BENEMÉRITA UNIVERSIDAD AUTÓNOMA DE PUEBLA

INSTITUTO DE FISIOLOGÍA

Laboratorio de Ecología de la Conducta

"La función comunicativa del bostezo y contagio de bostezo en el pez siamés (*Betta splendens*)"

TESIS

Que para obtener el grado de

DOCTORA EN CIENCIAS FISIOLÓGICAS

Presenta:

M.C. Alejandra Patricia Díaz Loyo

Director de tesis:

Dr. Alejandro C. Moyaho Martínez

Asesores de tesis:

Dr. Francisco Castelán

Dr. José Ramón Eguibar Cuenca

Dra. Amira del Rayo Flores Urbina

Dra. Laura Teresa Hernández Salazar

Puebla, Pue. Junio 2021

Dedico el presente trabajo a mi esposo, espero que tus ideas siempre vuelen muy alto.

AGRADECIMIENTOS

Agradezco a las siguientes instituciones por los apoyos económicos que recibí durante el doctorado:

- Beca CONACYT de doctorado 2017-2020 convocatoria 291197 número de becario 304699.
- Beca mixta CONACYT para realizar una estancia de investigación en la Universidad de Leiden, Países Bajos. Convocatoria 291250 programa 001692.
- A la Vicerrectoria de Investigación y Estudios de Posgrado de la BUAP por la beca VIEP 2039/2018 dentro del programa de Movilidad Académica y Estudiantil.
- Al International Society for Behavioral Ecology (ISBE) por el apoyo que recibí para presentar este trabajo en el ISBE 2018 en Minneapolis, Minnesota.
- A la Universidad Stanford y el laboratorio Schumer por el apoyo económico para realizar una estancia de investigación en sus instalaciones.

Estoy profundamente agradecida con el Dr. Alejandro Moyaho, por todas sus enseñanzas y su paciencia. Alejandro, no puedo dejar de agradecerte por todo lo que me has enseñado sobre ciencia, estadística, biología, e incluso de la vida. Gracias por permitirme ser parte de tu laboratorio, tus palabras siempre serán parte de mí. También le agradezco a la Dra. Evelyn Beristain Castillo, nunca me faltó su apoyo ni su amistad. Gracias por confiar en mí, por ayudarme a revisar mis métodos, y por siempre responder mis preguntas. Le agradezco al Biol.

José Luis Salazar Bautista el apoyo que me otorgó en todo momento, sin él me hubiera sido muy difícil completar la tesis.

También le agradezco a los revisores de este proyecto, el Dr. José Ramón Eguibar, la Dra. Amira del Rayo Flores Urbina, la Dra. Laura Teresa Hernández Salazar y el Dr. Francisco Castelán. Sus contribuciones siempre ayudaron a mejorar este proyecto.

Gracias a los investigadores que me permitieron hacer dos estancias de investigación en el extranjero: le agradezco a Jorg J.M.Massen, a Mariska Kret y Evy van Berlo de la Universidad de Leiden por la oportunidad de estudiar los orangutanes y su bostezo; también, gracias a Molly Schumer y Dan Powell por invitarme a colaborar con ellos en el estudio de los peces cola de espada. Aprendí mucho de ambas experiencias, y pude implementar algunos de los productos de este trabajo de tesis.

Gracias a mis compañeros de laboratorio: Paola y Natalie, con ellas nunca falta una buena historia; gracias a Gil y Gaby, ambos son personas increíbles y de gran corazón; gracias a Daniel, siempre me encantó escucharte hablar; gracias a Fer, por confiar tanto en mi y tenerme paciencia, aprendí mucho gracias a ti; gracias Layda, tu buen humor y optimismo son contagiosos; gracias a Astrid, recuerdo con mucho afecto tus deliciosos postres; gracias a Gregory y Francisco por ayudarme con algunas de las observaciones, ambos son muy inteligentes y tengo grandes expectativas para ustedes; gracias a Adzu, me encanta coincidir contigo en tantas cosas; gracias a Coco, quien se ha convertido en una amiga muy especial y una alegría constante en mi vida. Tu y Oli siempre tendrán un lugar en mi corazón.

Finalmente, le agradezco con todo mi corazón a mi familia. A mis padres, Delia Loyo y Arturo Díaz, que han creído en mi desde el inicio. Gracias por siempre acompañarme y ayudarme con todo, su formación y afecto son los pilares de mi persona. A mis eternos cómplices, Andrea y Arturo Díaz, algún día haré que admitan que me quieren. Gracias a Akira por ser parte de la familia, y ser la única persona que me confía en mi lo suficiente para hacerle un mohicano. Finalmente, le agradezco a Eloína, Humberto, Beto y Steph por todo su cariño, apoyo y confianza a lo largo de los años. Los quiero a todos.

RESUMEN

El bostezo es un comportamiento presente en todos los vertebrados en los que se ha buscado. Ocurre en contextos diversos, lo que sugiere que podría tener más de una función. Una de estas funciones podría ser de carácter comunicativo. Un estudio previo indicó que el bostezo del pez siamés parece comunicar un mensaje, cuya naturaleza aun es desconocida. En el estudio, motivo de esta tesis, me propuse determinar si el bostezo del pez siamés comunica la capacidad fisiológica de los individuos (hipótesis de capacidad fisiológica) o si comunica una invitación a la calma durante encuentros agonístico (hipótesis de apaciguamiento).

Para comprobar las predicciones que se desprenden de estas hipótesis, realicé pruebas de comportamiento agonístico entre pares de peces siameses. Las pruebas consistieron en enfrentar a dos peces a través de una división transparente, con un tercer pez como observador. De estas pruebas obtuve información sobre la frecuencia de bostezo y la actividad motora de los peces *(i.e.* desplazamiento dentro de la pecera experimental y frecuencia de desplantes agonísticos). Antes de aplicar la prueba de comportamiento, manipulé farmacológicamente la capacidad fisiológica de los peces para obtener peces con más y menos capacidad fisiológica. Estimé la capacidad fisiológica mediante el tiempo de resistencia en una prueba de nado contra una corriente de agua. De esta manera pude evaluar si el bostezo tiene relación los niveles de actividad motora (hipótesis de apaciguamiento) o con la capacidad fisiológica a los peces (hipótesis de capacidad fisiológica).

Inesperadamente, encontré que los tratamientos farmacológicos abatieron la frecuencia de bostezos casi por completo. Este resultado sugiere que un mecanismo inhibitorio del bostezo estaría relacionado al etanol (el vehículo del tratamiento farmacológico). En apoyo a esta explicación, registré bostezo en los peces observadores, a los cuales no expuse al etanol. Encontré que los peces observadores bostezaron con mayor frecuencia cuanto mayor fue su capacidad fisiológica ($z = 1.97$, g.l. = 13, $P = 0.048$). A continuación, comprobé si el bostezo se relaciona con la actividad motora de los peces, medida como el desplazamiento dentro de su compartimento durante la prueba. No encontré que la actividad motora tuviera efecto en la frecuencia de bostezo de los peces, y tampoco encontré diferencias en la actividad motora de los peces antes y después de bostezar.

Estos resultados me permiten concluir que la función del bostezo en el pez siamés es comunicar su capacidad fisiológica, y es poco probable que el bostezo comunique una invitación a la calma. Finalmente, no hallé evidencia que indicara la presencia de contagio de bostezo; sólo un caso que sugiere que la ventana de tiempo de contagio podría ser de 20 a 40 segundos.

Contenido

Abreviaturas usadas

CAS: Chemical Abstracts Service.

CTL: tratamiento control.

DEP: desviación estándar del promedio.

FLU: flutamida.

K: factor de condición de Fulton.

LE: longitud estándar.

MC: masa corporal.

ML: modelo lineal.

MLG: modelo lineal generalizado.

MLGM: modelo lineal generalizado mixto.

MLM: modelo lineal mixto.

MT : 17α-metiltestosterona.

NF: prueba de nado forzado.

OBS: peces observadores.

PC: prueba de comportamiento.

1. INTRODUCCIÓN

El bostezo es un comportamiento común a todos los vertebrados, pero su función aun se desconoce. Se ha descrito que ocurre en una gran variedad de situaciones, como en la transición entre la vigilia y el letargo, antecediendo a la alimentación y después de situaciones de estrés o tedio (Baenninger, 1987; Moyaho y col., 2002; Provine, 2005). El bostezo está presente en situaciones variadas, y los animales bostezan tanto en solitario como en situaciones sociales, por lo que se ha propuesto que el bostezo puede tener dos funciones dependiendo del contexto (Guggisberg y col., 2010; Moyaho y col., 2017; Zanella y col 2017, 2021): una función fisiológica (restablecimiento de la homeostasis de los individuos) y una función comunicativa (influencia en el comportamiento de otro individuo).

Las hipótesis de la función comunicativa del bostezo se han propuesto para explicar al bostezo que generalmente ocurre en tropas de primates en condiciones de cautiverio. Hay esencialmente tres posibles hipótesis de la función comunicativa del bostezo; **1)** la hipótesis de apaciguamiento, una invitación a la calma o reducción de la actividad (Sauer y Sauer, 1967); **2)** la hipótesis de agresividad, la intención de dañar a un rival (Darwin, 1873); **3)** la hipótesis de capacidad fisiológica, la comunicación de la capacidad fisiológica del individuo que bosteza (Moyaho y col., 2015). Esta última hipótesis surgió de un estudio realizado en roedores (Moyaho y col., 2015). Si el bostezo tuviera una función comunicativa en adición a su función fisiológica, deberíamos esperar que el bostezo fuera más frecuente en situaciones sociales. Una de las especies en las cuales se ha observado esto es el pez siamés (Baenninger, 1987).

Los machos del pez siamés (*Betta splendens*) bostezan con mayor frecuencia cuando están ante conespecíficos (*i.e.* individuos de la misma especie) que cuando están solos (Baenninger, 1987), lo que sugiere que el bostezo de estos peces puede cumplir una función comunicativa. En un estudio previo (Díaz-Loyo, 2016) encontré que en efecto el bostezo cumple una función comunicativa en estos peces, pues modificaron su comportamiento (*i.e.* desplantes agresivos) como respuesta a la observación de videograbaciones de conespecíficos bostezando. Así mismo, encontré evidencia que indica que es poco probable que el bostezo del pez siamés comunique agresión. Sin embargo, los resultados de aquel estudio no fueron suficientes para determinar si el bostezo comunica apaciguamiento o capacidad fisiológica.

El contagio de bostezo es otro aspecto de interés sobre el pez siamés. Dicho comportamiento se define como un aumento en la probabilidad de bostezar después de percibir a otro individuo hacerlo (Provine, 1986; Platek y col., 2003). Hasta el momento se ha reportado contagio de bostezo en varios grupos de vertebrados como los primates (Provine, 1986; Anderson y col., 2004), roedores (Moyaho y col., 2015) y aves (Miller y col., 2012), pero no en peces (Baenninger, 1987). Se sospecha que el contagio del bostezo requiere de capacidades cognitivas especiales que no se han atribuido a los peces (Platek y col., 2003; pero ver también Moyaho y col., 2015). Encontrar que existe contagio de bostezo en peces permitiría concluir que este comportamiento es filogenéticamente extenso y ancestral.

1.1. Bostezo

El bostezo se define como una apertura lenta de la boca, la retención de esta posición durante algunos instantes, y finalmente un cierre más rápido de la misma (Baenninger, 1997). Se han propuesto básicamente dos funciones del bostezo: una función fisiológica para el bostezo que ocurre generalmente en solitario, y una función comunicativa para el bostezo que ocurre en situaciones sociales (Guggisberg y col., 2010). En algunas especies diferentes tipos de bostezos han sido identificados (Vick y Paukner, 2010; Leone y col., 2014), los cuales corresponden a funciones distintas.

La secuencia de elementos que componen al bostezo ha sido descrita en varias especies de peces incluyendo el pez siamés (Simpson, 1968; Baenninger, 1987), el bostezo parece ser parte de los comportamientos que los machos de esta especie realizan durante peleas entre ellos. El bostezo también se ha reportado en el carrasco espinoso (Morris, 1955), y el pez espinoso de diez espinas (Morris, 1958), en ambos casos el autor sugiere que el bostezo es una actividad de desplazamiento (*i.e.* un comportamiento que ocurre cuando un animal está indeciso entre dos sistemas motivacionales distintos). En el caso del pez damisela (Myrberg, 1967) el autor no buscó una función del bostezo, pero lo reportó como un comportamiento que involucra actividad muscular intensa. Rasa (1971) observó bostezos en el pez el jaqueta coliamarilla y sugirió que este comportamiento sirve para calentar los músculos y preparar al pez para alguna actividad vigorosa.

En los peces como en otros animales ha sido difícil definir la función precisa del bostezo. Algunos autores atribuyeron una función fisiológica al bostezo de los peces, y otros encontraron que ocurre en contextos sociales. A continuación explicaré ambas hipótesis de la función del bostezo en vertebrados.

1.2. Hipótesis fisiológicas de la función del bostezo

Las hipótesis fisiológicas convergen en la idea de que el bostezo sirve para restaurar alguna condición fisiológica; es decir, para restaurar la homeostasis. Sin embargo, las evidencias experimentales a favor o en contra son escasas (Tabla 1.1). La hipótesis que más se ha investigado recientemente es la hipótesis que sugiere que el bostezo sirve para enfriar el cerebro (Gallup y Gallup, 2007; Gallup y col., 2009), pero la evidencia a favor es controversial (de Castro, 2009). Hasta ahora la hipótesis de la termorregulación del cerebro se ha puesto a prueba experimental en tres especies, los humanos (Gallup y Gallup, 2007), los pericos australianos (Gallup y col., 2009) y una cepa de ratas seleccionada para bostezar frecuentemente (Eguibar y col., 2017). Como evidencia a favor de esta hipótesis se ha presentado la correlación entre el número de neuronas corticales y la duración del bostezo en mamíferos (Gallup y col., 2016), en especies de felinos silvestres (Gallup y col., 2017), en razas de perros domésticos (Gallup y col., 2020) y comparando la misma correlación entre aves y mamíferos (Massen y col., 2021). Sin embargo, estos estudios han ignorado la duración del bostezo de otros vertebrados, y particularmente en los ectotermos; los animales que dependen del medio que les rodea para regular su temperatura corporal (Guschiana y Hardwood, 2006).

Los ectotermos emplean distintas estrategias conductuales para regular su temperatura corporal, por ejemplo desplazarse a zonas de sol y sombra. Algunos ectotermos como los reptiles disminuyen su temperatura corporal manteniendo la boca abierta (Smith, 1979), pero no se ha reportado que el bostezo también ocurra en estos animales cuando la temperatura corporal es alta. Si la función fisiológica del bostezo fuera la termorregulación del cerebro, esperaríamos que esta estrategia conductual de termorregulación sea más importante en ectotermos que en endotermos (*i.e.* los animales que si producen su propio calor corporal). Sin embargo, esto aun no se ha comprobado.

También se sabe que la frecuencia del bostezo en roedores está bajo control circadiano y antecede a los periodos de alimentación(Eguibar y col., 1984; Baenninger, 1987), después que roedores son expuestos a estímulos novedosos o estresantes (Moyaho y col., 2002), o durante momentos de aburrimiento, tedio o cansancio (Provine, 2005). Todos estos estudios sugieren que el bostezo podría tener alguna relevancia en la transición entre diferentes estados fisiológicos/anímicos (Moyaho y col., 2017). Sin embargo, la evidencia experimental es insuficiente para conocer la función fisiológica del bostezo.

Tabla 1.1. Hipótesis fisiológicas del bostezo, sus predicciones y evidencia encontrada.

Hipótesis	Promotor del bostezo		Consecuencia del bostezo		Evidencia global
	Esperado	Evidencia	Esperada	Evidencia	
Respiratoria Circulatoria	Hipoxia, Hipercapnia	Negativa	Incremento del oxígeno en sangre o en el cerebro	Faltante	Negativa
Espabilarse	Cansancio	Buena	Aumento de la actividad cerebral	Negativa	Negativa
Sueño y letargo	Cansancio	Buena	Cansancio	No concluyente	No concluyente
Termorregulación	Hipertermia del cerebro	No concluyente	Enfriamiento del cerebro	Faltante	No concluyente
Presión del oído medio	Cambio rápido en la presión del oído medio	Faltante	Igualar la presión del oído medio	Buena	No concluyente
Cambio de estado	-		Facilitar cambios de estado	Faltante	Faltante
Anticipación a eventos	Anticipación y espera	Buena	Mayor alerta	Faltante	No concluyente

Información tomada, traducida y modificada de Guggisberg y col., 2010.

1.2.1. Mecanismos fisiológicos del bostezo

Hasta ahora se ha descrito la participación de diversos neurotransmisores, neuropéptidos y neurohormonas en la regulación fisiológica del bostezo (Figura 1.1). Brevemente, las hormonas que participan en la regulación del bostezo incluyen al cortisol (Thompson, 2014), la testosterona (Holmgren y col., 1980, Graves y Wallen, 2006), la adrenocorticotropina (ACTH) (Ferrari y col., 1963) y la α -hormona estimulante de los melanocitos (α-MSH) (Ferrari y col., 1963). Éstas—ACTH y α-MSH—aumentan la frecuencia de

bostezo cuando se inyectan en el núcleo paraventricular del hipotálamo (Argiolas y col., 2000), aunque la lesión de este núcleo no inhibe totalmente el bostezo inducido por estas hormonas, lo que sugiere que otras vías o núcleos podrían también estar involucrados (Argiolas y col., 1987).

En cuanto al cortisol, la evidencia que vincula a la concentración de éste al bostezo es correlativa (Thompson, 2014). Puesto que es posible que la amígdala tenga una participación en el estrés y los niveles de cortisol (Hakamata y col., 2017), el hallazgo de Kubota y col. (2019) de que la estimulación con L-glutamato del núcleo central de la amígdala produce bostezos, cobra relevancia.

El mecanismo fisiológico por el cual esta hormona produce bostezos todavía se desconoce, aunque un estudio sugiere que podría actuar potenciando la acción estimuladora del bostezo de ACTH (Rodriguez-Sierra y col., 1981). El dimorfismo sexual (*i.e*. característica distintiva entre machos y hembras) del bostezo es un fenómeno frecuente (Graves y col., 2006; Leone y col., 2014), pero el mecanismo responsable todavía no ha sido estudiado. Holmgren y col., (1980) encontraron que la testosterona, pero no el estradiol, aumentaba la frecuencia de bostezo de ratas hembras y de ratas machos a las cuales se les han retirado los testículos. En primates también se ha observado que las hembras bostezan con una frecuencia similar a la de los machos cuando se les suplementa con andrógenos (Graves y col., 2006).

El sistema dopaminérgico es uno de los más importantes y mejor estudiados en relación con el bostezo. La dopamina induce el bostezo mediante un aumento en la concentración de

oxitocina, óxido nítrico y acetilcolina (Yamada y Furukawa, 1980; Mellis y col., 1996). Se ha encontrado que a concentraciones bajas la dopamina induce bostezo activando los receptores D_3, mientras que a concentraciones altas la intervención competitiva de los receptores D_2 inhibe al bostezo (Collins y col., 2005).

