF CHIMERA, monomers from the EF_Alr homology model and *E. faecalis* Alr experimental structure were superimposed to elucidate differences in overall folding, shown in **Figure 3.A**. The calculated Overall RMSD was found to be 0.426 Å and the Q-score 0.964 for the superposition, indicating that there is significant conservation of overall folding and secondary structure between both structures, including the active site regions. Additionally, the active site of the homology model was analyzed in order to identify the possible catalytic residues, and the residues responsible for stabilizing the PLP cofactor (shown in **Figure 3.B**). The EF_Alr homology model active site and preceding knowledge of the Alr mechanism, indicated two possible ionizable residues, Tyr268 and Lys40, shown with pink labels in **Figure 3.B**. Using the FindHBond tool in UCSF CHIMERA, seven residues were identified that formed hydrogen bonds with the PLP cofactor with an interatomic distance less than 3 Å: Tyr44, Arg140, His 170, Ser 208, Arg223, Tyr268, and Tyr357. These residues were all conserved among the different Alrs analyzed in the multiple sequence alignment (**Figure 1**) and likely function to stabilize the PLP cofactor in the active site.

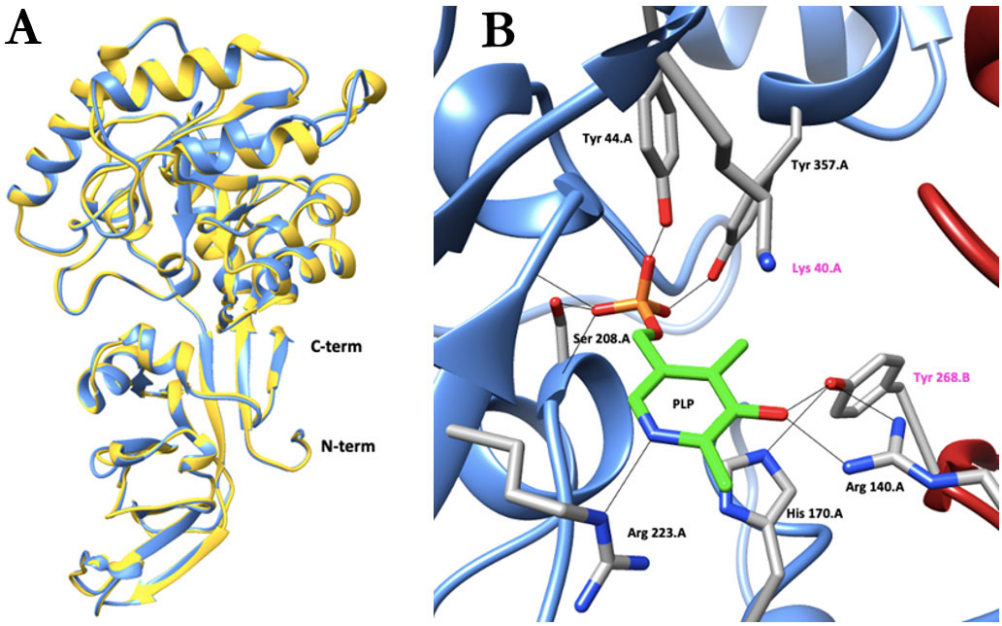


Figure 3. Comparison of the overall folding of Alanine Racemases and visualization of the active site (A) Superposition of EF_Alr monomer with *E. faecalis* experimental structure monomer. *E. faecium* Alr homology model monomer shown in yellow. *E. faecalis* Alr crystal structure monomer shown in blue.[13] (B) Proposed active site residues in the EF_Alr homology model. Chain A is shown in blue. Chain B, the partner monomer, is shown in red. Proposed catalytic residues are indicated by pink labels. Hydrogen bonds with an interatomic distance of less than 3.0 Å are depicted by black lines.

Determination of enzyme activity at different pH values

The EF_Alr was purified by using IMAC Ni-affinity chromatography. EF_Alr purity was confirmed through 10% SDS-Page (**Figure 4**). The EF_Alr was found to be banded at approximately 42.8 kD, concurring with the theoretical weight of the his-tagged enzyme. The Michaelis-Menten steady-state kinetic constants were determined by assaying the purified protein from pH 6.5 to 10.5 using different buffers. A buffer containing 50 mM sodium monobasic/dibasic phosphates and 100 mM NaCl was used for pH 6.5, 7, 7.5, 8, and 8.5, and a buffer containing 50 mM sodium carbonate/bicarbonate and 100 mM NaCl was used for pH 9, 9.5, and 10.5. The kinetic data were obtained in triplicate and fitted the Michaelis-Menten equation for each pH using GraphPad Prism7 to generate the steady-state kinetic constants (GraphPad Software, Inc). A representative Michaelis-Menten plot for pH 9 is shown in **Figure 5.A**. The steady-state kinetic constants are shown in **Table 4**. The highest catalytic efficiency, 8.61 ± 0.5 s^{-1} mM^{-1}, under experimental conditions was found at a pH of 9 (**Figure 5.B**).

