Determining the origin and maintenance of a sexual mimicry polymorphism in swordtail fish

How phenotypic diversity originates and is maintained are central questions in evolutionary biology. Polymorphisms in traits affecting survival and reproduction are common within species. One such polymorphism is sexual mimicry, where some individuals in the population imitate phenotypes of the opposite sex[1,2]. Such sexual mimicry has repeatedly evolved across the tree of life and can increase fitness by reducing conspecific aggression and/or by increasing access to mates[1,2]. While the same sexual mimicry traits often occur in multiple species within a clade, it is unclear how often these traits evolve independently, originate as ancestral polymorphisms, or move between species through introgressive hybridization[1]. Additionally, it is unknown if similar or different selective mechanisms maintain sexual mimicry polymorphisms in related species[2].

To understand the origin and maintenance of sexual mimicry polymorphisms, I will study a sexual mimicry trait that occurs in swordtail (*Xiphophorus*) fishes, which are an established behavioral and genomic model system[2,3,4]. Males in 11 *Xiphophorus* species are polymorphic for a dark pigmentation patch on their ventral sides (hereafter, false gravid spot or FGS; Fig. 1A-B), which mimics the pregnancy spot found in most female Poeciliid fish[2]. In a preliminary study, I mapped the genetic basis of the FGS in *X. birchmanni* to a narrow region on chromosome 2 using a genome-wide association study (GWAS; Fig. 1B). This region is upstream of the *kit-ligand*, a gene known to affect pigmentation in other vertebrates[5]. All significant SNPs fall within an inversion that distinguishes FGS and non-FGS individuals (Fig. 1C). However, it is unknown when, where, and how many times the FGS originated in *Xiphophorus*.

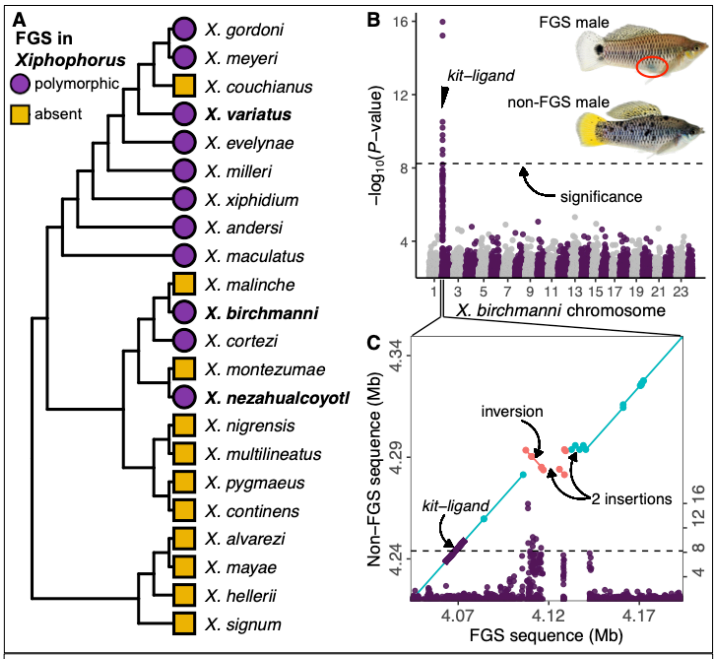

Fig 1. A) *Xiphophorus* phylogeny showing distribution of false gravid spot (FGS). **B)** GWAS for FGS in *X. birchmanni* implicates a single genomic region on chromosome 2. **C)** Alignment of FGS haplotype and non-FGS haplotype suggests structural rearrangements (inversions and insertions) upstream of the *kit-ligand* gene cause the FGS phenotype.

FGS frequencies range from ~10% in *X. nezahualcoyotl*, a species in which males and females are highly dimorphic due to other secondary sexual traits[2], to ~40% in *X. birchmanni* and >60% in *X. variatus*, species with more variable sexual dimorphism, which makes some males difficult to visually distinguish from females. Prior work in *X. nezahualcoyotl* demonstrated that FGS males experienced reduced aggression from other males but not increased female association (*i.e.* increased mating opportunities)[2]. However, it is unclear if the same or different selective mechanisms maintain the FGS in species with higher FGS frequencies.

My overall goal is to determine if the mechanisms governing the origin and maintenance of the false gravid spot (FGS) are similar or different in swordtail species. I propose the following specific aims:

- **Aim 1: Determine if the FGS originated multiple times independently, evolved as an ancestral polymorphism, or introgressed between species.** Because of the FGS's phylogenetic distribution and the known history of introgression in *Xiphophorus*[3,4], I hypothesize the FGS originated as an ancestral polymorphism in the *X. variatus* clade and later introgressed into the *X. birchmanni* clade.
- **Aim 2: Establish if the mechanisms of selection favoring the FGS are consistent across *Xiphophorus* species.** Because of their higher FGS frequencies and reduced sexual dimorphism, I hypothesize that FGS *X. birchmanni* and *X. variatus* experience greater female association in addition to reduced male aggression, whereas FGS *X. nezahualcoyotl* only experience reduced male aggression[2].