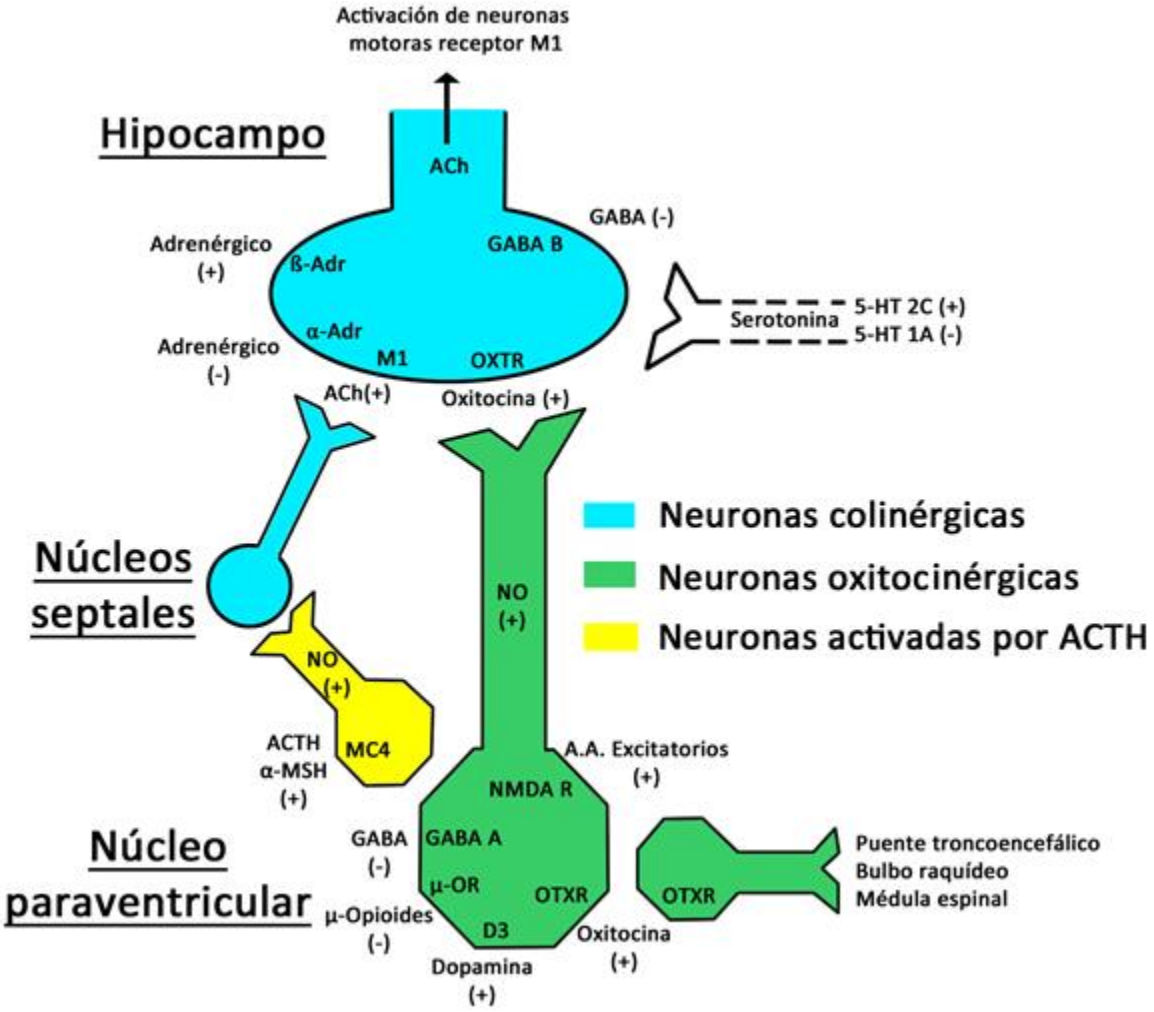


Figura 1.1 Representación esquemática de los neurotransmisores y receptores involucrados en el mecanismo de generación del bostezo. Tomado y traducido de Collins y Eguibar (2010). ACh = acetilcolina;α-Adr = receptor α-adrenérgico; β-Adr = receptor β-adrenérgico; M1 = receptor muscarínico M1; GABA = ácido gamma-aminobutírico; OXTR = receptor de oxitocina; NO = óxido nítrico; ACTH = adrenocorticotropina; α-MSH = α-hormona estimulante de los melanocitos; MC4 = receptor 4 de melanocortina; NMDA R = receptor de NMDA; μ-OR = receptor de μ-opioides; D3 = receptor D_3 de dopamina; $5\text{-HT}_{2C,\ 1A}$ = receptores de serotonina; (+) = la sustancia es promotora del bostezo; (-) la sustancia inhibe el bostezo.

Hasta el momento se han descrito al menos cuatro vías de regulación del bostezo (Collins y Eguibar, 2010): **1.** Dos grupos de neuronas oxitocinérgicas que proyectan desde el

núcleo paraventricular del hipotálamo a la región CA1 del hipocampo, al puente troncoencefálico, el bulbo raquídeo y la médula espinal; **2.** neuronas activadas por ACTH o α-MSH extienden su efecto desde el núcleo paraventricular al hipocampo mediante la activación de neuronas colinérgicas septo-hipocampales; **3.** la activación directa de neuronas colinérgicas hipocampales o septo-hipocampales; **4.** una vía serotoninérgica-colinérgica putativa.

A diferencia del bostezo espontáneo, la vía o vías por las cuales se genera el contagio del bostezo aun se desconocen. Evidencia previa sugiere que el sistema de neuronas espejo se activa en los sujetos que presencian bostezos (Haker y col., 2013), lo cual podría mediar el contagio reportado. El contagio de otros comportamientos como el rascado parece estar mediado por la actividad del núcleo supraquiasmático del hipotálamo (Yu-Qing y col., 2017). Estos autores sugieren que también otros comportamientos contagiosos podrían compartir la misma vía.

1.3. Hipótesis comunicativas de la función del bostezo

Este grupo de hipótesis se refieren al bostezo que ocurre en contextos sociales. Son básicamente tres las hipótesis que han sido propuestas para explicar la función del bostezo en tales contextos: 1) la hipótesis de la agresión (Darwin, 1873) sugiere que el bostezo comunica la intención de dañar a un rival; 2) la hipótesis del apaciguamiento propone que el bostezo comunica una invitación a reducir la actividad asociada a estados de vigilia intensa; 3) la hipótesis de la capacidad fisiológica sugiere que el bostezo comunica la capacidad fisiológica del individuo que bosteza. Encontré evidencia en un estudio previo que desfavorece a la hipótesis

de agresividad (Díaz-Loyo, 2016), antes encontré que cuando los peces siameses observan intentos de mordida -un comportamiento netamente agresivo - sus niveles de cortisol aumentan; no así cuando los peces observan bostezos, lo que sugiere que el mensaje contenido en el bostezo es distinto a la agresión. Por esta razón, y para fines de esta tesis, sólo explicaré las otras dos hipótesis.

1.3.1. Apaciguamiento

Definiré apaciguamiento como una disminución de la frecuencia de desplantes agresivos durante un conflicto agonístico *(i.e.* un encuentro entre dos animales que involucra intimidación, agresión, pelea o sumisión; Young, 2019). La hipótesis de que el bostezo sirve como tal surge de las observaciones de Sauer y Sauer en avestruces (1967). Estos autores observaron que los avestruces de un grupo silvestre bostezaban todos durante una ventana de tiempo estrecha, y que poco tiempo después disminuían su actividad hasta llegar al letargo. En otros animales sociales como las géladas (Leone y col., 2014) se ha encontrado que el bostezo antecede al letargo. Un estudio reciente en leones (Casetta y col., 2021) encontró que el bostezo promueve la sincronización en los niveles de actividad de la manada, y que la frecuencia de bostezo también era más frecuente en contextos de relajación. El bostezo, por lo tanto, podría servir como una invitación a la calma y reducir la actividad de un grupo.

1.3.2. Capacidad fisiológica

La capacidad fisiológica se refiere a la fuerza y habilidad que muestran los animales para resistir exitosamente a los efectos de cambios ambientales (*e. g*. cambios de temperatura,

intrusión de un rival). Los animales con mejor capacidad fisiológica suelen obtener y monopolizar recursos (*i.e.* hembras, alimento, territorio, etc.), y ganar conflictos agonísticos (Sinervo y col., 2000; Brownscombe y col., 2017). Por ejemplo, Sinervo y col. (2000) estimaron la capacidad fisiológica de una especie de lagartijas (*Uta stansburiana*) midiendo la resistencia física de los individuos en una banda móvil. Los autores encontraron que las lagartijas con el mayor nivel de testosterona también poseían la mejor capacidad fisiológica, la cual comunican con diferentes coloraciones. En otras especies los individuos podrían usar otro tipo de señales para comunicar su capacidad fisiológica durante encuentros agonísticos (*e.g*. bramidos; Reby y McComb, 2003) y evitar conflictos potencialmente peligrosos (Stegmann, 2005). A este respecto, se ha propuesto que el bostezo podría ser una de estas señales.

En una sublínea de ratas que fueron seleccionadas para bostezar mucho, Moyaho y col. (2015) encontraron que las ratas desconocidas entre sí se contagiaban el bostezo. El resultado fue sorpresivo, pues los autores esperaban que las ratas conocidas mostraran el contagio por ser más empáticas que las desconocidas. Debido a este resultado inesperado, los autores propusieron que el contagio observado era más bien una indicación de comunicación entre ratas que probablemente se "miraban" como adversarias (Moyaho y col., 2017). Por otra parte, la frecuencia de bostezo en ratas parece estar relacionada positivamente con los niveles de testosterona (Holmgren y col., 1980; Graves y col., 2006). La testosterona en machos puede ser a su vez el sustrato fisiológico de la capacidad fisiológica (Sinervo y col., 2000), ya que es responsable del desarrollo de la fuerza (Griggs y col., 1989) y resistencia físicas (Casto y col., 2020), y de acentuar el comportamiento agresivo (Rose y col., 1971). Por lo tanto, puede

anticiparse que un individuo con niveles altos de testosterona poseerá una capacidad fisiológica superior y podrá usar la frecuencia de bostezo para indicarlo (Moyaho y col., 2015), ya que ambos fenómenos (*i.e*. bostezo y capacidad fisiológica) están relacionados positivamente con la concentración de testosterona. Esta función podría ser diferente del bostezo que ocurre por contagio.

1.4. Contagio del bostezo

El contagio del bostezo es un aumento en la probabilidad de bostezar de un individuo después de percibir a otro haciéndolo (Provine, 1986; Platek, 2003). Se ha encontrado evidencia de que varias especies de vertebrados son susceptibles al contagio de bostezo (Tabla 1.2), principalmente primates, aunque no es el caso de otros grupos animales como los reptiles (Wilkinson y col., 2011) y peces (Baenninger, 1987).

Tabla 1.2. Especies en las cuales se ha reportado contagio del bostezo.

Especie	Autores del estudio
Chimpancés (*Pan troglodytes*)	Anderson y col., 2004
Macacos (*Macaca arctoides*)	Paukner y Anderson, 2006
Perros domésticos (*Canis familiaris*)[1]	Joly-Mascheroni, 2008
Geladas (*Theropithecus gelada*)	Palagi y col., 2009
Bonobos (*Pan paniscus*)	Demuru y Palagi, 2012
Pericos australianos (*Melopsittacus undulatus*)	Miller y col., 2012
Lobos (*Canis lupus*)	Romero y col., 2014
Ratas (*Rattus norvegicus*)[2]	Moyaho y col., 2015
Ovejas (*Ovis aries*)	Yanezawa y col., 2016
Orangutanes (*Pongo pygmeus*)	van Berlo y col., 2020
Elefantes (*Loxodonta africana*)	Rossman y col., 2020
Cerdos (*Sus scrofa*)	Norscia y col., 2021
Leones (*Panthera leo*)	Casetta y col., 2021

[1]El contagio lo evocó un individuo humano, no un conespecífico.
[2]Una sublínea de ratas criada selectivamente para bostezar.

Se ha sugerido que el contagio del bostezo requiere que los individuos posean habilidades cognitivas, en particular una capacidad empática (Platek y col., 2003, Palagi y col., 2009, Romero y col., 2014). Sin embargo, el hecho de que se haya encontrado contagio de bostezo en al menos una especie en la que se espera que los individuos tengan niveles básicos de empatía (*i.e.* las ratas) contrasta con la hipótesis. Por lo tanto, podría existir un mecanismo distinto, o adicional, que estuviera involucrado en el contagio del bostezo; o incluso, que no se requiriera de una capacidad empática cognitiva (*i.e.* la capacidad de conceptualizar el estado de otro individuo, Baron-Cohen y Wheelwright, 2004) como varios autores han insistido hasta ahora. Encontrar contagio de bostezo en otros grupos de animales como los peces permitiría concluir que esta capacidad es ubicua, especialmente si se asume que la empatía en ellos es limitada (Salena y col., 2021).

1.5. Betta splendens

El pez siamés (*Beta splendens)* es una especie de pez tropical originario de Asia –principalmente Tailandia, Vietnam y Cambodia– perteneciente a la familia Osphronemidae, popularmente conocida como gourami (Integrated Taxonomic Information System, consulta junio 2021). Estos son peces de agua dulce, perciformes (*i.e.* literalmente "peces con forma de perca"), con un órgano especializado llamado laberinto que usan para obtener oxígeno de la superficie del agua (Kang y Lee, 2010). Los peces siameses poseen un dimorfismo sexual marcado; los machos son más grandes, de colores más brillantes y con aletas más extensas que las hembras (Simpson, 1968). La cría selectiva hizo del pez siamés una especie agresiva, especialmente entre machos conespecíficos (Forsatkar y col., 2017). Muestran comportamiento agonístico muy frecuente caracterizado por una serie de pautas estereotipadas ya descritas (Simpson, 1968). Los peces siameses sólo bostezan en presencia de machos conespecíficos (Baenninger, 1987), lo que refuerza el carácter social del bostezo en ellos. Sin embargo, Baenninger (1987) sólo analizó la frecuencia del bostezo y no analizó si este tenía alguna función.

La evidencia con la que se cuenta sugiere que el bostezo del pez siamés tiene una función comunicativa, ya que los peces que observan a otros bostezando modifican su comportamiento agonístico. No obstante ese hallazgo, aun no es claro cuál es el mensaje que este comportamiento comunica. El bostezo del pez siamés durante conflictos agonistas podría comunicar apaciguamiento, es decir una invitación a reducir la actividad motora (*i.e.* la frecuencia de desplantes agonísticos y el desplazamiento dentro de la pecera) o la capacidad

fisiológica del bostezador (*i.e.* la capacidad de obtener y monopolizar recursos). Además, investigar si el bostezo se contagia en peces podría dar información comparativa valiosa sobre el rol de la empatía en el contagio del bostezo. Por lo tanto, en esta tesis me planteé responder las siguientes preguntas:

¿Comunica el bostezo del pez siamés una invitación a la calma o la capacidad fisiológica?

¿En el pez siamés el bostezo tiene la función de apaciguamiento? ¿En el pez siamés macho el bostezo funciona como una señal honesta de su capacidad fisiológica?

¿Existe contagio de bostezo en el pez siamés?

2. HIPÓTESIS

2.1. Hipótesis de la función comunicativa del bostezo

Hipótesis 1: El bostezo del pez siamés comunica apaciguamiento.

Predicciones de la hipótesis de apaciguamiento:

- En un encuentro entre dos peces (uno débil y uno fuerte físicamente), el pez débil tendrá una menor latencia y mayor frecuencia de bostezo en comparación con el pez fuerte.
- Un tercer pez (espectador) prefiriera estar con el pez, de un par, que hubiese bostezado con más frecuencia (es decir, el pez "más apaciguado").
- En un encuentro entre dos peces, después de bostezar, estos mostraran una reducción en la varianza de su actividad motora.

Hipótesis 2: El bostezo del pez siamés comunica capacidad fisiológica.

Predicciones de la hipótesis de capacidad fisiológica:

- Si dos peces poseen una capacidad fisiológica similar, éstos bostezaran más veces durante un encuentro agonístico, a comparación de dos peces con capacidad fisiológica muy diferente.

- En un encuentro agonístico el pez que hubiese bostezado menos también fuera el primero en retroceder y escapar.

- Un tercer pez (espectador) prefiriera aproximarse al pez, de un par, que hubiese bostezado menos veces (es decir, el pez con menor capacidad fisiológica).

2.2. Hipótesis sobre el contagio de bostezo en el pez siamés

Hipótesis: El bostezo se contagia en el pez siamés.

Predicción del contagio del bostezo del pez siamés:

- La frecuencia de bostezo de un pez que ocurra dentro de una ventana de tiempo de 4 minutos, siguientes al bostezo de otro pez, fuera significativamente mayor que si el bostezo del otro pez ocurre fuera de esa ventana de tiempo.

3. OBJETIVO GENERAL

Conocer la función comunicativa del bostezo en el pez siamés y buscar si existe contagio de bostezo en esta especie.

3.1. Objetivos particulares

- Determinar si existe una relación entre la capacidad fisiológica de los peces con la frecuencia de bostezo.
- Determinar si existe una relación entre la actividad motora de los peces (desplazamiento y frecuencia de desplantes) y cambios en la frecuencia de bostezo.
- Determinar si el contagio de bostezo ocurre entre peces siameses.
- Determinar el efecto que tiene presenciar bostezo en el comportamiento de individuos observadores.
- Proponer un procedimiento experimental que permita estudiar el contagio de bostezo en peces.

4. MATERIAL Y MÉTODOS

Para poner a prueba experimental las predicciones que se desprenden de las hipótesis que propuse, realicé pruebas de comportamiento en las cuales enfrenté pares de peces siameses (en adelante**, peces experimentales**) a través de una partición transparente. Así mismo, un tercer pez siamés participó como espectador (en adelante, **pez observador**). Antes de exponer a los peces experimentales a las pruebas, les administré diario y por vía de inmersión uno de tres tratamientos farmacológicos posibles:

i) (17S)-17-hidroxi-10,13,17-trimetil-2,6,7,8,9,11,12,14,15,16-decahidro-1H-ciclopenta[a]fenantren-3-ona (CAS 58-18-4) o 17α-metiltestosterona (en adelante MT) un andrógeno artificial;

ii) 2-metil-N-[4-nitro-3-(trifluorometil)fenill]propanamida (CAS 13311-84-7) o flutamida (en adelante FLU), un antagonista del receptor de andrógenos;

iii) etanol (CAS 64-17-5, en adelante CTL), como control.

Antes y después de la administración de los tratamientos farmacológicos medí el tamaño corporal y capacidad fisiológica, a través de una prueba de nado forzado, de cada pez experimental y observador.

Las pruebas de comportamiento consistieron en encuentros agonísticos entre pares de peces experimentales. Cada pareja de peces experimentales recibía uno de los seis tratamientos experimentales posibles (*i.e.* MT contra MT, MT contra FLU, MT contra CTL, FLU

contra FLU, FLU contra CTL, CTL contra CTL). Como mencioné antes, un tercer pez observador (OBS) estaba presente en cada prueba sin interactuar con los otros dos peces. Con las pruebas de comportamiento obtuve información sobre la frecuencia de bostezo y las pautas de comportamiento agonístico de los peces experimentales, además de la preferencia "por estar con alguien" del pez observador. Usé la información obtenida de las pruebas de comportamiento para establecer la función comunicativa del bostezo y para determinar si existe contagio de éste; los tratamientos farmacológicos solo fueron una estrategia para manipular la capacidad fisiológica de los peces. A continuación, describo con profundidad el procedimiento experimental que seguí (Figura 4.1).

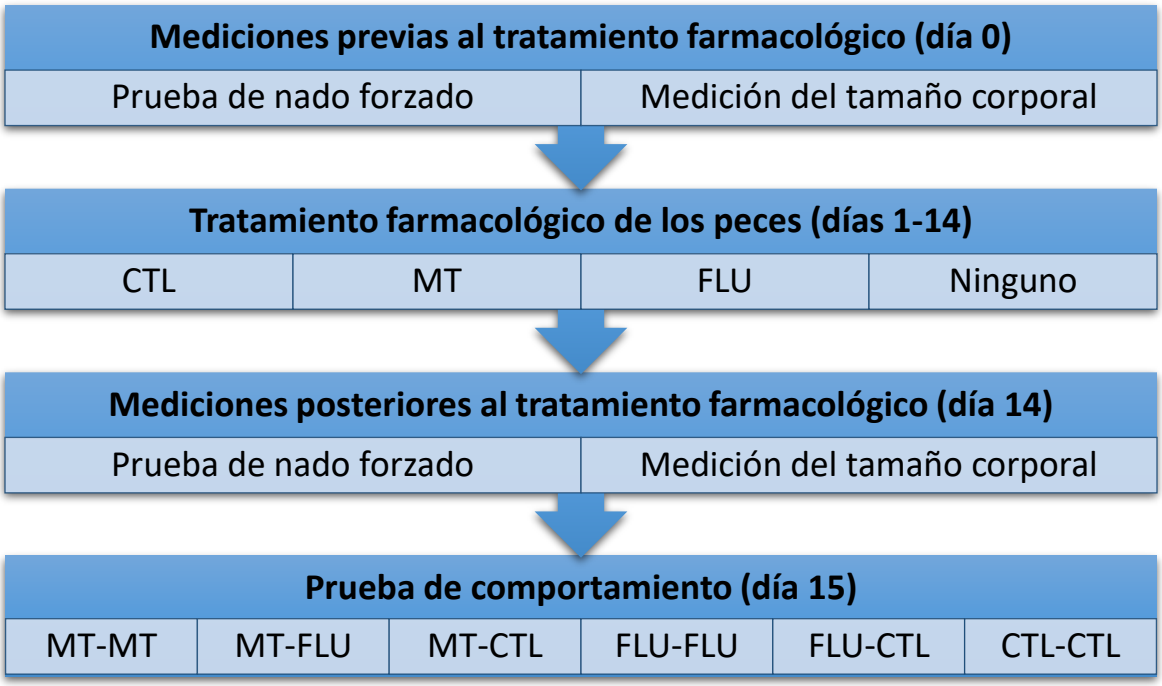


Figura 4.1. Resumen del procedimiento experimental que seguí en todos los bloques. Ver el texto para una explicación al respecto.