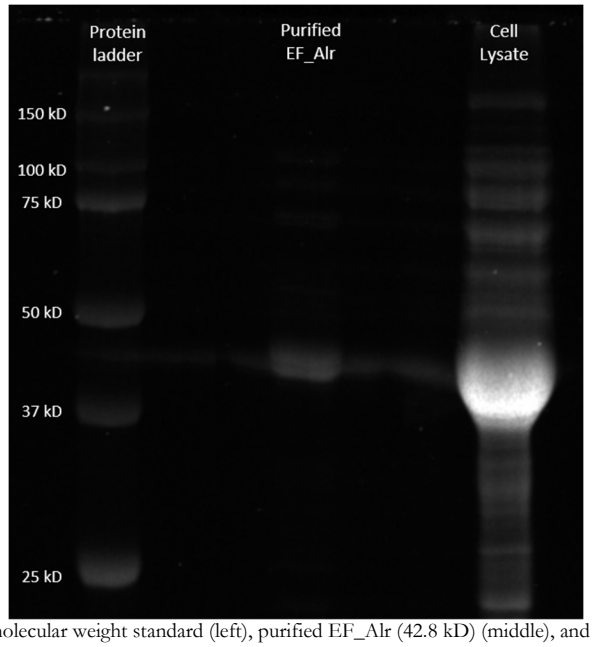


Figure 4. 10% SDS-PAGE including molecular weight standard (left), purified EF_Alr (42.8 kD) (middle), and lysate before loading Ni-column (right).

pH	k_{cat} (s^{-1})	$K_m \times 10^1$ (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
6.5	2.16 ± 0.1	12.1 ± 2	1.79 ± 0.3
7	2.29 ± 0.2	10.4 ± 4.0	2.20 ± 0.9
7.5	5.33 ± 0.2	9.63 ± 1	5.53 ± 0.6
8	2.98 ± 0.06	8.62 ± 0.6	3.46 ± 0.3
8.5	7.14 ± 0.2	8.53 ± 0.9	8.37 ± 0.9
9	6.39 ± 0.1	7.42 ± 0.4	8.61 ± 0.5
9.5	16.8 ± 0.5	30.0 ± 2	5.60 ± 0.4
10.5	18.1 ± 2.0	119 ± 20	1.52 ± 0.3

Table 4. Steady-state kinetic constants for EF_Alr at different pH values (triplicate). Error represents standard deviation of the mean.

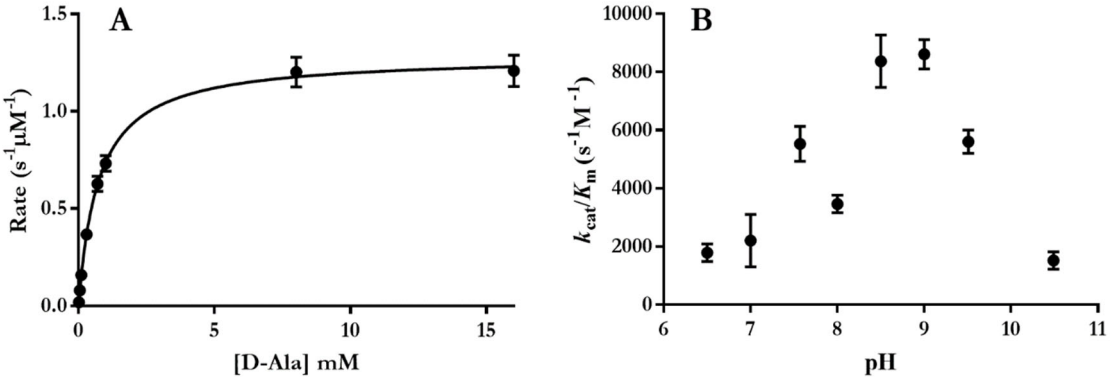


Figure 5. (A) Representative Michaelis-Menten plot for EF_Alr activity at pH 9 (50 mM sodium carbonate/bicarbonate and 100 mM NaCl). Reactions were performed at 30 °C. The steady-state kinetic constants for EF_Alr were determined by fitting the kinetic data (triplicate) to the Michaelis-Menten equation for different pH values. (B) The k_{cat}/K_m values were plotted against their corresponding pH values. Error bars represent standard deviation of the mean.

DISCUSSION

Due to the development of antibiotic resistance in bacteria there is a persistent need for the development of novel antibiotics. Alr is an appealing antibiotic drug target as it plays a fundamental role in bacteria and is absent in humans, yet the characterization of Alr from multidrug-resistant pathogen *E. faecium* has not been reported yet. Here we report the characterization of EF_Alr, an essential precondition for employing EF_Alr as a drug target for antibiotic drug development against the pathogenic VRE bacterium *E. facecium*. It is not possible to characterize an enzyme in any meaningful way without understanding its optimal pH conditions. The pH optimum for EF_Alr was found to be pH 9 while the pH optimum for Alr from various different bacterial species has been reported in the range of pH 8.3–10.5 (*E. coli* [pH 8.3], *B. stearothermophilus* [pH 8.5], *S. pneumoniae* [8.5], *P. putida* YZ-26 [pH 9.0], *M. tuberculosis* [9.0], *P. aeruginosa* PAOI [pH 10.0], *B. pseudofirmus* OF4 [pH 10.5]).[14–22] The maximum *kcat*/*K*m for EF_Alr at 30 °C was determined to be 8.61 $s^{-1}mM^{-1}$, higher than that found for Alr from *S. pneumoniae* [2.47 $s^{-1}mM^{-1}$] and *M. tuberculosis* [1.04 $s^{-1}mM^{-1}$].[16,18] The wide range in optimal pH and maximum catalytic efficiencies between Alr from different bacteria further stresses the importance of determining the pH optimum for EF_Alr.