Aim 1 Experimental Plan: To determine the origin of the FGS phenotype in swordtails, I will test 3 hypotheses: independent origin, ancestral origin, and clade-specific origin with subsequent introgression. To test if the FGS originated independently, I will map the FGS in *X. variatus*, which is 6-9 million generations diverged from *X. birchmanni*[3] and has a larger, darker FGS, and may thus represent an independent origin. I will then trace the evolution of the causative allele(s) within the genus to determine in which clade(s) the FGS originated, and if the FGS has moved between species through introgression.

I plan to determine the genetic basis of the FGS in *X. variatus* by assembling a new genome for an individual with each FGS phenotype and mapping the trait using GWAS. Following a similar approach to my prior work in *X. birchmanni*, I will use PacBio HiFi sequencing to generate one FGS and one non-FGS *X. variatus* genome assembly. This highly accurate long read sequencing technology will allow me to assemble *X. variatus* references that reflect possible structural rearrangements, such as those present at the FGS locus in *X. birchmanni* (Fig. 1C). I will then non-lethally sample 300 *X. variatus* males, phenotype for FGS, sequence these individuals at 1× coverage, and conduct a GWAS to identify genomic regions associated with the FGS phenotype[4]. Finding the same structural variants in *X. variatus* and *X. birchmanni* would indicate that the FGS has a shared genetic basis across *Xiphophorus*, while finding dissimilar patterns would suggest an independent evolution of the FGS.

To understand if the causative allele(s) originated as a polymorphism in the ancestor of all *Xiphophorus* with the FGS or moved into some species through hybridization, I plan to generate a local phylogenetic tree of the region(s) identified using GWAS and compare this tree to the genome-wide species tree[1,3]. Discordance between local and species trees, longer haplotypes, and short branch lengths would indicate introgression[1]. To generate the local tree, I will obtain 1 individual per FGS phenotype per species (33 individuals) from the *Xiphophorus* Genetic Stock Center. I will enrich for the region(s) identified through GWAS using a myBaits bead pulldown protocol[4], sequence these using PacBio HiFi, and build a tree from these regions[1]. My findings will help determine the relative importance of independent evolution, ancestral polymorphism, and introgression in the origin of shared sexual mimicry traits.

Aim 2 Experimental Plan: To determine if behavioral mechanisms of selection on the FGS are consistent across *Xiphophorus*, I will test if FGS males in *X. birchmanni* and *X. variatus* experience greater female association as well as reduced aggression from other males, and compare these results to previous experiments in *X. nezahualcoyotl*[2]. I will perform behavioral tests in the laboratory using 15 replicates[2] of the following groups of wild-caught individuals: one trait-matched (*e.g.* body length, dorsal fin height and color) pair of FGS and non-FGS males, one larger male, and one adult female.

To test for increased female association, I will use dichotomous choice tests with the FGS and non-FGS male confined to a randomly selected side of an observation tank[2]. I will measure the time that the female is within 1/5 tank-length of each male[2]. To test for reduced male aggression, I will place the FGS or non-FGS male in a tank with a larger male and quantify aggressive interactions (*e.g.* number of chases and displays) initiated by the larger male, with the order of trials being randomly determined. Finding increased female association with, and reduced male aggression towards, FGS *X. birchmanni* and *X. variatus* would suggest that additional mechanisms of selection occur in *Xiphophorus* species with higher FGS frequencies, while only detecting reduced male aggression would indicate that behavioral mechanisms of selection are consistent between species with different FGS frequencies.

Intellectual Merit: My study will address the origin and maintenance of adaptive diversity, which remains a fundamental question in evolutionary biology. By combining advances in sequencing with behavioral experiments in the laboratory, I will study the evolution of a sexual mimicry polymorphism at a genus-wide scale, which can increase understanding of the processes generating phenotypic diversity[1].

Broader Impacts: As part of this project, I will continue to mentor community college students from Cañada College and high school students through Stanford's RISE program, which recruits students from backgrounds that are underrepresented in science (see Personal Statement). I will continue to focus on education and outreach in science classrooms, for example, at Leland High School and Friendship Academy Middle School, where I have taught classes on sexual mimicry and hybridization.

[1] Palmer *et al.* 2020. *Nature Communications*, *11*. [2] Morris *et al.* 2010. *Behaviour*, *147*. [3] Cui *et al.* 2013. *Evolution*, *67*. [4] Powell *et al.* 2020. *Science*, 368. [5] Miller *et al.* 2007. *Cell*, *131*.