El proyecto fue revisado por el comité académico del Instituto de Fisiología de la Benemérita Universidad Autónoma de Puebla, y fue aprobado antes de comenzar las pruebas. Todas las pruebas experimentales se realizaron en el Laboratorio de Ecología de la Conducta del mismo instituto.

4.1. Diseño de experimentos

Utilicé un diseño de bloques aleatorizados. Usé cada semana de experimentos como un criterio de bloquización, con lo cual controlé el efecto que pudiera tener el tiempo transcurrido entre las series experimentales (Moyaho y Beristain-Castillo, 2019). El experimento contó con 6 tratamientos experimentales, que constituían a cada uno de los 9 bloques experimentales (Tabla 4.1).

Tabla 4.1. Diseño de experimento: bloques aleatorizados, que implementé para este estudio.

Bloque (semana)	CTL-CTL[1]	CTL-MT[2]	CTL-FLU[3]	MT-MT[4]	MT-FLU[5]	FLU-FLU[6]
1						
…						
9						

[1]Encuentro agonístico entre peces que recibieron etanol
[2]Encuentro agonístico entre un pez que recibió etanol y otro que recibió 17α-metiltestosterona
[3]Encuentro agonístico entre un pez que recibió etanol y otro que recibió flutamida.
[4]Encuentro agonístico entre dos peces que recibieron 17α-metiltestosterona
[5]Encuentro agonístico entre un pez que recibió 17α-metiltestosterona y otro que recibió flutamida.
[6]Encuentro agonístico entre dos peces que recibieron flutamida.

Conté con 197 machos del pez siamés repartidos en los 9 bloques experimentales, 22 peces por bloque excepto el bloque inicial que incluyó 21 peces. No era posible en un mismo día aplicar la prueba de nado forzado a los 22 peces de cada bloque, así que decidí dividirlos en 3 subgrupos (I, II y III). Los subgrupos I y III contaron con 7 peces cada uno, y el subgrupo II contó con los 8 peces restantes. Los subgrupos recibieron los mismos tratamientos de forma escalonada y en días sucesivos (Tabla 4.2). Realicé todas las pruebas experimentales en orden aleatorio, y los peces también fueron seleccionados de forma aleatoria.

Tabla 4.2. Cronograma de trabajo para cada bloque experimental.

	Día experimental																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
I[1]	NF[2]	Tratamiento farmacológico													NF	PC[3]		
II		NF	Tratamiento farmacológico													NF	PC	
III			NF	Tratamiento farmacológico													NF	PC

[1]I, II y III subgrupos en los cuales dividí a los peces de cada bloque.
[2]Prueba de nado forzado.
[3]Prueba de comportamiento.

4.2. Animales de estudio

Utilicé peces machos de la especie *Betta splendens*, o pez siamés. La edad de los peces varió entre 4 y 6 meses de edad, periodo en el cual los peces alcanzan la etapa adulta y también es cuando se ha estudiado el comportamiento agonístico de estos (Simpson, 1968). Los peces fueron adquiridos comercialmente en acuarios locales y mantenidos en el laboratorio durante

una semana previa al inicio de los experimentos; el objetivo de este procedimiento fue permitir que los peces se aclimataran a las condiciones en el laboratorio.

Antes de las pruebas experimentales los peces fueron alojados en peceras individuales, tres paredes d estaban cubiertas con papel *Kraft* opacas para evitar que interactuaran entre ellos, y para que su comportamiento agresivo disminuyera (Baenninger, 1987). Las peceras contenían 3 L de agua purificada (purificador de 5 etapas con luz ultravioleta marca Evans, modelo WP-1) y una piedra porosa conectada a una bomba que proveía intermitentemente aire al agua. Los peces fueron mantenidos bajo un fotoperiodo de 12 horas (las luces se encendían a las 7 am) y fueron alimentados dos veces al día con alimento comercial para peces siameses (BettaMin, marca Tetra). Usé a cada macho una sola vez para evitar que la experiencia previa modificara el comportamiento de los peces durante la prueba de comportamiento.

4.3. Mediciones previas al tratamiento farmacológico

4.3.1. Prueba de nado forzado previa al tratamiento farmacológico

Los experimentos de cada bloque comenzaban con la medición de la capacidad fisiológica de los peces experimentales y observadores usando una prueba de nado forzado (día 0 experimental). Esta prueba consiste en medir el tiempo proporcional que un pez nada contra una corriente constante de agua.

En esencia la prueba se trata de introducir a un pez en un canal de nado con forma de prisma cuadrangular (21 x 9 x 7 cm), con sus aristas construidas de madera sin tratar y las paredes recubiertas de malla suave color verde musgo (Figura 4.2 y 4.3). Los colores y materiales se aproximan tanto como es posible a las condiciones de hábitat natural de los peces (Brammah, 2015), logrando así disminuir el estrés que pudieran experimentar durante las manipulaciones experimentales. En un extremo del canal se coloca un espejo (9 x 7 cm) con una abertura circular lo suficientemente grande (2 cm de diámetro) como para insertar la boquilla de salida de una bomba de acuario (Micro Multifunction Pump, Moon's Aquariums). El espejo sirve para motivar a los peces a nadar contra una corriente para enfrentar a su reflejo (un adversario potencial), mientras que la bomba sirve para generar la corriente de agua. A cada lado de la abertura circular es añadido un rectángulo de acrílico transparente formando un ángulo oblicuo (3 x 7 cm). Estas piezas de acrílicos sirven para conducir al pez a la zona frente al espejo donde la corriente de agua es más intensa. Diseñé las características del canal de nado teniendo en cuenta el comportamiento y dimensiones del pez siamés; confirmé su funcionalidad en las pruebas piloto. E incluso, el aparato ya ha mostrado su utilidad experimental con otras especies de peces (Powell y col., 2020).

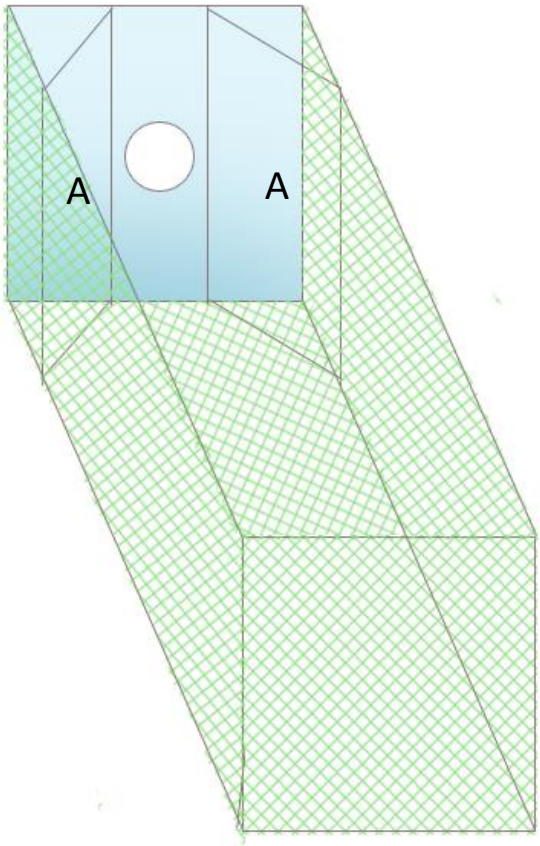


Figura 4.2. Ilustración del canal de nado que utilicé para medir la capacidad fisiológica de los peces experimentales y observadores. El espejo se encuentra en la parte superior de la ilustración, con la abertura circular marcada en el centro. A) Coloqué dos piezas de acrílico transparente a cada lado de la abertura circular para conducir al pez a la zona donde la corriente de agua es más intensa.

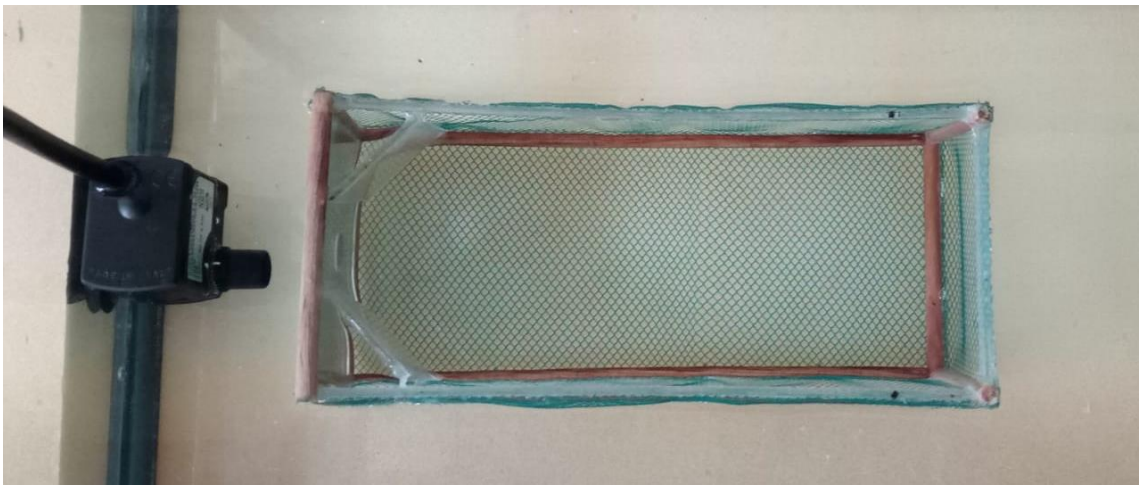


Figura 4.3. Fotografía del canal de nado, vista superior. La bomba y el espejo son visibles del lado izquierdo de la fotografía.

En cada prueba experimental colocaba el canal de nado dentro de una pecera de vidrio (40 x 25 x 15 cm) con 6 cm (profundidad) de agua nueva y limpia. En pruebas piloto observé que esta columna de agua era la suficiente para realizar la prueba, a la vez que evitara que los peces pudieran saltar y escapar (Figura 4.4). Al inicio de cada prueba experimental cubría el espejo con un acrílico opaco (8.5 x 7 cm) y luego colocaba al pez experimental dentro del canal de nado durante 10 minutos para que se acostumbrara a la condición novedosa. Pasado ese tiempo removía el acrílico opaco para permitir que el pez viera su reflejo; un minuto después encendía la bomba para generar la corriente de agua. La intensidad de la corriente de agua (45 mL/s) fue la necesaria para estimular al pez a nadar en contra, sin que sus aletas resultaran dañadas. La elección de esta intensidad surgió de los resultados de las pruebas piloto, en estas pruebas descarté que la prueba dañara a los peces piloto.

La prueba de nado forzado duraba 20 minutos (30 minutos en total contando el periodo de la aclimatación). Los peces experimentales de cada bloque fueron puestos a prueba en un orden aleatorio. Videograbé todas las pruebas experimentales para analizarlos posteriormente. Al final de cada prueba de nado forzado capturaba al pez con una red de acuario y lo colocaba en otra pecera para tomarle las mediciones morfológicas complementarias.

Figura 4.4. Fotografía representativa de una prueba de nado forzado. El pez se encuentra en la parte superior de la imagen, observando su reflejo.

4.3.2. Medición del tamaño corporal de los peces previo al tratamiento farmacológico

Al final de la prueba de nado forzado colocaba a cada pez en turno en una pecera de vidrio con agua limpia (15.5 x 5 x 10 cm) donde lo dejaba descansar por un periodo de 10 a 15 minutos. La pecera estaba rodeada de cartón para crear un ambiente calmado y así evitar estresar al pez. Para obtener la longitud estándar de cada pez introducía una red a la pecera y con ella empujaba al pez suavemente a una de las paredes, donde tenía puesta (por afuera) una regla común. Medía a cada pez la longitud que va del extremo de la boca al pedúnculo caudal, aproximando la medición al milímetro más cercano. Posteriormente obtuve la masa corporal de cada pez pesando un recipiente de plástico con agua con y sin el pez: la diferencia es una estimación de la masa corporal. Obtuve mediciones de la masa corporal de cada pez con una precisión de 0.01 g. Al final del experimento colocaba a cada pez (experimental u observador) en una pecera de alojamiento, acondicionada como describí anteriormente (sección 4.1). Veinticuatro horas después comenzaba el tratamiento farmacológico; esto para minimizar el efecto que la prueba inicial de nado forzado pudiera tener en la asimilación del tratamiento farmacológico.

4.4. Tratamiento farmacológico de los peces

Al día siguiente de la prueba de nado forzado comenzaba la aplicación del tratamiento farmacológico a los peces experimentales (día 1 experimental); cada uno de ellos fue asignado aleatoriamente a un tratamiento. Los tratamientos farmacológicos tenían el objetivo de modificar la capacidad fisiológica de los peces experimentales, para esto decidí utilizar un

andrógeno artificial (MT), un antagonista al receptor de andrógenos (FLU) el vehículo como control (CTL).

Cada día y durante 14 días añadía 50 µL de una solución stock del fármaco correspondiente a la pecera de cada pez experimental, de modo que la concentración final fuera la buscada: 1) MT: 9 x 10^{-9} M/g; 2) FLU: 1.1 x 10^{-7} M; 3) CTL: 2.85 x 10^{-4} M. Cada pez experimental recibió un solo fármaco durante los 14 días que duró el tratamiento farmacológico, y cinco peces de cada bloque recibieron todos el mismo fármaco (Tabla 4.3). Para cada tratamiento farmacológico conté con un pez que fungió como reserva, en caso de que alguno de los peces experimentales enfermara. Los siete peces observadores estuvieron en condiciones idénticas a las de los peces experimentales, pero no recibieron ningún tratamiento farmacológico.

Tabla 4.3. Resumen del plan de la aplicación del tratamiento farmacológico.

	Tratamiento farmacológico			
	MT	**FLU**	**CTL**	**Ninguno**
Número de peces	4	4	4	6
Número de reservas	1	1	1	1

4.5. Mediciones posteriores al tratamiento farmacológico

El día 14 experimental volvía a aplicar la prueba de nado forzado a todos los peces experimentales. Realizaba las pruebas siguiendo el mismo procedimiento que describí anteriormente (sección 4.3.1). Videograbé cada prueba experimental para el análisis posterior

de las pautas de comportamiento de interés (ver más abajo). Al final de cada prueba de nado forzado volvía a medir la longitud estándar y masa corporal de cada uno de los peces experimentales. Y lavaba la pecera de alojamiento de cada pez y llenaba con agua limpia (*i.e*. libre de tratamiento farmacológico).

4.6. Prueba de comportamiento

Al día 15 experimental realizaba las pruebas de comportamiento, ordenadas aleatoriamente, para determinar la función comunicativa del bostezo y el posible contagio de éste. Puse a prueba experimental a los pares de peces experimentales de acuerdo con los fármacos recibidos (*e.g.* control y flutamida), y en la presencia de un pez observador (Tabla 4.4). Con este fin fue construida una pecera con cinco compartimentos (Figura 4.5 y 4.6), dos de tamaño idéntico para los dos peces experimentales y un tercero para el pez observador. El compartimento del pez observador era lo suficientemente grande para abarcar los compartimentos de los dos peces experimentales. Entre los peces experimentales y el pez observador había un compartimento vacío que servía para minimizar el efecto de la presencia del pez observador en los peces experimentales; este es un procedimiento descrito con anterioridad (Dzieweczynski y col., 2006). Todos los compartimentos estaban sellados y, por lo tanto, no hubo comunicación química entre los peces. En los compartimentos de los peces experimentales puse una pared opaca de vidrio esmerilado (7 cm de longitud), cuyo espacio así formado servía como un escondite para el pez, en caso de buscar ocultarse del otro pez. El

compartimento del pez observador no estaba iluminado, lo que evitaba que los otros dos peces lo percibieran.

Tabla 4.4. Plan que describe las 6 pruebas de comportamiento que constituían cada bloque experimental.

Prueba	Compartimento 1	Compartimento 2	Compartimento del observador
1	MT[1]	MT	OBS[2]
2	MT	FLU[3]	OBS
3	MT	CTL[4]	OBS
4	FLU	FLU	OBS
5	FLU	CTL	OBS
6	CTL	CTL	OBS

[1] Peces que recibieron el tratamiento farmacológico de 17α-MT.
[2] Peces que no recibieron tratamiento farmacológico y fungieron como observadores.
[3] Peces que recibieron el tratamiento farmacológico de flutamida.
[4] Peces que recibieron el tratamiento farmacológico control (etanol).

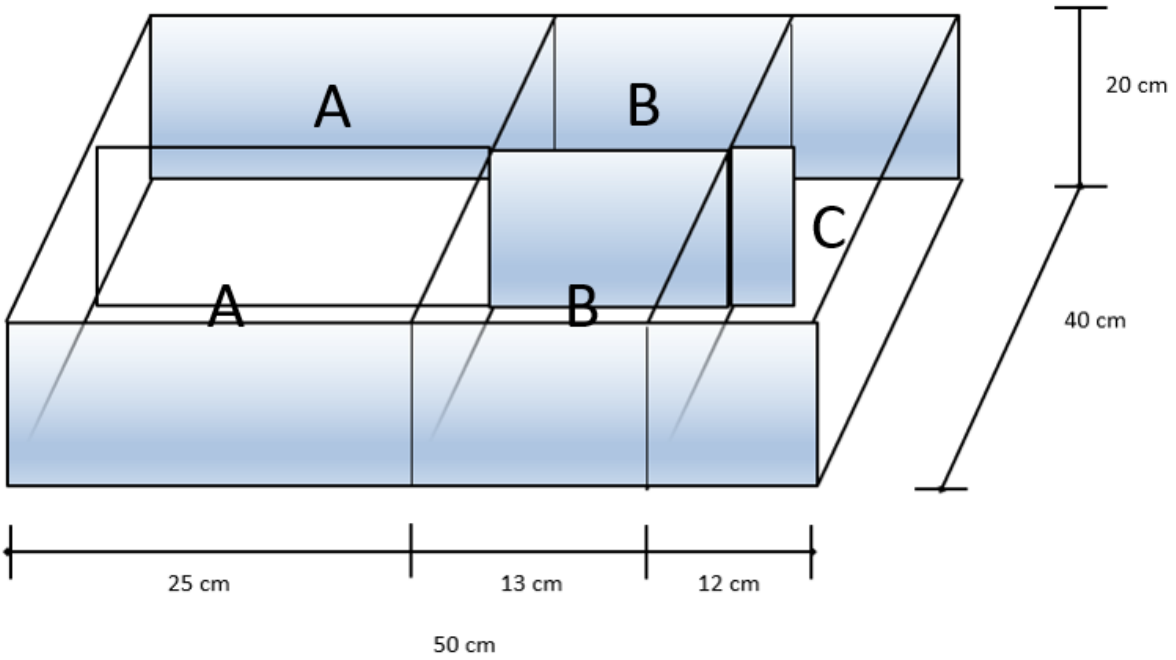


Figura 4.5. Representación esquemática de la pecera experimental. **A)** Los dos compartimentos de los peces experimentales; **B)** compartimento que solo contenía agua y servía para minimizar el efecto del pez observador en los peces experimentales; **C)** compartimento del pez observador.