Furthermore, computational methods were used to elucidate characteristics of EF_Alr sequence and structure. A multiple-sequence alignment of Alrs from six different bacterial species revealed that EF_Alr shares high sequence homology with other Alrs around the proposed active site residues. In the absence of a crystal structure, an EF_Alr homology model was constructed using the SWISS-MODEL server and served as a starting point for further elucidation of the EF_Alr structure. The model was statistically validated and displayed high stereochemical reliability (**Figure 3 and Table 2**). By overlaying the EF_Alr homology model with the experimental structure of Alr from *E. faecalis,* it was observed that both enzymes share exceedingly similar secondary and tertiary structures (**Figure 4.A**). Additionally, the EF_Alr homology model was used to construct a tentative active site for EF_Alr and identify putative active site residues and the residues that are responsible for stabilization of the PLP cofactor (**Figure 4.B**).

Previous reports have shown that the Lys40 and Try268 residues likely function as the catalytic acid and base in the racemization mechanism. This proposed mechanism would start with the formation of a Schiff base connecting PLP to Lys40. The lysine would then be substituted with the alanine substrate before the deprotonated Lys40 (for D-alanine) or deprotonated Tyr268 residue (for L-alanine) abstracts the α-hydrogen of the alanine to form a high-energy carbanionic intermediate. The intermediate

would eventually be protonated from the opposite side by either protonated Lys40 (for L-alanine) or protonated Tyr268 residue (for D-alanine), leading to the enantiomeric product.[31–33] The above mechanism was proposed based upon the known Alr mechanism for other bacterial species. Further studies need to be performed to validate our mechanistic hypothesis for this alanine racemization by EF_Alr.

As pathogenic bacteria continue to acquire resistance to clinically important antibiotics, research that characterizes antibiotic targets and designs new drugs will remain integral. This work encompasses important structural and kinetic information about alanine racemase from *E. faecium* that will be necessary for future exploration of novel inhibition strategies and the design of new antibiotics. Known sequence and structure similarities between alanine racemases could be exploited to create a broad-spectrum drug while knowledge of EF_Alr pH optimum and kinetics will be essential when evaluating inhibitory compounds. While the findings reported here could be utilized in many ways, the aim of this work is to build an informational foundation to aid in the development of new antibiotics so that the pathogenic impact of multidrug-resistant *E. faecium* may be diminished.

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PRESS SUMMARY

VRE bacterium *E. faecium* is a pathogen that poses a serious threat to public health as it is often the cause of life-threatening hospital-acquired infections that are difficult to treat with standard antibiotic therapies. In the midst of the COVID-19 pandemic this pathogen is even more concerning, as *E. faecium* infections are frequently acquired in hospitals through medical devices, such as ventilators. Due to this, there is an urgent need for the development of new antibiotic drugs that can target this pathogen. One possible antibiotic drug target is enzyme alanine racemase (Alr) which is critical to the survival of the bacterium. In this work, the

activity of Alr from *E. faecium (*EF_Alr) was characterized and a computational model of the enzyme structure was created. It was determined that EF_Alr has a pH optimum of pH 9. The computational model was used to analyze the possible structure and important components of this enzyme. The information reported in this work is the first steps towards the utilization of EF_Alr as an antibiotic drug target that could be used to treat infections from multidrug-resistant *E. faecium*.

Is Play Sexually Dimorphic in the Polygamous Squirrel Monkey?

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ABSTRACT
Play behavior is widespread in juvenile mammals and may be a mechanism for practicing skills needed in adulthood. In mammals characterized by strong adult male competition over females, juvenile males perform more social play than do females, and such play may assist in later mating competition. This study examined whether social play behavior is sexually dimorphic in a polygamous neotropical primate, the squirrel monkey (*Saimiri collinsi*), through a six-week field study of two groups of wild monkeys in Eastern Amazonia, Brazil. We hypothesized that males would conduct more rough-and-tumble play than females and that any sex-based play differences would be more evident in older juveniles. We video recorded juvenile play bouts and scored: age category (younger or older juvenile) and sex of players (male or female); and rough-and-tumble play behaviors (*i.e.*, bite, grab, and wrestle). Juvenile males initiated more play bouts than did females. Most players were older juvenile males, while older juvenile females were the least represented. Older juvenile play bouts occurred mostly among males, while younger juvenile bouts consisted of a more even sex distribution. While younger juveniles did not significantly affect the number of rough-and-tumble behaviors in bouts, the number of behaviors was significantly affected by the sex of older individuals. These results indicate that social play is sexually dimorphic in juvenile *S. collinsi*; specifically, males play more than females and sex differences are more pronounced in older cohorts.

KEYWORDS
Squirrel Monkeys; Mating System; Sexual Dimorphism; Juvenile Period; Development; Play Behavior; Social Behavior; Ethology

INTRODUCTION

Play behavior is widespread among vertebrates and even occurs in some invertebrates such as octopi.[1–3] Play is defined as any behavior that appears purposeless, is exaggerated in execution and timing, repeated, and not conducted under stress.[4] Due to the prevalence of juvenile play behavior in the animal kingdom, it is thought that play likely brings significant benefits to individuals.[2] While the adaptive function(s) of play remain largely unexplained, it is likely that play provides immediate (i.e. stress reduction)[5, 6] and future benefits (i.e. motor skill acquisition).[7, 8] Social play, a type of play which encompasses the interaction of two or more individuals, may function to help juveniles develop necessary adult social behaviors. According to the motor training hypothesis, juvenile social play functions as a way for individuals to practice important physical behaviors, such as fighting or mating.[9] Additionally, social play may help juveniles develop important social skills.[10]

The physical and social skills developed through social play behavior can benefit the sexes differently, depending on the intensity of the reproductive skew in a group. In polygynous and polygamous mammals, individuals of one sex, usually males, experience strong intrasexual mating competition and the best competitors experience greater reproductive success.[11-13] Juvenile social play may improve the reproductive success of individuals in these mating systems by providing opportunities to practice social behaviors such as fighting.[14] In species where males directly compete for females, juvenile play behavior tends to be sexually dimorphic such that males play more and conduct more rough-and-tumble (R&T) play, commonly known as "play fighting" than do females.[14–17] R&T play is described as mutual wrestling, grasping, pushing, and pulling.[18] Juvenile males that conduct R&T play behaviors may later benefit from a greater chance of winning fights against rivals,[14] having more efficient sexual behavior,[19] and effectively courting females.[20] Conversely, juveniles of monogamous species, such as the prairie vole (*Microtus ochragaster*) and the titi monkey (*Callicebus cupereus*), do not exhibit sexually dimorphic play.[21]

Squirrel monkeys, genus *Samiri*, are small (<1 kg) neotropical primates that live in multi-male multi-female groups consisting of 25-50 individuals.[22] Squirrel monkeys are polygamous and during the breeding season males aggressively compete over access to females.[23–25] Additionally, squirrel monkeys exhibit a long juvenile period that can last up to 3.5 years for females and 4.5 years for males.[22] Social play constitutes a significant portion of the daily activity budget of juveniles, and the most common play type is

R&T.[26, 27] Captive studies of squirrel monkeys (*Samiri boliviensis*) have indicated that males conduct more R&T play than females.[28, 29] However, these studies were mostly descriptive of general play patterns and did not include systematic data analysis to test hypotheses[28] . In addition, the captive studies were limited by small social group and sample sizes and lacked varying age cohorts.[29]. We conducted the present study on large social groups of wild *Saimiri collinsi* that contained several individual juveniles in distinct age cohorts. Because of squirrel monkeys' large groups that contain 15-20 juveniles, extended juvenile period, and strong male-male competition, these primates are a good model for studying sex differences in the play behavior of a polygamous species.

The purpose of this study is to determine if social play is sexually dimorphic in wild juvenile *S. collinsi*, a species inhabiting Eastern Amazonia, Brazil. Because *S. collinsi* is a polygamous primate, we hypothesize that juvenile play patterns will differ between males and females. First, we hypothesize that juvenile males will play more than juvenile females. Specifically, we predict that (1) males will initiate more play bouts, (2) participate in more bouts, and (3) exhibit more R&T play behaviors during bouts than will females. We also expect that juvenile males will play more with male partners than with female partners. Lastly, as juveniles approach adulthood they reach more sexually dimorphic roles. Therefore, any of the above sex differences in play behavior should be more apparent in older juvenile cohorts (2-3 years old) than younger juvenile cohorts (8-12 months old).

METHODS

Ethical Statement

All parts of this research complied with protocols approved by the Institutional Animal Care and Use Committee of California Lutheran University, by ICMBio in Brazil (permit number 32242-3) and by SisGen in Brazil (protocol number AA426B5). All procedures reported in this manuscript adhered to the American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates.