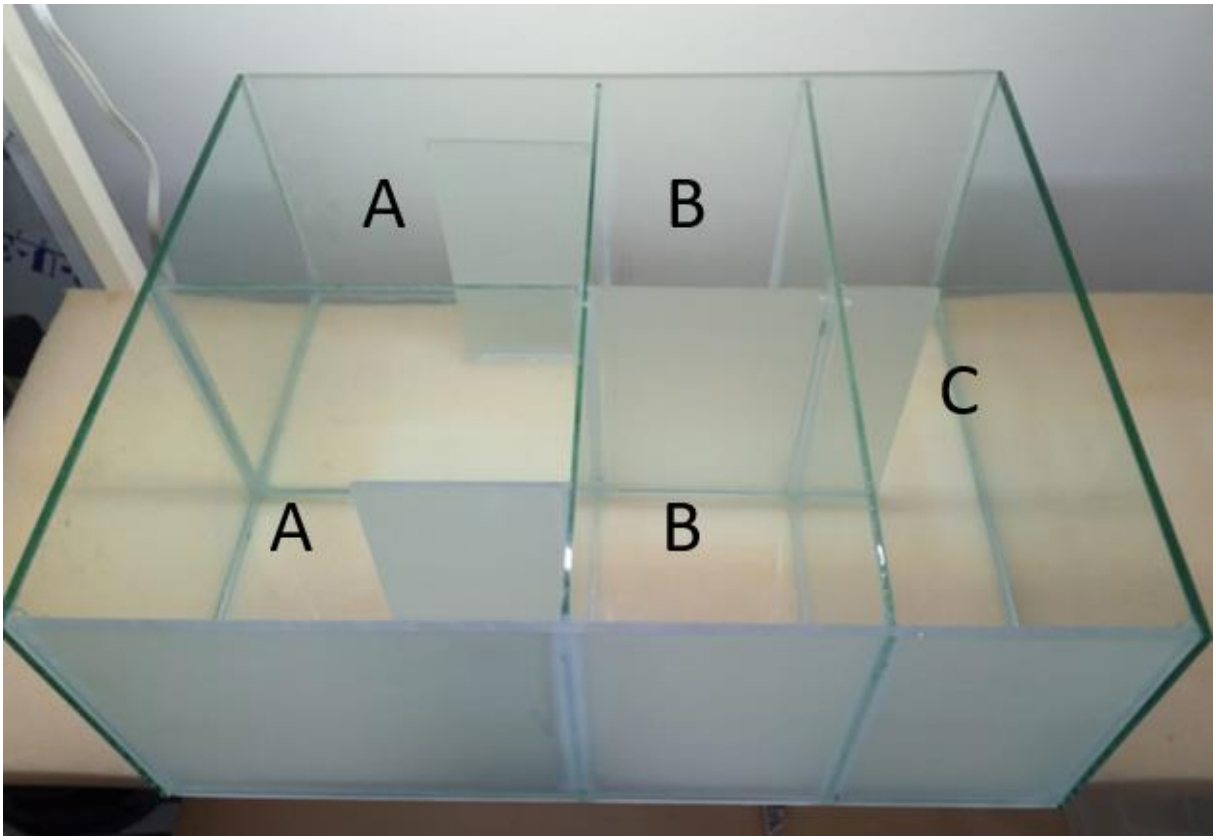


Figura 4.6. Fotografía de la pecera experimental. **A)** Los dos compartimentos de los peces experimentales; **B)** compartimento que solo contenía agua y servía para minimizar el efecto del pez observador en los peces experimentales; **C)** compartimento del pez observador.

Al inicio de cada experimento llenaba con agua limpia los 5 compartimentos, cuyas divisiones habían sido cubiertas previamente con acrílico opaco; luego colocaba a cada pez en su compartimento respectivo. Esperaba 10 minutos para permitir que los peces se aclimataran a la condición novedosa. Pasado este tiempo, removía los acrílicos opacos lo que permitía el contacto visual entre los peces. La prueba de comportamiento duraba 20 minutos durante los cuales videogrababa todos los sucesos para el análisis posterior del comportamiento de los peces. Al concluir la prueba regresaba a cada pez a su pecera de alojamiento, lavaba todo el material y preparaba el arreglo experimental para la siguiente prueba.

4.7. Descripción de las pautas de comportamiento

Cada prueba de nado forzado y de comportamiento fue videograbada para tener un registro permanente y facilitar su análisis posterior. Una persona ajena a las expectativas del experimento revisó los videos y registró el comportamiento de los peces usando las siguientes definiciones.

Bostezo. Una apertura lenta de la boca, la retención de esa posición durante algunos instantes y finalmente un cierre más rápido de la misma. La persona registraba la latencia de cada bostezo y la identidad del bostezador.

Tiempo de nado. Tiempo durante el cual el pez nadaba activamente contra la corriente de agua durante la prueba de nado forzado.

Tiempo de descanso. Tiempo durante el cual el pez era arrastrado por la corriente de agua, o el tiempo que permanecía inmóvil contra una de las paredes de malla del canal de nado.

Actividad motora del pez. Medí la actividad motora de los peces experimentales de dos formas: la distancia que los peces experimentales recorrían dentro de su compartimento durante la prueba de comportamiento, medido con el programa ToxTrac (Rodríguez y col., 2018) y la frecuencia de comportamientos agonísticos de los peces experimentales (*i.e.* mordidas y aperturas del opérculo, la membrana ósea que cubre las agallas). Por otra parte, definí la actividad motora de los peces observadores como el número de veces que cruzaban el centro de su compartimento durante las pruebas de comportamiento.

Escape del pez. Cuando uno de los peces experimentales se ocultaba tras la pared opaca en su partición.

Preferencia del observador. El pez experimental al que el pez observador se aproximaba por más tiempo; esto es, el lado de la pecera donde el pez observador pasaba más tiempo.

4.8. Análisis estadístico

Utilicé el programa estadístico R (R Core Team, 2020), y paquetes (descritos abajo) diseñados para operar con este programa, para hacer todos los análisis estadísticos. Consideré el valor de $P < 0.05$ como estadísticamente significativo. Utilicé modelos lineales generales (ML) cuando los datos cumplieron con los criterios de normalidad, lo cual comprobé con la aplicación de la prueba Shapiro-Wilk. Cuando los datos siguieron una distribución distinta: distribución gamma, apliqué modelos lineales generalizados (MLG), lo cual comprobé con el uso del paquete estadístico goft (Villaseñor y González-Estrada, 2015). En los casos en los que incluí efectos aleatorios, utilicé modelos lineales generales mixtos (MLM) y modelos lineales generalizados mixtos (MLGM), dependiendo de si los datos seguían o no una distribución normal. Para estos casos usé el paquete estadístico lme4. Cuando la variable de interés fue de naturaleza binomial, ajusté a los datos un modelo de regresión logística. Finalmente, utilicé el paquete ggplot2 para hacer las gráficas.

Variables independientes: El tratamiento con 17α-metiltestosterona, flutamida o etanol.

Variables dependientes: La actividad motora de los peces experimentales y observador, la frecuencia y latencia de bostezo de los peces experimentales y observador, la capacidad fisiológica de los peces experimentales y observador (medida como el tiempo de nado en la prueba de nado forzado) y la preferencia del pez observador.

Covariables: La masa corporal y longitud estándar de los peces.

5. RESULTADOS

Realicé los 9 bloques experimentales entre los meses de noviembre del 2018 y marzo del 2020. Conté con un total de 197 peces, aunque solo obtuve mediciones para 187 de ellos, pues 10 peces murieron o enfermaron antes del comienzo de las pruebas, en cuyo caso no formaron parte de los experimentos. Realicé un total de 53 pruebas de comportamiento, 6 en cada uno de los 9 bloques experimentales, excepto el séptimo bloque que solo contó con 5 pruebas de comportamiento porque tanto el pez experimental como su reemplazo enfermaron (Tabla 5.1). A continuación, describo los resultados de los experimentos.

Tabla 5.1. Desglose del número de experimentos realizados por bloque.

Bloque	Peces disponibles	Pruebas de comportamiento realizadas
1	21	6
2	22	6
3	22	6
4	22	6
5	22	6
6	22	6
7	22	5
8	22	6
9	22	6
Total	197	53

5.1. Resultados de las mediciones del tamaño corporal de los peces

Aquí reporto las mediciones del tamaño corporal de los 187 peces y sin distinción de tratamiento farmacológico (Tabla 5.2). La masa corporal de los peces se distribuye de forma normal, mientras que la longitud estándar se ajusta a una distribución gamma cuando los datos

son transformados (Anexo 1). Por lo tanto, en adelante utilizaré modelos lineales generales para los análisis que involucren la masa corporal y modelos lineales generalizados para los análisis que involucren la longitud estándar.

Tabla 5.2. Promedio y desviación estándar del promedio de todas las mediciones del tamaño corporal de los peces.

	Medición antes del tratamiento	**Medición después del tratamiento**
Longitud estándar (mm)	32.76 ± 0.18[1]	32.89 ± 0.19
Masa corporal (g)	0.98 ± 0.02	0.94 ± 0.02

[1] Promedio ± desviación estándar del promedio.

Analicé si existía una diferencia significativa entre el tamaño corporal de todos los peces tomado antes del tratamiento farmacológico y después de éste. Con este fin apliqué una prueba *t* de Student para muestras empatadas, cuyo resultado indicó que la masa corporal creció significativamente después de los 14 días de la aplicación del tratamiento farmacológico ($t = 8.66$, g.l. = 186, $P < 0.001$); g.l. se refiere al número de grados de libertad. Por lo que se refiere a la longitud estándar, la aplicación de un MLG a los datos no indicó alguna diferencia significativa entre las mediciones ($t = 1.68$, g.l. = 186, $P = 0.093$).

Evalué si el tratamiento farmacológico con MT, FLU o CTL (o su ausencia en el caso de los peces observadores) había tenido un efecto significativo en el tamaño corporal de los peces después de 14 días de tratamiento farmacológico. Para tal fin utilicé un MLM que apliqué a los datos de la masa corporal y un MLGM a la longitud estándar; incluí al factor bloque como un

efecto aleatorio en cada modelo estadístico. Ninguno de los tratamientos farmacológicos tuvo un efecto en el cambio de la masa corporal de los 187 peces (Tabla 5.3), aunque sí lo tuvo la masa corporal inicial (t = 31.98, g.l. = 98, P <0.001). Lo mismo ocurrió para la longitud estándar (Tabla 5.4), ya que solo la longitud estándar antes del tratamiento farmacológico tuvo un efecto significativo en la longitud estándar después del tratamiento (t = –15.47, g.l. = 98, P <0.001)

Tabla 5.3. Resumen estadístico de la aplicación del MLM a los datos de la masa corporal de los peces después de 14 días de tratamiento farmacológico.

Efectos aleatorios	**Varianza**				
Bloque	0.01				
Residual	0.01				
Efectos fijos	**Coeficiente**	**DEP**[1]	**g.l.**	**Valor *t***	**Valor *P***
CTL	0.07	0.03	67	2.31	0.023
MC[2] (antes)	0.89	0.03	98	31.98	**<0.001**
FLU	—0.01	0.01	176	—0.49	0.627
OBS	—0.01	0.01	174	—0.77	0.445
MT	—0.02	0.01	174	—1.26	0.210

[1] DEP, desviación estándar del promedio.
[2] MC, masa corporal.

Tabla 5.4. Resumen estadístico del ajuste del MLGM a los datos de la longitud estándar de los peces 14 días después del tratamiento farmacológico

Efectos aleatorios	**Varianza**				
Bloque	0.01				
Residual	0.01				
Efectos fijos	**Coeficiente**	**DEP[1]**	**g.l.**	**Valor *t***	**Valor *P***
CTL	1.38	0.04	67	35.60	<0.001
LE[2] (antes)	–0.45	0.03	98	–15.47	**<0.001**
FLU	–0.01	0.01	176	–0.57	0.568
OBS	–0.01	0.01	174	–0.44	0.660
MT	–0.01	0.01	174	–0.15	0.877

[1] DEP, desviación estándar del promedio.
[2] LE, longitud estándar.

5.1.1 Factor de condición de Fulton

Encontré una correlación evidente entre la longitud estándar y la masa corporal de los peces experimentales y observadores (n = 187), tanto antes del tratamiento farmacológico (r = 0.86, $P < 0.001$) como después del mismo (r = 0.90, $P < 0.001$). Decidí agrupar ambas mediciones en el llamado factor de condición de Fulton (Froese, 2006). Este factor (K) relaciona matemáticamente (Figura 5.1) la masa corporal y el volumen de un pez (aproximado a partir de la longitud estándar medida). En adelante incluiré los valores **K** en los análisis estadísticos en los cuales la participación del tamaño corporal de los peces esté contemplada.

$$K = \left(\frac{MC}{LE^3}\right) 100$$

Figura 5.1. Fórmula utilizada para calcular el factor de condición de Fulton.
MC = masa corporal (expresada en g); LE = longitud estándar (expresada en cm).

5.2. Análisis del comportamiento agonístico de los peces experimentales

Analicé la frecuencia de los intentos de mordida y las aperturas del opérculo que los peces experimentales (n = 104) realizaron durante las pruebas de comportamiento. Ambos comportamientos pertenecen al mismo sistema motivacional (*i.e.* agresión) y por eso decidí agruparlos en una sola categoría que llamé desplantes. Encontré que los valores de los desplantes no difieren de una una distribución gamma cuando son transformados (Anexo 2); así que procedí a ajustarles un MLGM manteniendo esa ventaja (Tabla 5.5). No obstante, los resultados indicaron que el tratamiento farmacológico no tiene un efecto significativo en la frecuencia de desplantes; tampoco lo tuvo **K**.

Tabla 5.5. Resumen estadístico del MLGM para los datos del efecto del tratamiento farmacológico en la frecuencia de desplantes de los peces experimentales.

Efectos aleatorios	**Varianza**				
Bloque	4.63×10^{-5}				
Residual	3.15×10^{-1}				
Efectos fijos	**Coeficiente**	**DEP**	**g.l.**	**Valor *t***	**Valor *P***
CTL	0.024	0.008	98	2.81	0.005
FLU	0.001	0.002	98	0.17	0.868
MT	0.001	0.002	98	0.16	0.874
K	–0.002	0.003	98	–0.79	0.428

5.3. Análisis de la frecuencia de bostezo de los peces

Registré 23 bostezos en 16 peces, 14 de los cuales fueron peces observadores y 2 peces que recibieron el tratamiento control. Durante los ensayos, 10 peces bostezaron una sola vez, 5 peces bostezaron 2 veces y 1 pez bostezó en 3 ocasiones.

Para analizar si la capacidad fisiológica tenía algún efecto en la frecuencia de bostezo de los peces, procedí como sigue. Primero estimé la capacidad fisiológica de 14 de los 16 peces que sí bostezaron usando los datos obtenidos en la prueba de nado forzado; las videograbaciones de la prueba de nado forzado de los 2 peces restantes se perdieron por problemas técnicos. Luego comprobé que el tiempo que los peces nadaron contra corriente tuviera una distribución normal, lo que así sucedió (W = 0.94, P = 0.362), y después apliqué un ML a los datos (Tabla 5.6). Sólo el resultado de la prueba de nado forzado antes del tratamiento tuvo un efecto significativo en el tiempo de nado después del tratamiento (t = 3.76, g.l. = 10, P = 0.004); los peces nadaron más tiempo, cuanto más habían nadado en la prueba anterior. Extraje los residuales de este modelo estadístico; es decir, la variación que las variables predictivas no explicaron. Asumí que tal variación residual podía tomarse como un indicador de la capacidad fisiológica de los peces, y así la usé en los análisis posteriores.

Tabla 5.6. Resumen estadístico del ajuste del ML a los datos del tiempo que los peces nadaron contra una corriente de agua, después de la aplicación del tratamiento farmacológico.

Efectos fijos	Coeficiente	DEP[1]	g.l.	Valor *t*	Valor *P*
CTL	–0.60	1.05	10	–0.57	0.580
t[2] nado (antes)	0.83	0.22	10	3.76	0.004
K	0.18	0.40	10	0.46	0.654
OBS	0.20	0.17	10	1.18	0.264

[1] DEP, desviación estándar del promedio.
[2] t, tiempo.

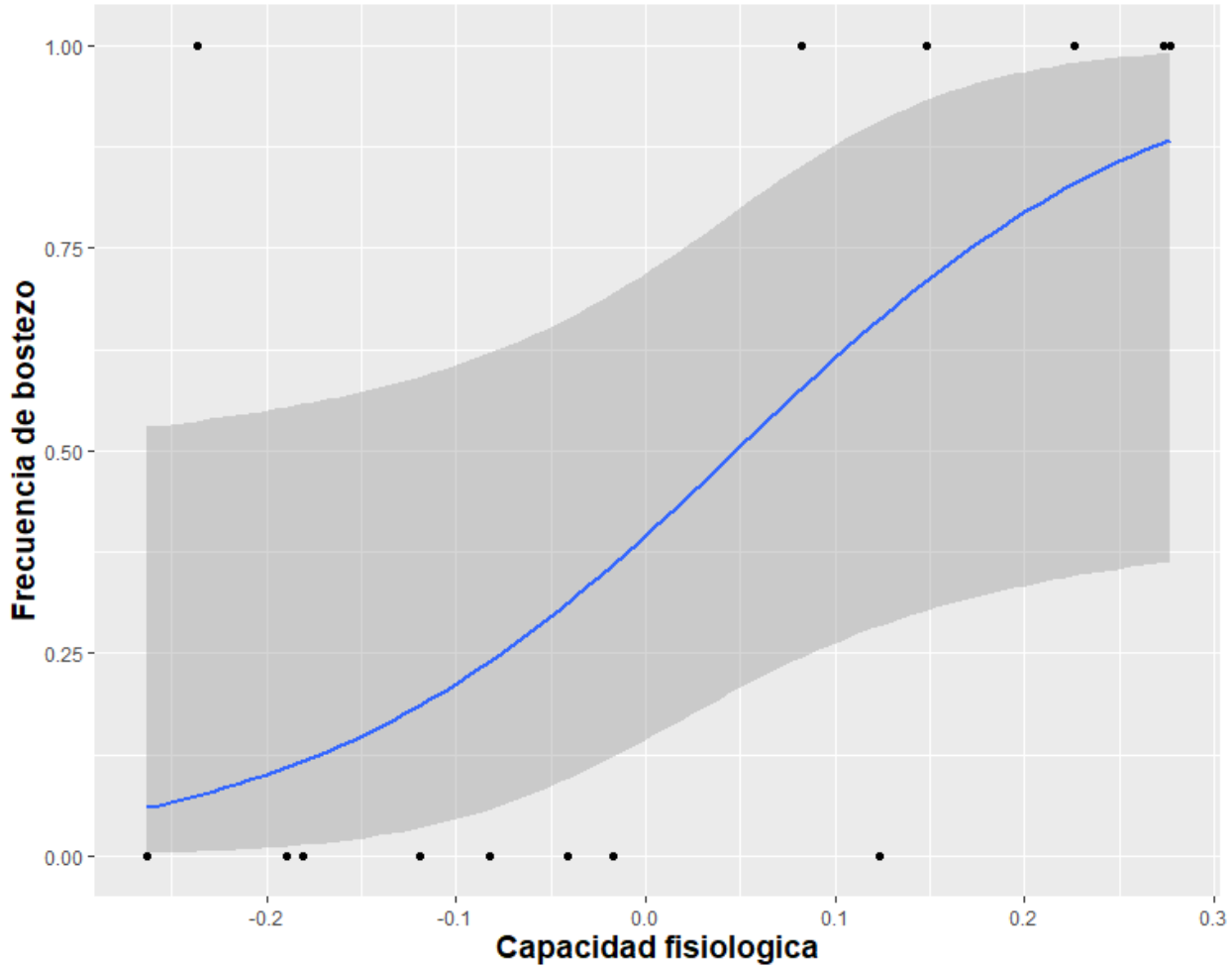
Para analizar estadísticamente el efecto de la capacidad fisiológica en la frecuencia de bostezo de los peces agrupé a los peces en dos categorías: peces que bostezaron una sola vez

(8 peces) y peces que bostezaron más de una vez (6 peces). Enseguida apliqué un modelo de regresión logística a la frecuencia de bostezo teniendo a la capacidad fisiológica de los peces como la variable predictiva. El resultado indicó que los peces bostezaron tanto más, como su capacidad fisiológica crecía (Tabla 5.7; z =1.97, g.l. = 13, P = 0.048; Gráfica 5.1).

Tabla 5.7. Resumen estadístico del ajuste de un modelo de regresión logística a la frecuencia de bostezo en función de la capacidad fisiológica de los peces.

Efectos fijos	**Coeficiente**	**DEP[1]**	**g.l.**	**Valor *z***	**Valor *P***
Intercepto	–0.42	0.69	13	–0.61	0.543
Capacidad fisiológica	8.85	4.49	13	1.97	0.048

[1] DEP, desviación estándar del promedio.