Study Site and Subjects

We conducted a 6-week study in June and July of 2019 in the community of Ananim, 150 km east of Belém, Brazil (01° 11' S and 47° 19' W). The site consists of approximately 800 hectares of primary and secondary rainforest.[30] The climate is highly seasonal with the wet season occurring from January to June and the dry season from July to December.[30]

We observed two *S. collinsi* social groups (N=73.4 contact hours), each consisting of 40-50 individuals (approximately 22% adult males, 35% adult females, and 43% juveniles). Each social group was distinguished based on home range location and the presence of individuals with unique physical features (e.g., missing fur or visible injury). Because of long-term studies conducted in the area, both groups were habituated to human observers.[30] This population of *S. collinsi* mate in July and August and the subsequent birthing season occurs during January and February.[31] Infants, classified as individuals who are nutritionally dependent on their mother, are weaned at 6 to 8 months of age.[31] Nutritionally independent individuals who have not yet reached sexual maturity are hence forth referred to as juveniles. No infants were present during data collection as they were weaned prior to the start of the study. We subdivided juveniles into a J1 cohort (younger juveniles: 8 to 12 months of age) and a J2 cohort (older juveniles: 2 to 3 years of age). Individual juveniles were not recognized; however, due to strict seasonal breeding, the juvenile age cohorts were identifiable by differences in head shape, body size, and fur color.[31] Specifically, J1s were characterized by a smaller body size, larger head to body ratio, more oblong head shape, and pronounced white facial markings when compared to J2s. Juveniles were sexed based on visual identification of their genitalia. Based on non-systematic observations, we assessed that there were more J2s than J1s present in both social groups, but males (approximately 10 to 12 individuals) and females (approximately 10 to 13 individuals) were roughly equally represented within each age cohort.

Behavioral Data Collection

We followed study groups six days a week from 07:00 to 15:00 hours, alternating days between the groups. We collected all-occurrence samples using continuous observations of play bouts.[32] We recorded the bouts (N=56 bouts observed) with a Sony HDRCX405 HD Handycam video recorder (N=0.98 hours of footage). When an individual initiated a play bout or when we first observed a bout already in progress, we began recording and ceased when all individuals involved exhibited play termination. Play bout recordings were later watched in slow motion using Microsoft's Windows Media Player. The following variables were scored for each bout: identity (sex and age cohort) of the bout initiators, number and identity of all players, and the behaviors executed by each player (see **Table 1** for ethogram). Initiation attempts were considered successful if they resulted in a play bout between the initiating individual and the recipient of the invitation. Using a digital voice recorder, we also collected *ad libitum* observations[32] of attempted initiations seen live between July 3 and July 15. For all observed initiation attempts, we noted the following variables: identity of the initiator, initiation behavior used, identity of the initiation recipient, and whether the initiation resulted in a play bout.

Behavior Name	Description
Chase	The actor runs after the partner.
Pounce	The actor jumps onto the partner and quickly jumps off.
Grab	The actor grasps and pulls on the partner's tail or head using one or both arms[29]
Bite	The actor bites an area of the partner's body without hurting them.
Wrestle	Actor and partner are involved in mutual grasping, pulling, and pushing of various body parts. Can be conducted on an even surface (i.e., the forest floor) or suspended by the forelegs or tail from a branch.
Terminate Play	The actor halts play behavior with the partner and conducts other non-play behavior (i.e., rest, forage, or travel).

Table 1. Rough-and-tumble play ethogram of *Samiri collinsi* used in the present study.

Data Analysis

We first examined age and sex differences in the total number of players, total number of initiators, and total number of R&T behaviors using 2x2 Chi-square test of counts with Yate's corrections. For the purposes of our analyses, we classified total R&T behaviors as the combined observations of biting, grabbing, and wrestling (**Table 1**).

Additionally, we used Poisson regression analysis in R Studio[33] using *glm* to determine how each age/sex cohort predicted R&T behaviors per bout. Using the counts of each player age and sex (where "M" indicates male and "F" indicates female) group present in a bout, we modelled the number of total R&T behaviors exhibited during the bout (**Equation 1**). We examined coefficient significance ($p<0.05$) to determine the effect of each player type's presence on the total R&T behaviors exhibited in a bout.

$$ln(R\&T) = \beta_0 + \beta_1(J1M) + \beta_2(J1F) + \beta_3(J2M) + \beta_4(J2F) \quad \textbf{Equation 1.}$$

Finally, to explore player pairings in bouts, we compared proportion tables for all possible sex groupings (i.e., all males, all females, males, and females) of players among bouts of each age grouping (i.e., J1 only, J2 only, J1 and J2). Because several of these variables included counts of zero, it was not possible to examine these differences using statistical tests.

RESULTS

Identity of Players and Initiators

Age and sex significantly affected the number of players in bouts ($\chi^2=22.5$, $p=0.000002$). Male players were observed more often (78% of total players observed) than were females (22% of total observed players). J2s were also more common (62% of total players observed) than were J1 players (38% of total players observed), but J2 players were mostly male. Additionally, J2 males were the most observed player cohort in play bouts (**Figure 1**). While J2s showed a large sex difference in players, among J1s, male and female players were relatively equally represented (**Figure 1**).

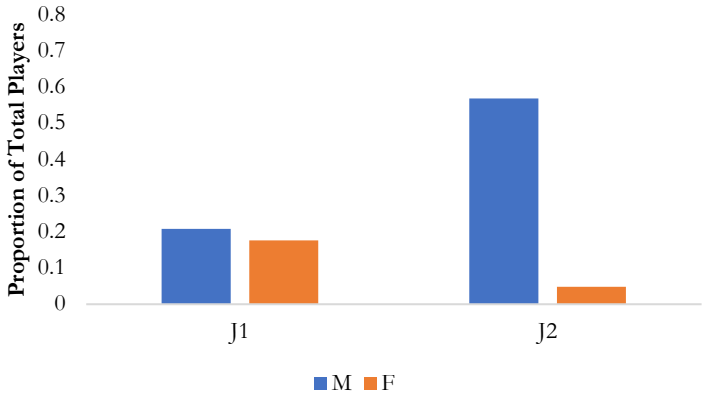

Figure 1. The proportion of players in bouts by age-sex class (J1=younger juveniles; J2=older juveniles; M=males; F=females; N=125 players).

Additionally, the number of initiations significantly varied by age and sex ($\chi^2=9.29$, $p=0.002$). Overall, males initiated play more often (93% of observations) than did females (7% of observations) and J2s initiated more often (76% of observations) than J1s (24% of observations). Among J2s, males initiated more than females (**Figure 2**). J2 males conducted most initiations among all age-sex groups, while J2 females initiated the least. However, among J1s, males and females initiated equally.

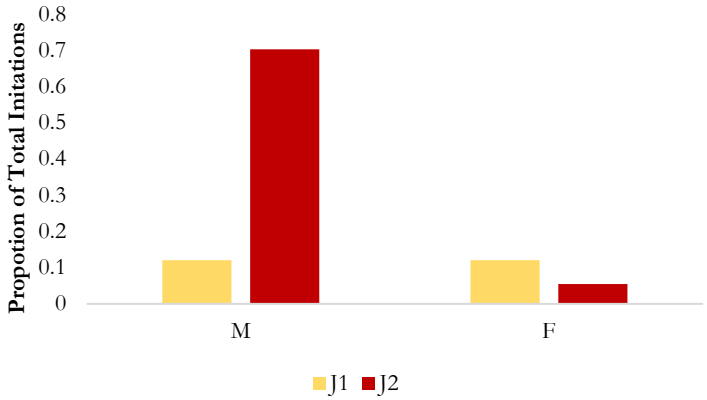

Figure 2. The proportion of play initiations performed by each age-sex class (J1=younger juveniles; J2=older juveniles; M=males; F=females; N=91 initiations).

Play Behaviors

Age and sex also significantly affected the number of R&T behaviors that players exhibited during bouts (χ^2=125.9, p<0.001). Males and J2s conducted more behaviors than did females and J1s. J2 males showed the most R&T behaviors, while J2 females showed the fewest. Among J1s, males exhibited twice as many behaviors as females (**Figure 3**).

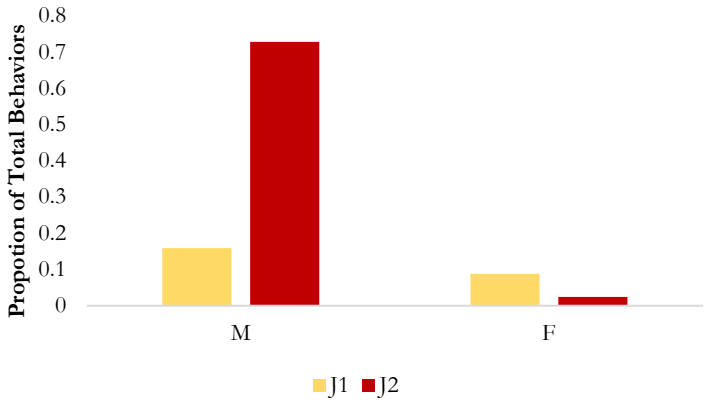

Figure 3. The proportion of total R&T behaviors (bite, grab, wrestle) shown by each age-sex group (J1=younger juveniles; J2=older juveniles; M=males; F=females; N=660 behaviors).

The Poisson regression indicated that the number of players of each age and sex cohort also significantly affected the number of R&T behaviors. The number of male and female J1s did not significantly affect the number of behaviors (p>0.05) in a bout; however, the number of J2s did (**Table 2**). For each J2 male in a bout, the number of expected R&T behaviors increased (estimated as 1.25 behaviors per individual). However, as the number of J2 females in bouts increased the number of R&T behaviors per bout decreased (estimated as -0.62 behaviors per individual).

		Estimated Value	Standard Error	z value	p value
Intercept (β_0)		2.233	0.140	15.911	p<0.001*
J1 male		0.001	0.078	0.116	0.908
J1 female		0.033	0.076	0.445	0.656
J2 male		0.222	0.066	3.382	0.001*
J2 female		-0.477	0.154	-3.104	0.002*

Table 2. Summary table of Poisson regression coefficient analysis (* indicates significance).