Gráfica 5.1. Gráfica que ilustra el cambio en la frecuencia de bostezo debido al incremento en la capacidad fisiológica de los peces. Los puntos en la parte inferior de la gráfica corresponden a los peces que bostezaron una sola vez, mientras que los puntos en la parte superior corresponden a los peces que bostezaron dos o tres veces. La línea azul representa el mejor ajuste de acuerdo con un modelo de regresión logística, y la franja de color gris oscuro corresponde a los intervalos de confianza al 95% de la curva.

De modo similar, ajusté un modelo de regresión logística a la frecuencia de bostezo, esta vez incluyendo a la actividad motora de los 14 peces observadores como la variable explicativa (Tabla 5.8). No encontré evidencia que sugiriera que la actividad motora de los

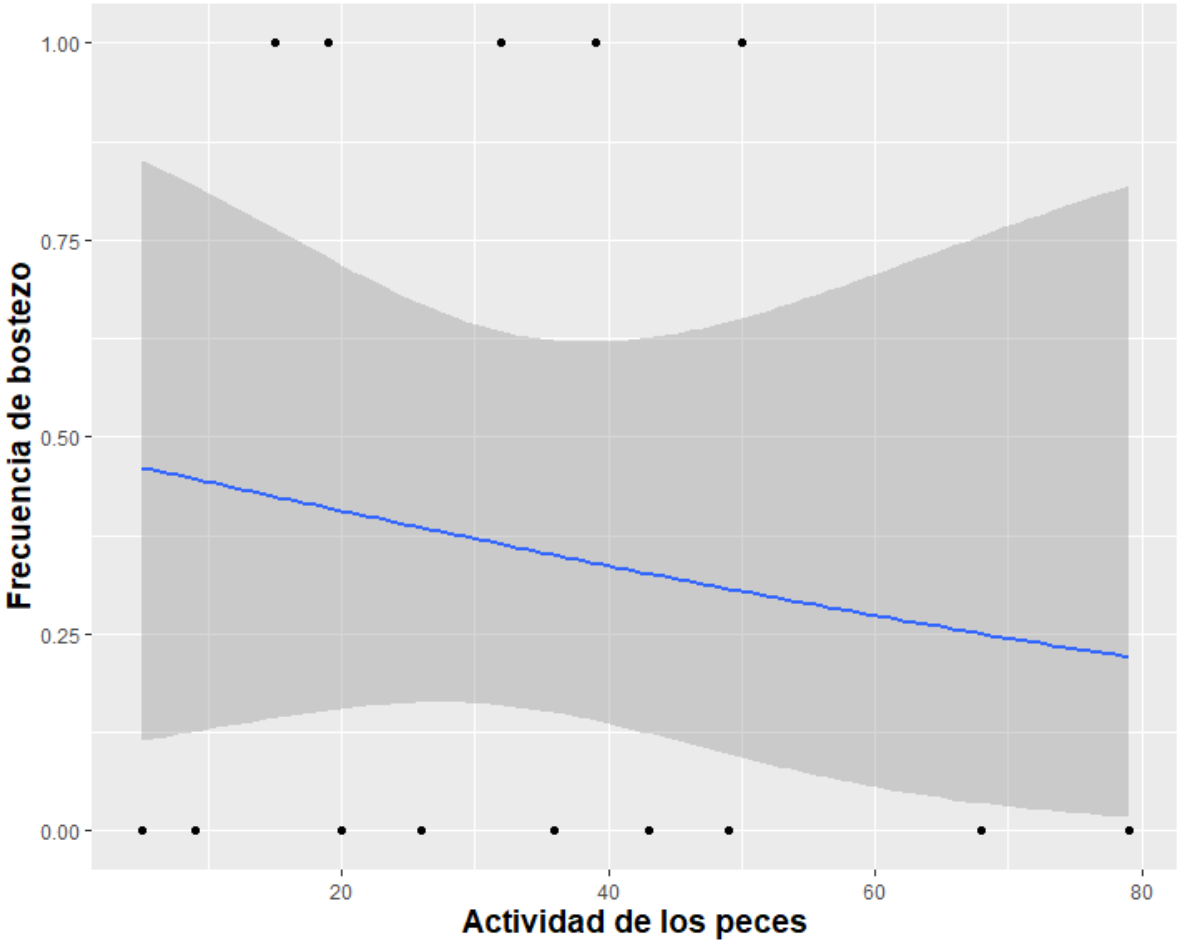
peces afectara de modo significativo la frecuencia de bostezo (z = – 0.53, g.l. = 13, P = 0.596;

Gráfica 5.2).

Tabla 5.8. Resumen estadístico del ajuste de un modelo de regresión logística a la frecuencia de bostezo en función de la actividad motora de los peces observadores.

Efectos fijos	Coeficiente	DEP[1]	g.l.	Valor *z*	Valor *P*
Intercepto	–0.08	1.09	13	–0.07	0.942
Actividad motora	–0.01	0.03	13	–0.53	0.596

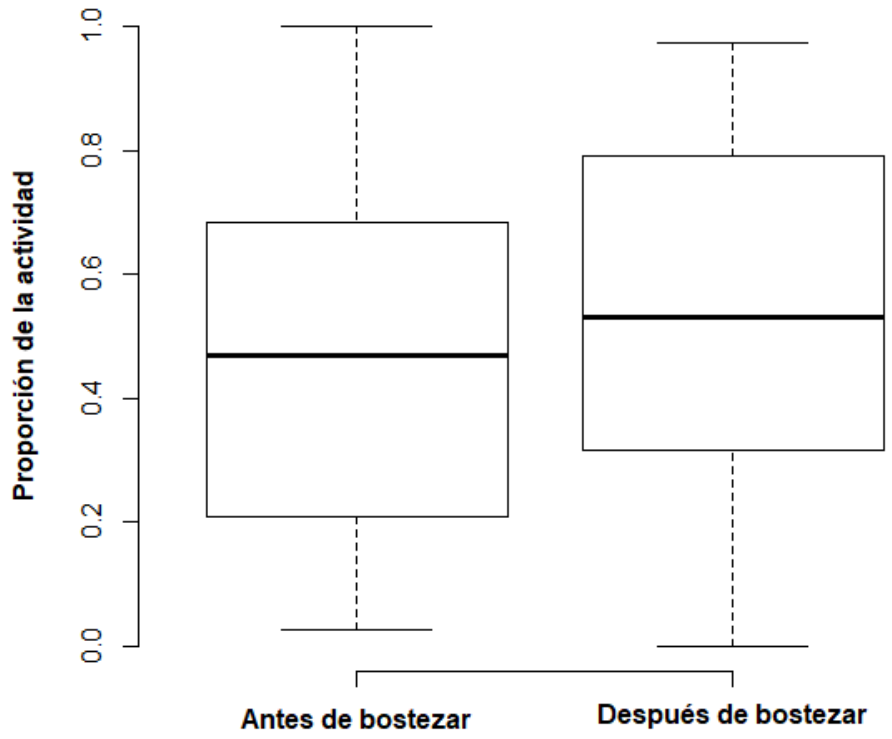
[1] DEP, desviación estándar del promedio.



Gráfica 5.2. Gráfica que ilustra la relación entre la frecuencia de bostezo y la actividad motora de los peces. Los puntos en la parte inferior de la gráfica corresponden a los peces que bostezaron una sola vez, mientras que los puntos en la parte superior corresponden a los peces que bostezaron dos o tres veces. La línea azul representa el mejor ajuste de acuerdo con un modelo de regresión logística, y la franja de color gris oscuro corresponde a los intervalos de confianza al 95% de la curva.

Investigué si el bostezo de los peces observadores tenía algún efecto en la actividad motora de los mismos peces, es decir, si los peces se apaciguaban después de bostezar. Con esta finalidad, calculé la proporción de la actividad motora del pez antes y después de bostezar, comprobé la normalidad de los datos, y después les apliqué una prueba *t* de Student para muestras empatadas. No encontré alguna evidencia que indicara que el bostezo hubiese

afectado la actividad motora de los peces observadores (t = 0.009, g.l. = 13, P = 0.993); es decir, los peces observadores mostraron los mismos niveles de actividad motora antes y después de bostezar (Gráfica 5.3).

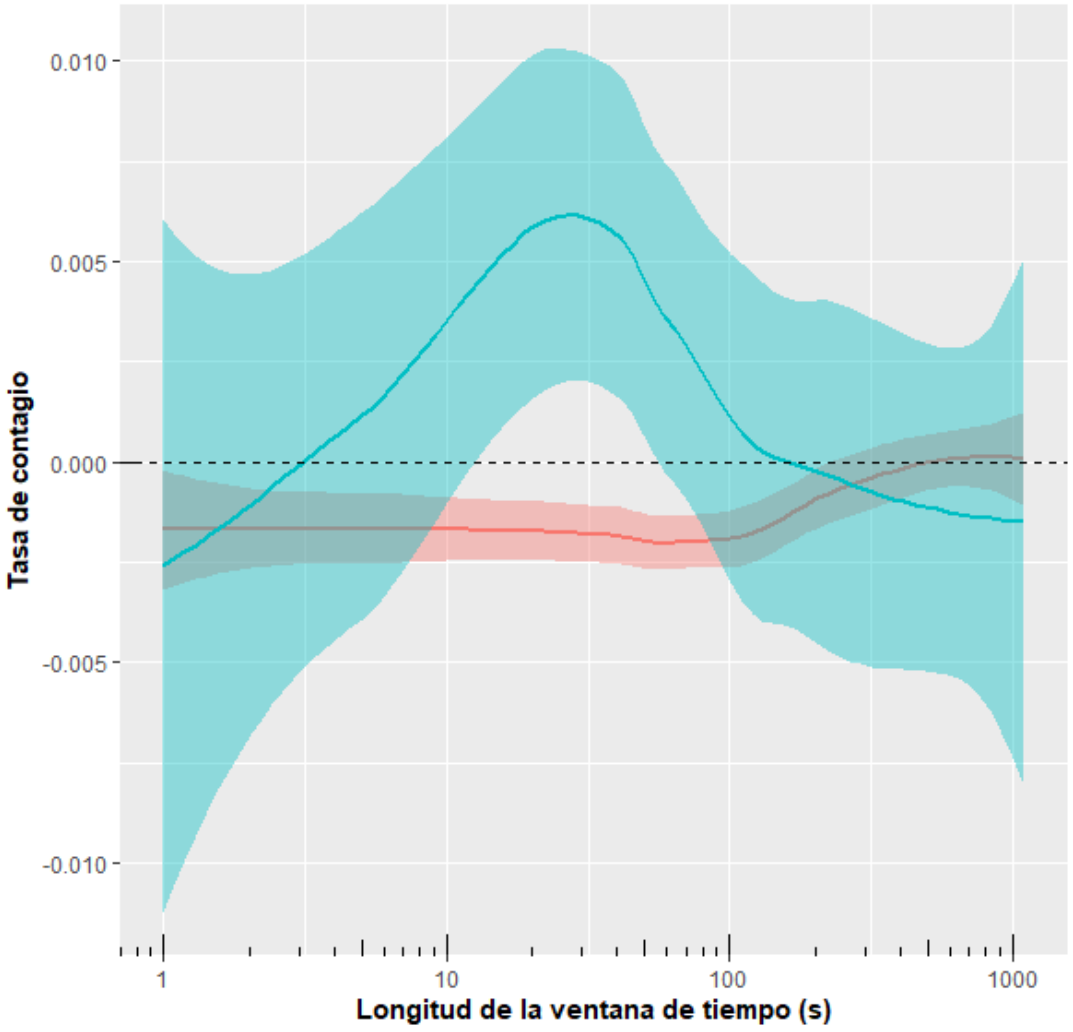


Gráfica 5.3. Gráfica de cajas que ilustrativa la proporción de la actividad motora de los peces observadores, antes y después de bostezar. La línea gruesa en cada caja representa a la mediana (*i.e.* segundo cuartil), las líneas inferior y superior de las cajas representan el primer y tercer cuartil, respectivamente. Los bigotes representan 1.5 veces el rango intercuartilar (*i.e.* la diferencia entre el tercer y el primer cuartil).

5.4. Análisis del contagio del bostezo

Sólo dos peces bostezaron en una misma prueba, dos veces cada uno; un pez recibió el tratamiento control y el otro era un pez observador. No obstante, calculé la probabilidad de

que estos bostezos hubiesen ocurrido por contagio. Seguí el procedimiento sugerido por Moyaho y col (2019), el cual calcula la probabilidad de contagio de un bostezo utilizando ventanas de tiempo de duración creciente; aquí usé ventanas de tiempo de 1, 5, 10, 20, 30, 40, 50, 60, 90, 120, 180, 240, 300, 600, 900 y 1100 s. Además, el método calcula la probabilidad de contagio asignando aleatoriamente la ubicación temporal de los bostezos, y la usa como un control. Después hice las curvas de contagio (Gráfica 5.4), como sugieren Moyaho y col. (2019). La tasa de contagio fue más alta entre 20 y 40 s después de presenciar un bostezo, lo que indica que hay una probabilidad mayor de contagio en este rango de tiempo que fuera de él.



Gráfica 5.4. Curvas de contagio para los bostezos observados (línea azul) y los bostezos control (curva roja). Las bandas de color alrededor de las líneas representan los intervalos de confianza al 95% respectivos. La escala de la abscisa es logarítmica.

.

6. DISCUSIÓN

El trabajo que aquí presento tuvo como objetivo primordial esclarecer la función comunicativa del bostezo en el pez siamés. Los resultados de un estudio previo en estos peces, indicaron que el comportamiento de un pez observador se modificaba en respuesta a la presentación de bostezos. Sin embargo, esos resultados fueron insuficientes para saber si el mensaje contenido en el bostezo, es una invitación a la calma o la indicación de una capacidad fisiológica superior del bostezador (Díaz-Loyo, 2016). Los resultados de este trabajo, diseñado para determinar la veracidad de una y otra hipótesis, sugieren que la función del bostezo en el pez siamés es comunicar la capacidad fisiológica de éste a un pez rival. La evidencia obtenida no es consistente con la hipótesis del apaciguamiento como función comunicativa del bostezo.

En general, encontré que el tratamiento farmacológico que recibieron los peces experimentales tuvo efectos distintos a los esperados. Específicamente, no encontré un efecto del tratamiento farmacológico en el tamaño corporal de los peces, y tampoco en la frecuencia de desplantes. Sin embargo, los mismos tratamientos suprimieron la frecuencia de bostezo en los peces experimentales.

Es probable que el tiempo de aplicación del tratamiento farmacológico (14 días) no fuera suficiente para producir cambios notables en el tamaño corporal de los peces. No hay trabajos previos que hayan sido planeados para evaluar la tasa de crecimiento de los peces siameses entre 4 y 6 meses de edad, por lo que no sabemos si los cambios en el tamaño corporal de los peces en 14 días fueron muy pequeños para ser medidos. Un estudio midió la

tasa de crecimiento de los peces de 0 a 105 días, cuando el crecimiento es más acelerado, y en pocas ocasiones encontraron crecimiento significativo entre mediciones sucesivas de 15 días (Kumar y col., 2016). Por otro lado, se ha encontrado que otros fármacos como la serotonina y la fluoxetina modifican el comportamiento agonístico de los peces siameses después de 14 y 6 días de aplicación, respectivamente (Clotfelter y col., 2007; Forsatkar y col., 2014). Sin embargo, en otro estudio se reportaron diferencias en el comportamiento agresivo del pez siamés después de 21 días de exposición a flutamida (Dzieweczynski y col., 2018). Estos estudios sugieren que el tiempo de aplicación del tratamiento farmacológico debió ser suficiente para haber producido cambios en la frecuencia de desplantes; no parece que fuera necesario usar un protocolo de una duración mayor, aunque es posible que una concentración diferente de los fármacos hubiera modificado los resultados.

A pesar de la carencia de efecto en el tamaño corporal y los desplantes, el tratamiento farmacológico sí tuvo un efecto en la frecuencia de bostezo de los peces. De los 16 peces que bostezaron 14 fueron peces observadores; es decir, peces que no recibieron tratamiento farmacológico. La escasez de bostezo pudo ser causada por el etanol que formó parte de todos los tratamientos farmacológicos. Esta posibilidad es compatible con el hecho de que la mayoría de los bostezos fueron observados en los peces observadores.

Se sabe que, al menos en los mamíferos, el etanol está relacionado con el sistema dopaminérgico y que interactúa con los receptores D_3 (Leggio y col. 2014). Los receptores D_3, particularmente en el núcleo paraventricular, constituyen uno de los sistemas mejor estudiados

para inducir bostezo (Blin y col., 1990; Collins, 2005; Collins y Eguibar, 2010). Se ha reportado que el etanol produce bostezos en otros vertebrados, como los monos Rhesus (Czoty y col. 2018) y las ratas (Heaton y Varrin, 1991), aunque se desconoce lo que ocurre en peces. La aparente contradicción entre los resultados reportados para mamíferos y lo que yo obtuve aquí, podría resolverse con base en dos aspectos: i) el bostezo provocado por la estimulación de los receptores D_3 es dependiente de la dosis; concentraciones altas inhiben el bostezo mediante la activación de los receptores D_2 (Collins y col., 2005, 2007). Un estudio posterior podría estar encaminado en describir una posible relación dosis-respuesta entre la frecuencia de bostezo de los peces y la dosis de etanol; ii) es posible que el mecanismo fisiológico que desencadena el bostezo en los peces sea distinto al de otros vertebrados. Estudios previos han revelado la existencia de una homología entre el sistema dopaminérgico del cerebro de los teleósteos y el cerebro de los mamíferos (Matsui, 2017), incluso, se ha reportado la presencia de receptores D_1 y D_2 en el cerebro de los peces (O'Connel y col., 2011; Messias y col., 2016), pero no de receptores D_3. La evidencia que obtuve en el presente estudio podría sugerir que el bostezo de los peces no depende de la activación mediante etanol de los receptores D_3, pero si ser inhibido por la activación de los receptores D_2.

El tratamiento farmacológico inhibió la frecuencia de bostezo de los peces experimentales, lo cual impidió corroborar las predicciones que propuse inicialmente. Sin embargo, busqué extraer información compatible con la que había planteado obtener con las predicciones iniciales. Considero que la información así obtenida proporciona evidencia

experimental consistente con las hipótesis de la función comunicativa del bostezo del pez siamés y que, por lo tanto, confiere validez a los objetivos del estudio.

Si la hipótesis del apaciguamiento fuera cierta, esperaría encontrar que los peces siameses menos activos también fueran aquellos que bostezaran más. Sin embargo, no encontré relación entre la actividad motora de los peces observadores y su frecuencia de bostezo. Más aun, encontré que los peces observadores mantuvieron sus niveles de actividad después de bostezar, contrario a lo que esperaría que sucediera si el bostezo del pez siamés comunicara una invitación a la calma. Estudios previos en avestruces (Sauer y Sauer, 1967) revelaron que estas aves disminuyen su actividad después de bostezar, incluso, otros estudios han permitido relacionar al bostezo con la transición entre diferentes niveles de actividad (Baenninger, 1997; Leone y col., 2014, Casetta y col., 2021). No obstante, yo no encontré que los peces siameses modifiquen su actividad motora después de bostezar. Sin embargo, no tengo la certeza plena de que los peces experimentales no hayan percibido a los peces observadores y, por lo tanto, no puedo saber si los bostezos de éstos tuvieron algún efecto en los peces experimentales. Pese a este inconveniente, la evidencia que reuní sugiere que es poco probable que los peces siameses bostecen con la intención de apaciguar a un rival.