Bout Composition

The age of players affected play bout partner pairings. In J1s, all-female bouts were observed (17% of observations); however no all-female bouts were observed in J2s (**Figure 4**). Among J2 bouts, males played almost exclusively with other males (96% of observations). J1s also exhibited all-male bouts (33% of observations), but these were less common than in J2 bouts. Mixed-sex bouts were the most observed pairing among J1s and mixed-age bouts. In mixed-age bouts, pairings consisted of J2 males playing

with J1 females (31% of observations), J1 males playing with J2 females (38% of observations), and other pairings of multiple J2s and J1s of both sexes (31% of observations).

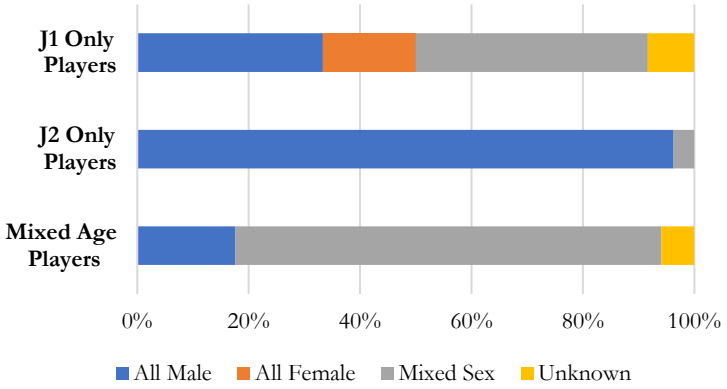

Figure 4. The total proportion of partner pairings by age cohort (J1=younger juveniles; J2=older juveniles; N=56 play bouts).

Initiation Success

While the sex of initators did not affect the outcome of initations, the sex of the recipient did. There was no difference in the success of initations among male and female intiators (χ^2=1.168, p=0.289; **Figure 5A**); however, the sex of the recipient significantly affected outcome. Individuals that attempted to initiate play with male juveniles had more successful play initiations than when trying to initiate play with females (χ^2=0.0002, p=0.989; **Figure 5B**).

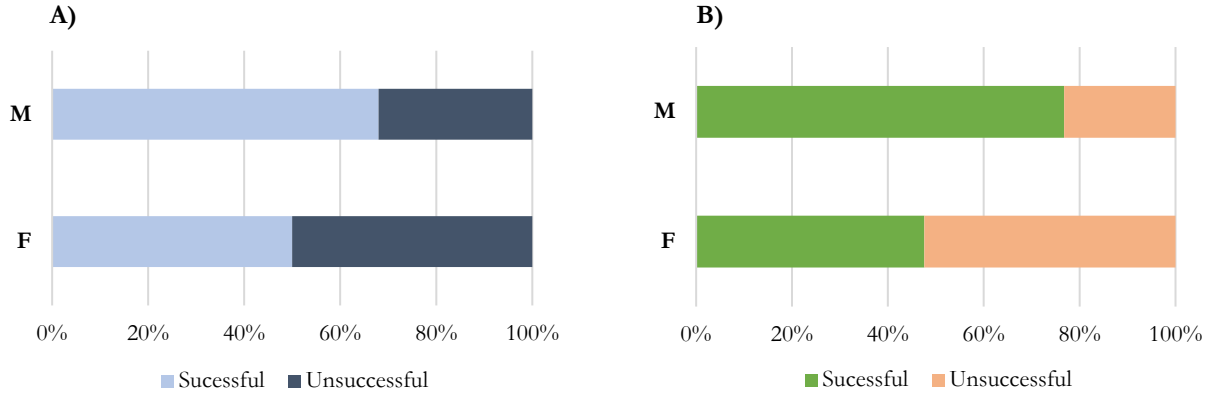

Figure 5. The total proportion of initation outcome based on A) initators of each sex class (N=91 initiations) and B) initation recipients of each sex class (N=77 iniations).

DISCUSSION

The purpose of this study was to determine if the polygamous primate *Samiri collinsi* exhibits sexually dimorphic social play. In support of our hypotheses, we found that juvenile males and females differed significantly in their play patterns and that these sex differences were more evident with age. Specifically, older juvenile males constituted the majority of total play bout participants, initiators, and conducted most of the R&T behaviors observed, while older juvenile females accounted for the lowest observations for all listed variables. In general, males participated in more bouts, initiated more bouts, and exhibited more total R&T behaviors than females did. Additionally, we found that males played most often with other males. Our results indicate that juvenile social play in wild *S. collinsi* is sexually dimorphic and supports captive observations of *S. boliviensis* and other primate species characterized by high mating competition.[16, 28, 34] Our study provides systematic data that confirm general play patterns described in captive studies of squirrel monkeys, by observing larger group sizes, varying age cohorts, quantifying specific behaviors, and describing bout compositions.[27,28] These differences suggest that juveniles of competitive mating systems may benefit from the practice of motor and/or social skills that may assist them in future mating competition.