Por otro lado, encontré que los peces con capacidad fisiológica mayor también fueron los que bostezaron con más frecuencia. Esta relación es consistente con lo sugerido anteriormente para una cepa de ratas criada por su frecuencia elevada de bostezo (Moyaho y col., 2015). La capacidad fisiológica es una condición (estado) interna que permite a los

animales (generalmente machos) afrontar conflictos agonísticos, ya sea como resistencia física (la medición que yo hice en la prueba de nado forzado), fuerza física, o velocidad de respuesta, entre otros. En general, estas características dependen de los niveles de testosterona del individuo (Sinervo, 2000). Con el fin de evitar un conflicto potencialmente costoso, los animales suelen comunicar su capacidad fisiológica a un rival, ya sea a través de pautas elaboradas de comportamiento (Reby y McComb, 2003) o de rasgos morfológicos conspicuos (Sinervo, 2000). Para que esta estrategia de comunicación sea estable (*i.e.* los individuos de una población la han adoptado) la señal empleada debe ser honesta; es decir, que esté respaldada (*e.g.* masa muscular, testosterona, etc.), lo cual generalmente implica un costo (Smith, 1994). Por ejemplo, el costo del bostezo puede ser dividido en dos partes: i) el que está asociado a la concentración de testosterona (Graves y Wallen, 2006), pues ésta tiene un efecto inmunosupresor. Por lo tanto, una frecuencia relativamente alta de bostezo también dejaría al animal más vulnerable a infecciones, entre otras cosas; ii) el bostezo es un comportamiento estereotipado, y una vez que se inicia no puede detenerse. Un animal que está bostezando es vulnerable a sufrir agresiones físicas por animales rivales, especialmente si el bostezo está acompañado del cierre de los ojos. Así, el bostezo parece cumplir con las características de una señal honesta en los peces siameses; el bostezo parece ser un indicador de la capacidad fisiológica, apoyado ésto por la relación que encontré entre la frecuencia de bostezo y la capacidad fisiológica de los peces. Por lo tanto, la hipótesis de la capacidad fisiológica parece explicar mejor la función comunicativa del bostezo en el pez siamés.

Esto confirmaría observaciones previas que mencionan al bostezo del pez siamés como una pauta de desplante agonístico entre machos (Simpson, 1968; Beanninger, 1987). El comportamiento agonístico del pez siamés está ampliamente ritualizado e incluye el despliegue de las aletas de los peces (Simpson, 1968). La longitud de las aletas de los machos del pez siamés es un carácter dimórfico y que depende de los niveles de andrógenos (Kumar y col., 2016) pero no parece participar en las peleas entre machos (Allen y Nicoletto, 1997). Los autores sugieren que la longitud de las aletas de los machos podría ser un carácter favorecido por la selección de las hembras. Por lo tanto no esperaríamos que la longitud de las aletas sea una señal de la capacidad fisiológica de los peces, mientras que el bostezo si parece serla.

Finalmente, calculé la probabilidad de contagio del bostezo en una ocasión, cuando dos peces bostezaron durante el mismo ensayo. El resultado del método que utilicé (Moyaho y col., 2019) sugiere que es probable que los bostezos observados en esos peces hayan sido producto del contagio. El mismo resultado anticipa que el contagio del bostezo del pez siamés debería ocurrir dentro de una ventana de entre 20 y 40 segundos. Desde luego, solo pude calcular el contagio de bostezos en una pareja de peces, por lo que no puedo generalizar el hallazgo. Por lo demás, este resultado sugiere que el método de análisis de contagio puede ser aplicado en estudios posteriores en el pez siamés para confirma la ventana óptima de contagio de 20 a 40 segundos.

7. CONCLUSIÓN

Con base en la evidencia aquí reunida, concluyo que el bostezo del pez siamés comunica la capacidad fisiológica del bostezador. Luego de contrastar experimentalmente la hipótesis de la capacidad fisiológica con la hipótesis del apaciguamiento, encontré evidencia desfavorable para la segunda. En cambio, la hipótesis de la capacidad fisiológica resultó favorecida porque hallé que los peces que más bostezaron también fueron los que más capacidad fisiológica mostraron. También obtuve evidencia, aunque escasa, de que los peces siameses podrían bostezar contagiosamente entre 20 y 40 segundos después de presenciar un bostezo; estudios posteriores podrían ser planeados para corroborar si en efecto existe contagio del bostezo dentro de esta ventana de tiempo.

8. PERSPECTIVAS

Sería importante continuar este trabajo y realizar el estudio farmacológico del efecto del etanol en el bostezo del pez siamés. En el futuro se puede realizar la curva dosis-respuesta de la frecuencia de bostezo de los peces en función de la concentración de etanol. También, sería importante confirmar si estos peces poseen receptores D_3, y por lo tanto, si el mecanismo fisiológico del bostezo es análogo al de otros vertebrados.

También se puede extender las observaciones y manipulaciones experimentales que realicé a las hembras del pez siamés. Un aspecto interesante sería averiguar si las hembras prefieren, de entre un par de machos, al individuo que bostece más o a aquel que bostece menos.

Finalmente, queda pendiente confirmar la presencia del contagio del bostezo en el pez siamés, y también confirmar si la ventana de tiempo de contagio que propongo (20 a 40 s) es la correcta.

9. REFERENCIAS

Allen, J. M., & Nicoletto, P. F. (1997). Response of Betta splendens to computer animations of males with fins of different length. *Copeia*, *1997*(1), 195-199.

Anderson, J. R., Myowa–Yamakoshi, M., y Matsuzawa, T. (2004). Contagious yawning in chimpanzees. *Proceedings of the Royal Society of London B: Biological Sciences*, *271*(Suppl 6), S468-S470.

Anías J., Holmgren M., Urbá-Holmgren R., y Eguibar J.R. (1984). Circadian variation of yawning behavior. *Acta Neurobiol Exp*, *44*, 179-86.

Argiolas, A., Melis, M. R., Mauri, A., & Gessa, G. L. (1987). Paraventricular nucleus lesion prevents yawning and penile erection induced by apomorphine and oxytocin but not by ACTH in rats. *Brain research*, *421*(1-2), 349-352.

Argiolas, A., Melis, M. R., Murgia, S., & Schiöth, H. B. (2000). ACTH-and α-MSH-induced grooming, stretching, yawning and penile erection in male rats: site of action in the brain and role of melanocortin receptors. *Brain research bulletin*, *51*(5), 425-431.

Baenninger, R. (1987). Some comparative aspects of yawning in Betta splendens, Homo sapiens, Pantheraleo, and Papio sphinx.. *Journal of Comparative Psychology*, *101*(4), 349.

Baenninger, R. (1997). On yawning and its functions. *Psychonomic Bulletin y Review*, *4*(2), 198-207.

Baron-Cohen, S., & Wheelwright, S. (2004). The empathy quotient: an investigation of adults with Asperger syndrome or high functioning autism, and normal sex differences. *Journal of autism and developmental disorders*, *34*(2), 163-175.

Blin, O., Masson, G., Azulay, J. P., Fondarai, J., & Serratrice, G. (1990). Apomorphine-induced blinking and yawning in healthy volunteers. *British journal of clinical pharmacology*, *30*(5), 769-773.

Brammah, M. (2015). The Betta Bible: The Art and Science of Keeping Bettas.

Brownscombe, J. W., Cooke, S. J., Algera, D. A., Hanson, K. C., Eliason, E. J., Burnett, N. J., ... & Farrell, A. P. (2017). Ecology of exercise in wild fish: integrating concepts of individual physiological capacity, behavior, and fitness through diverse case studies. *Integrative and comparative biology*, *57*(2), 281-292.

Casetta, G., Nolfo, A. P., & Palagi, E. (2021). Yawn contagion promotes motor synchrony in wild lions, *Panthera leo*. *Animal Behaviour*, *174*, 149-159.

Casto, K. V., Edwards, D. A., Akinola, M., Davis, C., & Mehta, P. H. (2020). Testosterone reactivity to competition and competitive endurance in men and women. *Hormones and behavior*, *123*, 104665.

Collins, G. T., Witkin, J. M., Newman, A. H., Svensson, K. A., Grundt, P., Cao, J., & Woods, J. H. (2005). Dopamine agonist-induced yawning in rats: a dopamine D3 receptor-mediated behavior. *Journal of Pharmacology and experimental therapeutics*, *314*(1), 310-319.

Collins, G. T., Newman, A. H., Grundt, P., Rice, K. C., Husbands, S. M., Chauvignac, C., ... & Woods, J. H. (2007). Yawning and hypothermia in rats: effects of dopamine D3 and D2 agonists and antagonists. *Psychopharmacology*, *193*(2), 159-170.

Collins, G. T., & Eguibar, J. R. (2010). Neurophamacology of yawning. In *The mystery of yawning in physiology and disease* (Vol. 28, pp. 90-106). Karger Publishers.

Clotfelter, E. D., O'Hare, E. P., McNitt, M. M., Carpenter, R. E., & Summers, C. H. (2007). Serotonin decreases aggression via 5-HT1A receptors in the fighting fish Betta splendens. *Pharmacology Biochemistry and Behavior*, *87*(2), 222-231.

Czoty, P. W., John, W. S., Newman, A. H., & Nader, M. A. (2018). Yawning elicited by intravenous ethanol in rhesus monkeys with experience self-administering cocaine and ethanol: Involvement of dopamine D3 receptors. *Alcohol*, *69*, 1-5.

Darwin, C. (1873). *The Expression of the Emotions in Man and Animals: London, Murray, 1872*. Culture et civilisation.

de Castro Siqueira, L. (2009). Yawning and thermoregulation in budgerigars: lack of support from results. *Animal Behaviour*, *78*(6), e1-e2.

Demuru, E., y Palagi, E. (2012). In bonobos yawn contagion is higher among kin and friends. *PLoS One*, *7*(11), e49613.

Díaz Loyo, A. P. (2016). *La función comunicativa del bostezo en Betta splendens* (Tesis de maestría, Benemérita Universidad Autónoma de Puebla).

Dzieweczynski, T. L., Eklund, A. C., & Rowland, W. J. (2006). Male 11-ketotestosterone levels change as a result of being watched in Siamese fighting fish, *Betta splendens*. *General and comparative endocrinology*, 147(2), 184-189.

Dzieweczynski, T. L., Portrais, K. B., Stevens, M. A., Kane, J. L., & Lawrence, J. M. (2018). Risky business: Changes in boldness behavior in male Siamese fighting fish, Betta splendens, following exposure to an antiandrogen. *Environmental Pollution*, *235*, 1015-1021.

Eguibar, J. R., Uribe, C. A., Cortes, C., Bautista, A., & Gallup, A. C. (2017). Yawning reduces facial temperature in the high-yawning subline of Sprague-Dawley rats. *BMC neuroscience*, *18*(1), 1-8.

Forsatkar, M. N., Abedi, M., Nematollahi, M. A., y Rahbari, E. (2014). Effect of testosterone and fluoxetine on aggressive behaviors of fighting fish, *Betta splendens. International Journal of Aquatic Biology*, 1(6), 289-293.

Forsatkar, M. N., Nematollahi, M. A., Amiri, B. M., & Huang, W. B. (2014). Fluoxetine inhibits aggressive behaviour during parental care in male fighting fish (Betta splendens, Regan). *Ecotoxicology*, *23*(9), 1794-1802.

Forsatkar, M. N., Nematollahi, M. A., & Brown, C. (2017). Male Siamese fighting fish use gill flaring as the first display towards territorial intruders. *Journal of Ethology*, *35*(1), 51-59.

Froese, R. (2006). Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. *Journal of applied ichthyology*, *22*(4), 241-253.

Gallup, A. C., & Gallup Jr, G. G. (2007). Yawning as a brain cooling mechanism: nasal breathing and forehead cooling diminish the incidence of contagious yawning. *Evolutionary Psychology*, *5*(1), 147470490700500109.

Gallup, A. C., Miller, M. L., & Clark, A. B. (2009). Yawning and thermoregulation in budgerigars, Melopsittacus undulatus. *Animal Behaviour*, *77*(1), 109-113.

Gallup, A. C., Church, A. M., & Pelegrino, A. J. (2016). Yawn duration predicts brain weight and cortical neuron number in mammals. *Biology letters*, *12*(10), 20160545.

Gallup, A. C. (2017). Yawn duration predicts brain volumes in wild cats (Felidae). *International Journal of Comparative Psychology*, *30*.

Gallup, A. C., Moscatello, L., & Massen, J. J. (2020). Brain weight predicts yawn duration across domesticated dog breeds. *Current Zoology*, *66*(4), 401-405.

Gallup, A. C., & Meyers, K. (2021). Seeing others yawn selectively enhances vigilance: an eye-tracking study of snake detection. *Animal Cognition*, 1-10.

Graves, F. C., y Wallen, K. (2006). Androgen-induced yawning in rhesus monkey females is reversed with a nonsteroidal anti-androgen. *Hormones and behavior*, 49(2), 233-236.

Griggs, R. C., Kingston, W. I. L. L. I. A. M., Jozefowicz, R. F., Herr, B. E., Forbes, G. I. L. B. E. R. T., & Halliday, D. A. V. I. D. (1989). Effect of testosterone on muscle mass and muscle protein synthesis. *Journal of Applied Physiology*, *66*(1), 498-503.

Guggisberg, A. G., Mathis, J., Schnider, A., y Hess, C. W. (2010). Why do we yawn?. *Neuroscience and Biobehavioral Reviews*, 34(8), 1267-1276.

Guschina, I. A., & Harwood, J. L. (2006). Mechanisms of temperature adaptation in poikilotherms. *Febs Letters*, *580*(23), 5477-5483.

Hakamata, Y., Komi, S., Moriguchi, Y., Izawa, S., Motomura, Y., Sato, E., ... & Tagaya, H. (2017). Amygdala-centred functional connectivity affects daily cortisol concentrations: a putative link with anxiety. *Scientific reports*, *7*(1), 1-11.

Haker, H., Kawohl, W., Herwig, U., & Rössler, W. (2013). Mirror neuron activity during contagious yawning—an fMRI study. *Brain imaging and behavior*, *7*(1), 28-34.

Heaton, J. P. W., & Varrin, S. (1991). The impact of alcohol ingestion on erections in rats as measured by a novel bio-assay. *The Journal of urology*, *145*(1), 192-194.

Holmgren, B., Urba-Holmgren, R., Aguiar, M., y Rodriguez, R. (1980). Sex hormone influences on yawning behavior. *ActaNeurobiolExp* (Wars), 40(2), 515-519.

Joly-Mascheroni, R. M., Senju, A., y Shepherd, A. J. (2008). Dogs catch human yawns. *Biology Letters*, 4(5), 446-448.

Kang, C. K., & Lee, T. H. (2010). The pharyngeal organ in the buccal cavity of the male Siamese fighting fish, Betta splendens, supplies mucus for building bubble nests. *Zoological science*, *27*(11), 861-866.

Kubota, N., Amemiya, S., Yanagita, S., Nishijima, T., & Kita, I. (2019). Central nucleus of the amygdala is involved in induction of yawning response in rats. *Behavioural brain research*, *371*, 111974.

Kumar, S., Behera, S., Nagesh, T. S., Gogoi, R., & Prakash, C. B. (2016). Effect of 17a-Methyltestosterone on the growth performance of a fighting fish (Betta splendens). *J. Exp. Zool. India*, *19*, 89-93.

Leggio, G. M., Camillieri, G., Platania, C. B., Castorina, A., Marrazzo, G., Torrisi, S. A., ... & Salomone, S. (2014). Dopamine D3 receptor is necessary for ethanol consumption: an approach with buspirone. *Neuropsychopharmacology*, *39*(8), 2017-2028.

Leone, A., Ferrari, P. F., y Palagi, E. (2014). Different yawns, different functions? Testing social hypotheses on spontaneous yawning in *Theropithecus gelada*. *Scientific reports*, 4

Massen, J. J., Hartlieb, M., Martin, J. S., Leitgeb, E. B., Hockl, J., Kocourek, M., ... & Gallup, A. C. (2021). Brain size and neuron numbers drive differences in yawn duration across mammals and birds. *Communications Biology*, *4*(1), 1-10.

Matsui, H. (2017). Dopamine system, cerebellum, and nucleus ruber in fish and mammals. *Development, growth & differentiation*, *59*(4), 219-227.

Melis, M. R., Succu, S., & Argiolas, A. (1996). Dopamine agonists increase nitric oxide production in the paraventricular nucleus of the hypothalamus: correlation with penile erection and yawning. *European Journal of Neuroscience*, *8*(10), 2056-2063.

Messias, J. P., Santos, T. P., Pinto, M., & Soares, M. C. (2016). Stimulation of dopamine D1 receptor improves learning capacity in cooperating cleaner fish. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1823), 20152272.

Miller, M. L., Gallup, A. C., Vogel, A. R., Vicario, S. M., y Clark, A. B. (2012). Evidence for contagious behaviors in budgerigars (*Melopsittacus undulatus*): an observational study of yawning and stretching. *Behavioural processes*, 89(3), 264-270.

Morris, D. (1955). The reproductive behaviour of the river bullhead *(Cottusgobio L.*), with special reference to the fanning activity. Behaviour, 7(1), 1-32.

Morris, D. (1958). The reproductive behaviour of the ten-spined stickleback (Pygosteuspungitius L.). Behaviour. Supplement, III-154.

Moyaho, A., Rivas-Zamudio, X., Ugarte, A., Eguibar, J. R., y Valencia, J. (2015). Smell facilitates auditory contagious yawning in stranger rats. *Animal cognition*, 18(1), 279-290.

Moyaho, A., Urbina, A. F., Guzmán, E. M., & Walusinski, O. (2017). Yawning: a cue and a signal. *Heliyon*, *3*(11), e00437.

Moyaho, A., & Beristain-Castillo, E. (2019). Experimental Design: Basic Concepts.

Moyaho, A., Díaz-Loyo, A. P., Juárez-Mora, O. E., & Beristain-Castillo, E. (2019). A Laboratory Method to Measure Contagious Yawning in Rats. *JoVE (Journal of Visualized Experiments)*, (148), e59289.

Myrberg Jr, A. A., Brahy, B. D., y Emery, A. R. (1967). Field observations on reproduction of the damselfish, *Chromis multilineata* (Pomacentridae), with additional notes on general behavior. Copeia, 819-827.

Norscia, I., Coco, E., Robino, C., Chierto, E., & Cordoni, G. (2021). Yawn contagion in domestic pigs (*Sus scrofa*). *Scientific Reports*, 11(1), 1-12.

O'Connell, L. A., Fontenot, M. R., & Hofmann, H. A. (2011). Characterization of the dopaminergic system in the brain of an African cichlid fish, Astatotilapia burtoni. *Journal of Comparative Neurology*, *519*(1), 75-92.

Palagi, E., Leone, A., Mancini, G., y Ferrari, P. F. (2009). Contagious yawning in gelada baboons as a possible expression of empathy. *Proceedings of the National Academy of Sciences*, 106(46), 19262-19267.

Palagi, E., Guillén-Salazar, F., & Llamazares-Martín, C. (2019). Spontaneous yawning and its potential functions in South American sea lions (Otaria flavescens). *Scientific reports*, *9*(1), 1-8.

Paukner, A., y Anderson, J. R. (2006). Video-induced yawning in stumptail macaques (*Macaca arctoides*). *Biology Letters*, 2(1), 36-38.

Platek, S. M., Critton, S. R., Myers, T. E., y Gallup, G. G. (2003). Contagious yawning: the role of self-awareness and mental state attribution. *Cognitive Brain Research*, 17(2), 223-227.

Provine, R. R. (1986). Yawning as a stereotyped action pattern and releasing stimulus. *Ethology*, 72(2), 109-122.

Provine, R. R. (2005). Yawning: the yawn is primal, unstoppable and contagious, revealing the evolutionary and neural basis of empathy and unconscious behavior. *American scientist*, 93(6), 532-539.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rasa, O. A. E. (1971). The Causal Factors and Function of Yawning in *Microspathodon Chrysurus* (Pisces: Pomacentridae). Behaviour, 39(1), 39-5

Reby, D., & McComb, K. (2003). Anatomical constraints generate honesty: acoustic cues to age and weight in the roars of red deer stags. *Animal behaviour*, *65*(3), 519-530.

Rodriguez, A., Zhang, H., Klaminder, J., Brodin, T., Andersson, P. L., & Andersson, M. (2018). ToxTrac: a fast and robust software for tracking organisms. *Methods in Ecology and Evolution*, *9*(3), 460-464.

Rodriguez-Sierra, J. F., Terasawa, E., Goldfoot, D. A., & DeWied, D. (1981). Testosterone potentiation of the effectiveness of ACTH1–24 on the induction of the stretch-yawning syndrome (SYS) in male guinea pigs. *Hormones and behavior*, *15*(1), 77-85.

Romero, T., Ito, M., Saito, A., y Hasegawa, T. (2014). Social modulation of contagious yawning in wolves*. PLoS One*, 9(8), e105963.

Rose, R. M., Holaday, J. W., y Bernstein, I. S. (1971). Plasma testosterone, dominance rank and aggressive behaviour in male rhesus monkeys. *Nature*.