One of the prominent functions of play may be to provide opportunities for juveniles to practice motor skills that are vital for adulthood.[35, 36] In species with strong reproductive skew, early juvenile differences in play may reflect the need for different mating-related adult behaviors. Because male squirrel monkeys experience a high degree of mating competition and prevent non-

resident males from entering the group, play behavior may function to help males develop mating related behaviors, such as fighting.[24, 25] Most of the play observed in juvenile male *S. collinsi* was R&T, and this type of play is very similar to adult fighting behavior in a variety of species.[37, 38] This evidence suggests that play may function to help improve fighting tactics in species that are intensely competitive over mates. R&T play in juvenile males also may be associated with developing other mating behaviors, such as mounting, that could improve fitness. Interestingly, if males were to physically practice sexual behaviors necessary for adulthood, then those behaviors should be apparent in play. However, while sexual play behaviors, such as mounting, were rarely observed in our study, R&T play was prominent among males. Therefore, if play does function to improve copulation behaviors it is likely through the development of motor skills, such as coordination and quick reflexes, rather than the practicing of mounting behavior. While this connection is not thoroughly understood, there are examples of R&T play being related to copulatory behavior. In chimpanzees (*Pan troglodytes*), for instance, high rates of social play exhibited by juvenile males were associated with earlier mounting attempts.[39] Due to competition, males' access to females may be limited; therefore, having well developed copulation behaviors may increase their chance of producing offspring.

Practicing social skills may also be an important function of juvenile play behavior. We found that males played most often with other males, which agrees with studies of other reproductively skewed primates.[14, 34] High numbers of male-only bouts could have occurred because females stopped playing in the later juvenile period. However, early juvenile initiation evidence suggests that males do prefer to play with other males.[28] Additionally, we found that initiations toward males were more successful and that initiation success was not affected by the sex of the initiator. In other words, males were more likely to participate in play when invited. Our study also points to male-only bouts as including higher counts of R&T behaviors. This pattern suggests that because males express more R&T behaviors, they may gain more benefit from these pairings. Males likely play more with other males because they will be interacting with males more often in a future competitive setting. In another squirrel monkey species (*S. sciureus*), almost all juvenile males disperse from their natal group to seek access to mates.[40] Sub-adult males need to navigate social interactions with unfamiliar males in order to join new troops, because males live together on the periphery of a core female group for most of the year.[23, 25] Social play among juvenile males may assist in developing the social skills required to reduce tension in unfamiliar social interactions with other males. Our findings that male-male play is most apparent during the older juvenile stage, where males are closer to dispersal age, also supports the idea that practicing male-male social interactions is an important function of play. For example, in rhesus macaques (*Macaca mulatta*), juvenile males changed their play tactics to better suit play with unmatched male partners, while females did not alter their behavior as often.[20] Being able to reduce tension during social interactions with unfamiliar males may help sub-adult males join new troops, and therefore gain access to potential mates.

Finally, our findings suggest that the function of play behavior may change throughout the juvenile period. While play in *S. collinsi* was not sexually dimorphic in younger juveniles, this pattern was different in older juveniles. Possibly, early juvenile social play provides a necessary function to all individuals, such as the general development of motor skills. For example, high rates of juvenile social play in ground squirrels (*Spermophilus beldingi*) were correlated to greater proficiency in motor skills, such as improved balance.[7] Additionally, low sexual dimorphism in play is also observed in species with low mating competition, such as monogamous species.[21] This further suggests that non-dimorphic play behavior provides some sort of necessary benefit to individuals of both sexes, such as increased juvenile survival. Young juvenile mountain goats (*Oreamnos americanus*) that had higher rates of play were more likely to survive their first year of life.[41] When dimorphic behavior emerges closer to adulthood, play may develop a more specific function that is related to future mating competition, such as practicing fighting and social flexibility.[19, 20]

In conclusion, our results indicate that juvenile play behavior in *S. collinsi* is sexually dimorphic and that the differences in play vary based on juvenile age. The finding that males engage in more play than females and play most with other males supports the trend that play patterns are associated with the competition in a species' mating system. Juvenile play in highly competitive mating systems may provide males benefits that assist in mating competition, but also function to develop necessary motor skills in juveniles of both sexes.[7, 19] Because play patterns vary based on the age of individuals and a species' mating system, it is likely that play has numerous functions. Play behavior is widespread and it is possible that play provides a general benefit that could be shared by all species, in addition to other functions that are species-specific. Further understanding of the context and characteristics of play behavior can help uncover its purpose in animals.

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PRESS SUMMARY

Play behavior is widespread in juvenile mammals, including in humans, and is considered an important mechanism for practicing skills needed later in life. In species where males compete for mates, juvenile males often play more than do females. It is thought that more juvenile play may prepare males for later mating competition behaviors, such as fighting and sexual behaviors. Here, we studied whether the play behavior of wild juvenile squirrel monkeys (*Samiri collinsi*), a small Amazonian primate characterized by strong mating competition, differed by their sex and age. We found that juvenile males initiated more play bouts, participated in more bouts, and exhibited more play behaviors than females. Additionally, juvenile males played most often with other males while females played with members of both sexes. However, these differences in play behavior were more pronounced for older juveniles. Our results indicate that play in wild squirrel monkeys is affected by the age and sex of juveniles and suggest that juvenile play may function to help males prepare for mating competition.

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