Rossman, Z. T., Padfield, C., Young, D., Hart, B. L., & Hart, L. A. (2020). Contagious yawning in African elephants (*Loxodonta africana*): responses to other elephants and familiar humans. *Frontiers in Veterinary Science*, *7*, 252.

Salena, M. G., Turko, A. J., Singh, A., Pathak, A., Hughes, E., Brown, C., & Balshine, S. (2021). Understanding fish cognition: a review and appraisal of current practices. *Animal Cognition*, 1-12.

Sauer, E. F., y Sauer, E. M. (1967). Yawning and other maintenance activities in the South African Ostrich. *The Auk*, 571-587.

Simpson, M. J. A. (1968). The display of the Siamese fighting fish, *Betta splendens*. *Animal Behaviour Monographs*, 1, i-73.

Sinervo, B., Miles, D. B., Frankino, W. A., Klukowski, M., y DeNardo, D. F. (2000). Testosterone, endurance, and Darwinian fitness: natural and sexual selection on the physiological bases of alternative male behaviors in side-blotched lizards. *Hormones and Behavior*, 38(4), 222-233.

Smith, E. N. (1979). Behavioral and physiological thermoregulation of crocodilians. *American Zoologist*, *19*(1), 239-247.

Smith, J. M. (1994). Must reliable signals always be costly?. *Animal behaviour*, *47*(5), 1115-1120.

Stegmann, U. E. (2005). John Maynard Smith's notion of animal signals. *Biology and Philosophy*, *20*(5), 1011-1025.

Thompson, S. B. (2014). Yawning, fatigue, and cortisol: expanding the Thompson Cortisol Hypothesis. *Medical hypotheses*, *83*(4), 494-496.

van Berlo, E., Díaz-Loyo, A. P., Juárez-Mora, O. E., Kret, M. E., & Massen, J. J. (2020). Experimental evidence for yawn contagion in orangutans (*Pongo pygmaeus*). *Scientific reports*, 10(1), 1-11.

Vick, S. J., & Paukner, A. (2010). Variation and context of yawns in captive chimpanzees (Pan troglodytes). *American Journal of Primatology: Official Journal of the American Society of Primatologists*, *72*(3), 262-269.

Villaseñor, J. A., & González-Estrada, E. (2015). A variance ratio test of fit for Gamma distributions. *Statistics & Probability Letters*, *96*, 281-286.

Wallbrunn, H. M. (1958). Genetics of the Siamese fighting fish, Betta splendens. *Genetics*, *43*(3), 289.

Wilkinson, A., Sebanz, N., Mandl, I., y Huber, L. (2011). No evidence of contagious yawning in the red-footed tortoise *Geochelone carbonaria. Current Zoology*, 57(4), 477-484.

Yu, Y. Q., Barry, D. M., Hao, Y., Liu, X. T., & Chen, Z. F. (2017). Molecular and neural basis of contagious itch behavior in mice. *Science*, *355*(6329), 1072-1076.

Yamada, K., & Furukawa, T. (1980). Direct evidence for involvement of dopaminergic inhibition and cholinergic activation in yawning. *Psychopharmacology*, *67*(1), 39-43.

Yonezawa, T., Sato, K., Uchida, M., Matsuki, N., y Yamazaki, A. (2016). Presence of contagious yawning in sheep. *Animal Science Journal*.

Young C. (2019) Agonistic Behavior. In: Vonk J., Shackelford T. (eds) Encyclopedia of Animal Cognition and Behavior. Springer, Cham. https://doi.org/10.1007/978-3-319-47829-6_320-1

Zannella, A., Norscia, I., Stanyon, R., & Palagi, E. (2015). Testing yawning hypotheses in wild populations of two strepsirrhine species: Propithecus verreauxi and Lemur catta. *American journal of primatology*, *77*(11), 1207-1215.

Zannella, A., Stanyon, R., & Palagi, E. (2017). Yawning and social styles: Different functions in tolerant and despotic macaques (*Macaca tonkeana* and *Macaca fuscata*). *Journal of Comparative Psychology*, 131(3), 179.

Zannella, A., Stanyon, R., Maglieri, V., & Palagi, E. (2021). Not all yawns tell the same story: The case of Tonkean macaques. *American Journal of Primatology*, e23263.

10. ANEXOS

Anexo 1. Pruebas de ajuste a una distribución de la longitud estándar y masa de los peces experimentales y observadores

Tabla 8.1. Resumen de los resultados de la aplicación de la prueba Shapiro-Wilk a las mediciones de la masa corporal de los peces, antes y después del tratamiento farmacológico.

	W	P
Masa corporal antes	0.99[1]	0.361
Masa corporal después	0.99	0.313

[1] Se refiere al estadístico de la prueba.

Tabla 8.2. Resumen de los resultados de la aplicación de la prueba Villaseñor-González, para corroborar si los datos, una vez transformados, siguen una distribución gama. Los datos, tomados antes y después del tratamiento farmacológico, corresponden a la longitud estándar de los peces.

	V	P
Longitud estándar antes	1.99[1]	0.159
Longitud estándar después	1.67	0.237

[1] Se refiere al estadístico de la prueba.

Transformación de los datos de longitud estándar: la raíz cuadrada de cada medición restada de 7

Anexo 2. Prueba de ajuste a una distribución gamma de los desplantes agonísticos de los peces experimentales.

Resumen de la prueba Villaseñor-González de la frecuencia de desplante agonístico elevado al cuadrado*: V* = – 2.32, *P* = 0.101

Anexo 3. Publicaciones que se desprenden del presente trabajo.

Las siguientes publicaciones están relacionadas con el trabajo experimental que realicé como estudiante del Instituto de Fisiología de la Benemérita Universidad Autónoma de Puebla

Moyaho, A., Díaz-Loyo, A. P., Juárez-Mora, O. E., & Beristain-Castillo, E. (2019). A Laboratory Method to Measure Contagious Yawning in Rats. *JoVE (Journal of Visualized Experiments)*, (148), e59289.

Este artículo propone un método novedoso para el estudio del contagio del bostezo. Los autores desarrollamos este método para aplicarlo a los datos que obtuve en esta tesis. Utilicé el procedimiento matemático propuesto para obtener la tasa de contagio del bostezo a diferentes ventanas de tiempo y construir curvas de contagio del bostezo. El uso de este método sugiere la presencia del contagio del bostezo en esta especie, lo cual tendría implicaciones de gran impacto para la hipótesis de la empatía. Además, las curvas de contagio que obtuve proporcionan información sobre la posible ventana de contagio en esta especie. Esto resultará de importancia para futuros estudios del contagio del bostezo.

van Berlo, E., Díaz-Loyo, A. P., Juárez-Mora, O. E., Kret, M. E., & Massen, J. J. (2020). Experimental evidence for yawn contagion in orangutans (Pongo pygmaeus). *Scientific reports*, *10*(1), 1-11.

Este artículo es producto de una estancia de investigación en la Universidad de Leiden en los Países Bajos. Este trabajo surge del interés en establecer la presencia del contagio de bostezo en una especie de primates cercana al ser humano, pero en la cual no se había encontrado evidencia de contagio del bostezo. En el estudio analizamos si la cercanía social tenía alguna importancia en el contagio del bostezo. También, los resultados de ambos trabajos se complementan en una visión más clara del bostezo y su contagio en diferentes especies.

Powell, D. L., García-Olazábal, M., Keegan, M., Reilly, P., Du, K., Díaz-Loyo, A. P., ... & Schumer, M. (2020). Natural hybridization reveals incompatible alleles that cause melanoma in swordtail fish. *Science*, *368*(6492), 731-736.

Este artículo es producto de una colaboración con la Universidad de Stanford en EUA. Mi contribución consistió en aplicar la prueba de nado forzado a peces cola de espada, esto para determinar si la presencia de melanoma afecta la capacidad fisiológica de los peces. La prueba de nado forzado, un desarrollo original de este trabajo de tesis, puede ser aplicada a otras especies de peces y responder preguntas diversas. Por lo tanto, considero que el desarrollo de la prueba de nado forzado es uno de los productos de este trabajo de tesis.

Video Article

A Laboratory Method to Measure Contagious Yawning in Rats

Alejandro Moyaho[1], Alejandra P. Díaz-Loyo[1], Oscar E. Juárez-Mora[1], Evelyn Beristain-Castillo[2]

[1]Laboratorio de Ecología de la Conducta, Instituto de Fisiología, Benemérita Universídad Autónoma de Puebla Pue. Mexico

[2]Facultad de Medicina, Benemérita Universidad Autónoma de Puebla Pue. Mexico

Correspondence to: Alejandro Moyaho at alejandro.moyaho@correo.buap.mx

URL: https://www.jove.com/video/59289
DOI: doi:10.3791/59289

Keywords: Behavior, Issue 148, sensory modalities, contagious yawning, unfamiliar rats, familiar rats, olfactory cues, sensory cues, animal communication

Date Published: 6/14/2019

Citation: Moyaho, A., Díaz-Loyo, A.P., Juárez-Mora, O.E., Beristain-Castillo, E. A Laboratory Method to Measure Contagious Yawning in Rats. *J. Vis. Exp.* (148), e59289, doi:10.3791/59289 (2019).

Abstract

Communication is an essential aspect of animal social life. Animals may influence one another and come together in schools, flocks, and herds. Communication is also the way sexes interact during courtship and how rivals settle disputes without fighting. However, there are some behavioral patterns for which it is difficult to test the existence of a communicatory function, because several types of sensory modalities are likely involved. For example, contagious yawning is a communicatory act in mammals that potentially occurs through sight, hearing, smell, or a combination of these senses depending on whether the animals are familiar to one another. Therefore, to test hypotheses about the possible communicatory role of such behaviors, a suitable method is necessary to identify the participating sensory modalities.

The method proposed here aims to obtain yawn contagion curves for familiar and unfamiliar rats and evaluate the relative participation of visual and olfactory sensory modalities. The method uses inexpensive materials, and with some minor changes, it can also be used with other rodent species such as mice. Overall, the method involves the substitution of clear dividers (with or without holes) with opaque dividers (with or without holes) that either allow or prevent communication between rats placed in adjacent cages with holes in adjoining sides. Accordingly, four conditions can be tested: olfactory communication, visual communication, both visual and olfactory communication, and neither visual nor olfactory communication. As social interaction occurs between the rats, these test conditions simulate what may occur in a natural environment. In this respect, the method proposed here is more effective than traditional methods that rely on video presentations whose biological validity can raise concerns. Nonetheless, it does not discriminate between the potential role of hearing and roles of smell and vision in yawn contagion.

Video Link

The video component of this article can be found at https://www.jove.com/video/59289/

Introduction

Traditionally, communicatory behavior has been studied from two perspectives. From one perspective, ethologists observe and record the behavior of animals in natural settings and attempt to recognize its adaptive value[1]. The particular sense or senses involved have not been the primary interest of these studies. From another perspective, physiologists are more interested in unraveling the mechanisms by which animals communicate[1]; hence, laboratory studies have provided methods to address the role that sensory modalities play in communication[2,3]. These two perspectives are indeed complementary, because knowledge of both adaptive value and immediate mechanisms is necessary to gain a comprehensive understanding of communicatory behaviors in the social life of animals.

Yawning behavior is a conspicuous component of the behavioral repertoire in several species of vertebrates[4], ranging from fish to primates[5]. It can be described as a slow opening of the mouth and maintenance of its open position, followed by a more rapid closure of the mouth[5]. The duration of the whole sequence depends on the species; for example, primates yawn for longer durations than non-primate species[6]. In many species, with humans being the exception, males tend to yawn more frequently than females[7]. This feature might underpin the possible communicatory function of yawning, although regular patterns of yawning and its daily frequency may also suggest a physiological function. In rats, spontaneous yawning follows a circadian rhythm, with peaks of high frequency occurring in the morning and afternoon[8,9].

One interesting feature of yawning behavior is that it can be a contagious act (when the releasing stimulus of a behavior happens to be another animal behaving in the same way[10]) in several species of vertebrates[11,12,13,14,15,16], including birds[17] and rodents[18]. Furthermore, recent evidence has indicated that contagious yawning may reflect a communicatory role, because the yawning of one rat can affect the physiological state of another when exposed to olfactory cues[19]. However, whether or not yawning has a communicatory role is still under debate[20,21], and analyzing contagious yawning is an essential first step to solve this issue.

On the other hand, contagious yawning has been linked to an animal's ability to empathize with the perspectives of other animals; hence, closely related individuals are more likely to show contagion[4]. This hypothesis has been frequently tested in laboratory conditions in which animals are presented with yawn stimuli on video[12,13]; hence, contagion can only occur through visual cues. Other investigations have assessed yawn contagion in more natural conditions using groups of animals[14,15]. A major problem of this is that socially interacting animals often respond to

cues and exchange signals that are conveyed through combinations of sensory modalities. Disentangling the actual senses involved in a given behavior from their combined effects is not always an easy task. Typically, researchers pharmacologically or surgically hinder an animal's use of a given sense, then infer the role of that sense in the relevant behavior[2,3,18,22]. Fortunately, there are other methods in which only physical barriers are used to either allow or impede communication between animals[23,24,25], thereby achieving greater biological validity.

The method proposed here has been specifically designed to study contagious yawning in familiar and unfamiliar rats in a social setting. According to the empathetic hypothesis, the former group should be more susceptible to contagious yawning. The method does not require the animals to be surgically or pharmacologically deprived of any senses. Instead, it works by placing the rats in adjacent cages with holes and physically obstructing their communication using either clear or opaque dividers with or without holes. Thus, four test conditions can be examined: (1) olfactory communication (OC, perforated opaque divider), (2) visual communication (VC, nonperforated clear divider), (3) visual and olfactory communication (VOC, perforated clear divider), and (4) neither visual nor olfactory communication (NVOC, nonperforated opaque divider). Therefore, researchers can compare the relative contributions of olfactory, visual, and to some extent, auditory cues in yawn contagion. This approach is not new, as similar methods have been used to isolate the senses involved in certain communicatory behaviors in animals such as lizards[23] and mice[26]. In fact, Gallup and colleagues[27] have used a similar method to demonstrate the role of visual cues in contagious yawning in budgerigars. The main features of these methods are simulation of a social context and the minimal stress inflicted on the animals. Furthermore, the use of interacting animals increases the biological validity of the conclusions.

There are several ways to measure contagious yawning[25,28]. Dr. Stephen E. G. Lea (personal communication, 2015) helped us numerically adapt a method previously employed by primatologists[13,14] for an earlier analysis of the data used here[18]. Presented in this protocol is an enhanced version of this method with a wider range of applications. It consists of weighting the total number of a rat's yawns, within and outside of a given time window, by the proportion of observation time corresponding to the yawns within and outside the time window.

For example, if it is assumed that rats A and B are observed for 12 min, their yawning is recorded to the nearest minute, and a 3 min time window is set to measure contagious yawning. Next, the following sequences of yawns for each of those rats are considered: rat A (0,0,0,1,0,0,2,0,0,0,2,1) and rat B (**0**,1,1,0,1,1,0,0,0,**0**,**0**,3). It should be noted that each number (0-3) corresponds to the number of yawns scored at each min. For rat A, during minutes 1, 10, and 11 (numbers in bold type), rat B does not yawn within the preceding 3 min (the chosen time window) or within that minute. In those minutes, rat A yawns a total of 2 times. Therefore, the yawn rate of rat A without any yawn stimulus (non-post-yawn yawn rate) is 2/3 (i.e., 0.67 yawns/min). In the remaining 9 min, rat B yawns at least one time in either the same minute or the 3 previous minutes. Rat A yawns a total of four times in those 9 min. Therefore, the yawn rate of rat A in response to a yawn stimulus (post-yawn yawn rate) is 4/9 (i.e., 0.44 yawns/min). The application of the same procedure to rat B yields a non-post-yawn yawn rate of 2/3 (i.e., 0.66) and post-yawn yawn rate of 5/9 (0.55).

On the other hand, if yawning is recorded to the nearest decimal of a minute, yawn contagion will result in an adjusted post-yawn time. For example, if the following yawn times are recorded over a 12 min observation period for rats A and B: rat A (2.3, 5.1, 5.8, **10.4**, **10.8**, **11.1**) and rat B (1.2, 2.4, 4.5, 5.1, 11.2, 11.6, 11.8). For rat A, the time periods over which rat B does not yawn within the past 3 min range from 0 to 1.2 min and from 8.1 to 11.2 min (i.e., 3.1 min), which yields a total of 4.3 min of non-post-yawn time. The number of times that rat A yawns during those times is three (numbers in bold type), so the non-post-yawn yawn rate is 3/4.3 (i.e., 0.69), while the post-yawn yawn rate is 3/7.7 (i.e., 0.38; the denominator from 12-4.3 min). Similarly, for rat B, the time periods over which rat A does not yawn within the past 3 min range from 0 to 2.3 min and from 8.8 to 10.4 min, which yields a total of 3.9 min. The number of times rat B yawns within those periods is one, so the non-post-yawn yawn rate is 1/3.9 (i.e., 0.25). Accordingly, the post-yawn yawn rate is 6/8.1 (i.e., 0.74).

While a near-contemporaneous match in behavior is an ideal criterion to demonstrate the presence of a contagion, aspects such as the constraints on what an individual attends to, time of reaction to a stimulus, distribution of the behavior over time (e.g., yawning may occur in episodes), and time to acclimatize to the experimental setting all give rise to species differences, making it difficult to use a unique time window. This may be the reason why researchers have used time windows that vary from seconds[5] to several minutes[11], which creates problems when comparing results[28]. Because of this, it is proposed to repeat the procedure described above for a range of time windows to obtain yawn contagion curves and compare the yawn contagion curves between species.

Equivalent yawn contagion curves can be compared by randomly distributing the number of yawns observed for each rat over the observation period. Thus, the proposed method to measure yawn contagion offers two types of controls: the (1) yawn rate occurring outside of the time window (non-post-yawn time) and (2) artificial yawn contagion curve obtained from the random distribution of the number of yawns. Therefore, this approach to analyze yawn contagion is a step forward from other procedures, such as those comparing the percentage or frequency of yawning within a single time window to that occurring outside this window[25], without taking into account the actual times frames. The method is complemented by an R-based program[29] to conveniently and objectively calculate the probability of contagious yawning for one or more time windows.

To illustrate the usefulness of this method and advantages of the R-based program, a data set from a previously published study[18] is used. The experimental condition consisted of 144 male rats allocated to either a familiar or unfamiliar condition. The rats in each experimental condition were subdivided into four subgroups of nine pairs and exposed to any of the four test situations described above. The yawning behaviors of the rats in each experimental condition and test situation were then recorded over a period of 60 min.

Protocol

The experimental protocols and animal husbandry were conducted in accordance with institutional guidelines.

1. Materials

1. Find a complete list of the materials used to implement the method in the **Table of Materials**. Use **Figure 1** and seek expert advice to construct an inverted T-shaped table, observation cages, and cage dividers. Follow the safety indications for the use of sharp tools and potentially dangerous materials.
2. Make an inverted T-shaped table by gluing two rail-like wooden bars (45 cm length, separated 0.6 cm from each other) onto the top and in the middle of a piece of wood (100 cm x 45 cm x 1.5 cm). Then, place a second piece of wood (50 cm x 45 cm x 0.6 cm) vertically between the rail-like bars. Ensure that this second piece of wood prevents the pair of rats on one side from seeing the other pair on the opposite side (**Figure 1**).
3. Use glass and acrylic to create eight observation cages. Ensure that each cage (19 cm wide, 19 cm long, 10 cm high, 3 mm thick) has 24 equidistant holes (5 mm diameter) arranged in three rows in the middle of one of its sides. Make this side of acrylic (3 mm thick) and shorten the height of the opposite side by 0.7 cm to allow the rat to breathe.
    1. Make sure each observation cage has a sliding lid made of acrylic to prevent the rat from rearing and distracting itself during the 60 min observation period.
4. Make four dividers out of acrylic (19 cm wide, 30 cm high, 3 mm thick). Drill 24 holes (5 mm diameter) that match those in the observation cages, each in one clear divider and one opaque divider.
5. Prepare data sheets in advance to record the occurrence of yawns. Include, in the heading of each data sheet, the name of the observer, experimental condition (i.e., familiar or unfamiliar rats), relevant test situation (OC, VC, VOC, NVOC), the date, and initial and final times of the observation. Divide the rest of the data sheet into two columns, each with the number of the rat written in the upper part, to record the yawning behavior of the rats.

2. Procedure

1. House 144 male rats in groups of four in plastic cages (home cages) from weaning until they reach 2.5 months in age. Do not use female rats, because they tend to show greater variation in behavior due to hormonal cycles, which may be confused with the effects of the test situation. Make sure that the rats in each cage are not siblings.
   NOTE: It might be difficult to obtain all the rats together. In that case, create blocks of at least 8 familiar rats and 8 unfamiliar rats so that every test situation is represented once in each experimental condition[30].
2. To identify the rats, mark them with symbolic numbers on the tails using a commercial marker. For example, represent the numbers 1 to 4 by combining dots and lines (e.g. ,one dot for number 1 and one line for number 4). Identify familiar and unfamiliar rats using different colors.
   NOTE: Handle the rats following safety directions provided by animal facility staff, and comply with recommendations concerning the correct use of laboratory animals at the institution where experiments will be performed.
3. Randomly choose which home cages will house the group of familiar rats and which will house the unfamiliar group. For example, suppose there are 16 available rats living in four home cages: number the home cages 1 to 4, then use R (download it at http://cran.r-project.org/) as follows [after the prompt (>)]:
   > sample(4,4)
   [1] 4 3 2 1
    1. Group the familiar (or unfamiliar) rats in home cages 4 and 3, and group the unfamiliar (or familiar) rats in home cages 2 and 1. Make sure the animal facility staff maintains the identity of each cage and handles the rats in the same manner as all other rats in the animal facility.
    2. Use the R program again and randomly choose rats to form each pair in each experimental condition. Ensure that the rats in each pair of familiar rats originate from the same home cage, and ensure that the opposite is true for the pairs in the unfamiliar group. Randomly choose pairs of rats for each test situation in each experimental condition.
4. Perform two test sessions per day with two of the eight possible test situations (four for each familiar and unfamiliar rats) per test session (i.e., 60 min observation period). Conduct the two test sessions consecutively within 3 h.
   NOTE: Make sure to conduct all test sessions (four pairs of rats per day) either in the morning or afternoon to avoid any confounding factors. Run a complete replicate (eight test situations) of the experiment over two consecutive days.
    1. Make sure to use the test situations in a random sequence for each replicate of the experiment. For example, use numbers 1 to 8 to identify each test situation, then use R as follows:
       >sample(8,8)
       [1] 8 7 4 6 5 1 2 3
    2. Allocate test situations 8 and 7 to test session 1, test situations 4 and 6 to test session 2, and so on. Then, randomly choose the side of the inverted T-shaped table where each pair of rats (test situation) will be placed.
5. Repeat the same procedure (steps 2.3 to 2.4.2) for a second replicate (i.e., block) of the experiment.
   NOTE: If the aim of the study is to test the effects of a social condition (i.e., two interacting rats) on yawning frequency, use one rat placed in an observation cage next to an empty observation cage as a control for each of the four test situations. Perform this experiment 2x for each test situation for a control group of eight rats.
6. Set up the test session by transferring the first four rats from the animal facility to the observation room, where they will remain for 15 min to acclimate to the novel environment. Transport the rats in individual cages and keep them separated from each other during transportation and in the observation room.
   NOTE: Follow the safety directions for transporting animals provided by the animal facility staff and use the indicated clothes to work with animals in the laboratory. The rats will not have access to food and water while they are in the observation room.
7. After the acclimatization period has passed, place the inverted T-shaped table on a larger rectangular table. Make sure there is a ceiling lamp that sufficiently lights the room when making observations.
8. Put filter paper on the bottom of each observation cage and place the cages in pairs on each side of the inverted T-shaped table. Position the corresponding divider in between each pair of cages.

NOTE: The filter paper makes it easier to clean the cages and provides a rough surface on which the rats can move without slipping.

9. Strategically place two digital camcorders such that each records the yawning behavior of each pair of rats. Make sure the camcorders are safely fixed to tripods and correctly orientated to the observation cages. Connect the camcorders to a desktop computer to simultaneously monitor the behavior of the rats.
   NOTE: Store the digital information on flash drives to permanently archive the experimental sessions. Use flash drives with a high storage capacity.
10. Following the acclimatization period, place the rats in the observation cages according to the allocation previously determined. Set the automatic focus of the camcorder and start the video recording simultaneously with a stopwatch. Stop the video recording at the end of the 60 min observation period.
    NOTE: While the experimenters may be outside of the room during the test session and observing remotely, it is recommended that one person is in the observation room (as far away as possible from the experimental setting) while monitoring the behavior of the rats to ensure that the test session proceeds without interruption.
11. When the observation is over, return the rats to their home cages in the animal facility. Ask the animal facility staff to ensure that the rats have access to food and water again. Thoroughly clean the observation cages using a nontoxic detergent and prepare the experimental set-up to perform the second and final test sessions of the day.
    NOTE: Clean the wood to remove any scents left by the rats when placed in the observation cages, which could affect the behavior of the next pair of rats tested.
12. Train one to two volunteers partially blind to the assigned treatments to identify and record yawning using an all-occurrence sampling method. Make sure the observers use the definition of yawning described in the introduction section.
13. Play back and project each video on a computer screen using any standard playback system. Ask the observer to view each video and observe and record yawning behavior using the previously prepared data sheets, then allow him/her to review the videos at slower speeds to enhance the ability to observe and measure yawning.
    1. Ask the observers to score yawning using a notation system similar to the following: use vertical lines with the minute written as a superscript to represent the occurrence of a yawn (e.g., $|^{3}\,||^{6}\,|^{9}$, indicating one yawn at minute 3, two yawns at minute 6, and one yawn at minute 9). Score yawning as a sequence (e.g.,1.2, 2.2, 3.2, 5.0, 5.8) to record it with greater precision (minutes rounded to one decimal place).
    2. Alternatively, directly input data from the videos into a computer using standard data collection programs. Make sure the observer is acquainted with such programs.
       NOTE: If necessary, allow the observer to view each video in more than one session. Ensure that one observer views all the videos corresponding to one block of experiments. If two different observers viewed these videos, there may be a risk that differences in their abilities to observe and record yawning will confound the effects of the test situations. It is important to score the intra-observer reliability between different observers on 10%-20% of the videos to ensure that yawning is annotated in the same way.

3. Data processing

1. Transcribe the temporal sequence of yawns in each rat from the data sheets to a spreadsheet. Ensure there is one column for each rat with a short heading (e.g., fr1.l and fr1.r to indicate the first pair of familiar rats on the left and right sides, respectively). Ensure that all columns are the same length by filling the empty cells with either 0 or NA (see below).
   NOTE: Use the general guide provided as a supplementary document to fully understand the following steps to process the data, measure contagious yawning, and obtain contagious yawning curves.
   1. If yawning was recorded to the nearest minute, type in the number of yawns at each relevant minute and use 0 (no yawn) to fill in the cells when no yawn occurred in a given minute. Save the worksheet as a text file (.txt).
   2. If yawning was recorded to the nearest decimal of a minute, type in the sequence top down and use "NA" to fill in the empty spaces of columns (rats) in which the numbers of yawns (rows) is lower than that of the column with the maximum number (rows) of yawns recorded for a rat. Save the worksheet as a text file (.txt).
      NOTE: As R has the ability to work with empty cells, the worksheet can be saved with columns of different lengths. Use the help options provided by R to handle missing data.

2. Initiate R. Import the data from the file in which they were previously located.
3. Save the program codes with the extension ".R" (provided as supplementary material), then download the specific program code (see general guide) depending on whether yawns were recorded as integers or fractional numbers. Run the program for each pair of rats and desired number of time frames.
   1. Run the program again, now using a random distribution of the number of yawns from each rat (see general guide). Follow the same steps (steps 3.3 to 3.3.1) for all experimental conditions (i.e., familiar and unfamiliar rats) and/or all the test situations. Proceed as indicated in the general guide and export the results to Excel.
      NOTE: Instead of importing a previously saved program, as suggested above using a text editor, copy and directly paste the program into R's workspace.

4. To create yawn contagion curves, use the data previously saved in an spreadsheet. Subtract the non-contagion rates from the contagion rates for each pair of rats, time window, and test situation. Separate the analyses of the observed data and artificial (randomly distributed) data.
   1. Next, calculate confidence intervals (CI) for each time window and test situation using a bootstrap procedure (see the general guide). Separate the analyses for familiar and unfamiliar rats. Then, combine the artificial data from the four test situations for each experimental condition and calculate the CI for each time window using a bootstrap procedure.
   2. Next, create plots for each test situation and experimental condition (see **Figure 2** and **Figure 3**). Finally, perform a multiple regression analysis to compare the intensity of yawn contagion between test situations (see below).

Representative Results

The rats were selected from a previously produced sub-line of Sprague-Dawley rats that were selected for frequent yawning (approximately 22 yawns per hour[31]). However, the nine pairs of unfamiliar and nine pairs of familiar male rats (between 2.5 and 3 months of age) used per test situation yawned approximately 12 times per hour, on average[18]. Therefore, the test situations to measure yawn contagion partially inhibited yawning behavior.

Yawn contagion was measured over a range of time windows that varied from 1 to 10 min. **Figure 2** shows the mean difference in the yawn rates between unfamiliar male rats in contagion and non-contagion conditions for each time window and test situation. These yawn contagion curves indicate that only OC rats (in cages with a perforated, opaque divider) showed yawn contagion, which is evident from time window 4 and onwards, because the CI of the averages does not overlap with the CI of the randomly allocated numbers of yawns over the 60 min period. The band indicated, as expected, that a random allocation of the number of yawns in each rat in the observation period produced yawn rates that oscillated at approximately zero, without showing any apparent pattern.

The multiple linear regression model fitted to the data indicated that the curves from the four test situations were significantly different ($F_{1,3}$ = 11.5, $p < 0.0001$). Specifically, yawn contagion was stronger in OC rats than: VC rats (rats in cages with a nonperforated, clear divider; t = -3.8, $p < 0.001$), VOC rats (rats in cages with a perforated, clear divider; t = -5.74, $p < 0.0001$), and NVOC rats (rats in cages with a nonperforated, opaque divider; t = -2.64, $p < 0.01$). In all cases, the degrees of freedom were 695, since the analysis took into account not only the four test situations, but also the 10 time windows. As these conditions generated autocorrelated measurements, an autocorrelation term was added (ARMA, autoregressive moving average) to the statistical model. The overall trends over the 10 time windows also differed statistically from a 0 slope ($F_{1,1}$ = 11.99, $p < 0.0001$). **Figure 3** shows the same analysis as that in **Figure 2**, applied to familiar male rats. In this case, none of the four test situations stimulated yawn contagion because their CI overlapped with the randomly generated CI band. There were no differences among the four test situations ($F_{1,3}$ = p = 0.14); although, the overall increase in yawn contagion over the 10 time windows differed from a 0 slope ($F_{1,1}$ = 9, $p < 0.01$).

The role of olfaction in the social life of mammals may be the reason why only the OC rats showed yawn contagion and yawned more frequently than the VC rats[18]. Visual cues may not have facilitated yawning, because albino rats do not see as well as non-albino rats. However, the VC rats yawned twice as much as the group of rats individually placed in an observation cage next to an empty cage[18]. This finding definitively supports the social nature of yawning and suggests that yawn contagion in rats is dependent on olfactory cues. However, the latter only supports the degree of familiarity between pairs of rats as being involved in yawn contagion, and auditory cues are the likely channel by which yawn contagion occurs[18].

Overall, these are unexpected results according to the prediction that familiar rather than unfamiliar rats will show contagious yawning. Although the results presented do not use a positive control (we have no data in which contagious yawning is observed in closely related individuals), it is believed that this method is unbiased for contagious yawning in unfamiliar rats. Several elements support such a claim. First, familiar and unfamiliar rats did not differ in the average frequencies of yawning[18]; thus, the differences in yawn contagion detected here did not seem to depend on how frequently they yawned. Second, the fact that the same numbers of yawns per rat were randomly allocated to the observation period and that this did not produce yawn contagion in any group renders the finding of contagious yawning in unfamiliar rats more robust. Finally, a cross-correlation analysis (to detect contemporaneous matching of behavior) was previously applied[18] to the same dataset used here, and the result coincided with what was found. Therefore, this method is sensitive enough to detect and measure changes in contagious yawning.

Alternatively, it may be questioned whether what was found here is contagious yawning. Such a concern is not exclusive to this study. There are two perspectives from which contagious yawning and its causality have aroused concerns. First, empathy does not seem to be the only factor underlying contagious yawning[18], as species not expected to show empathy and/or mental state attribution skills such as sheep and rats can still show contagious yawning. Second, there is growing concern that what is called contagious yawning might not be as such. For many decades (Thorpe, 1956; cited previously[10]), contagious, stereotyped behavior patterns and contagious yawning in particular have been regarded as an expression of social facilitation. More recently, Kapitány and Nielsen[25] suggested through the use of simulated data that what is a perceptual pattern-recognition error may be incorrectly called contagious yawning. Therefore, the problem may not lie in the way contagious yawning is being measured but rather in how it is defined.

In summary, the method proposed here is useful to detect yawn contagion. In addition, it is useful to discard some sensory cues, thereby reducing the number of sensory cues to only one or two. Further experiments to test specific predictions are necessary to distinguish the effects of one sense from the others.

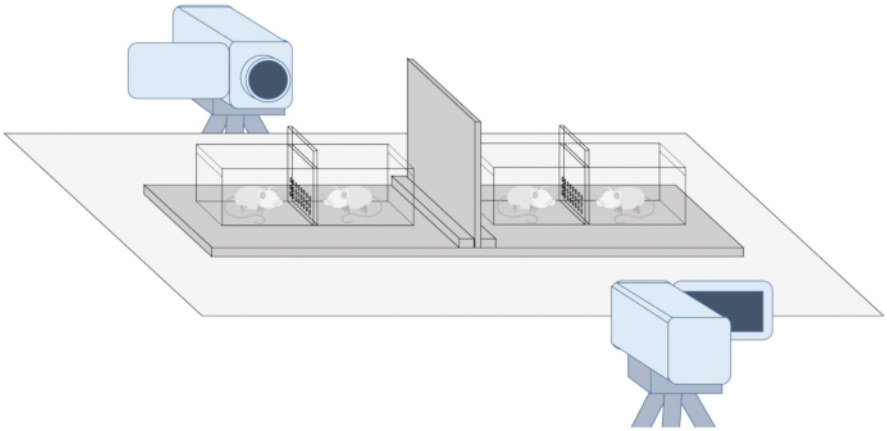


Figure 1: Schematic of the observation set-up to measure contagious yawning. Four individual cages were arranged in pairs on each side of the inverted T-shaped table. In each pair, the cages were facing each other with acrylic dividers in between. The specific test situation determines whether the divider in between the cages has holes and whether it is clear or opaque. The camcorders were strategically placed to record any instance of yawning behavior. Please click here to view a larger version of this figure.

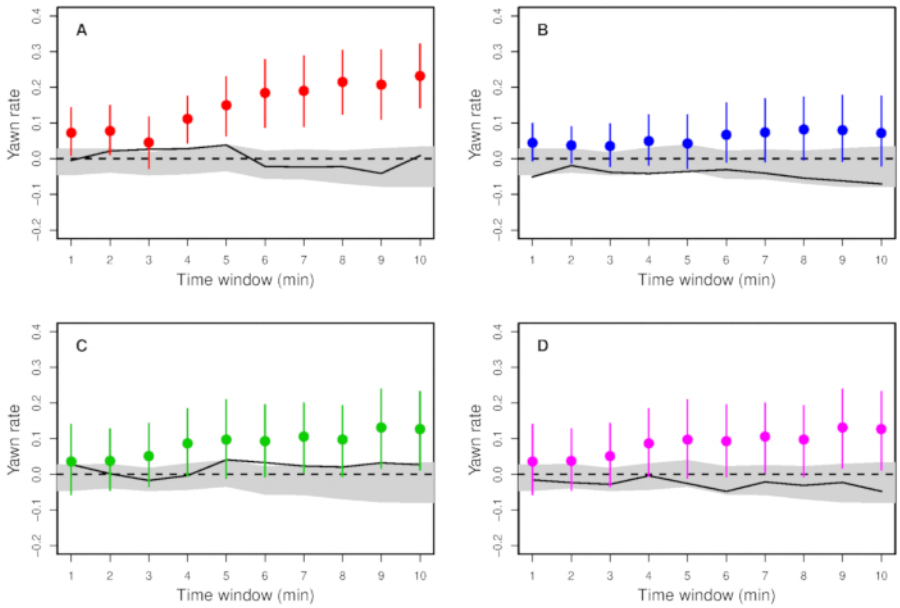


Figure 2: Yawn contagion curves for nine pairs of unfamiliar male rats exposed to (A) olfactory cues, (B) visual cues, (C) olfactory and visual cues, and (D) neither visual nor olfactory cues. Each circle represents the average difference in yawn rate with 95% confidence intervals (CI) from a rat that yawned in the same minute or in the minute (time window) preceding the yawn of the other rat and when it did not yawn. Yawn rate values above the dashed line indicate yawn contagion, whereas values below the dashed line indicate non-yawn contagion. The solid line joining the points at each time window is used to indicate the average difference in yawn rates at each time window when the number of yawns of each rat was randomly allocated to the 60 min observation period. The dark gray band indicates a 95% CI obtained by combining the random dataset from the four test situations at each time window (a continuous shade is used to facilitate the interpretation of the figure). One pair of rats exposed to olfactory cues was removed from the analysis because neither of the rats yawned. Please click here to view a larger version of this figure.

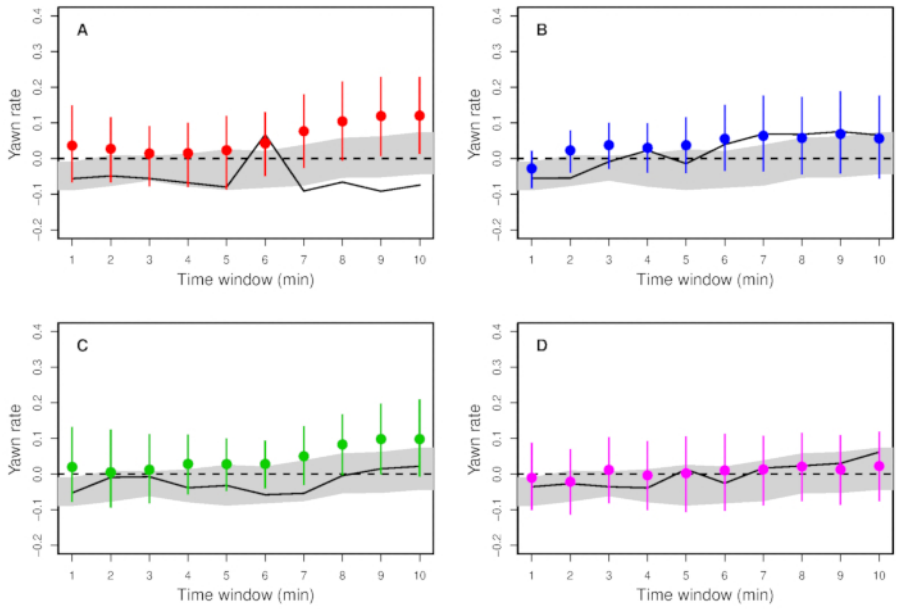


Figure 3: Yawn contagion curves for nine pairs of familiar male rats exposed to (A) olfactory cues, (B) visual cues, (C) olfactory and visual cues, and (D) neither visual nor olfactory cues. Each circle represents the average difference in yawn rate with 95% confidence intervals (CI) from a rat that yawned in the same minute or in the minute (time window) preceding the yawn of the other rat and when it did not yawn. Yawn rate values above the dashed line indicate yawn contagion, whereas values below the dashed line indicate non-yawn contagion. The solid line indicates the average difference in yawn rates at each time window when the number of yawns of each rat was randomly allocated to the 60 min observation period. The dark gray band indicates a 95% CI obtained by combining the random dataset from the four test situations at each time window. One pair of rats exposed to visual cues was removed from the analysis because one of the rats did not yawn. Please click here to view a larger version of this figure.

Discussion

There are critical steps in the method that should be taken into account to obtain successful results. Familiar rats must share home cages for at least 1.5 months after weaning and before running the experiments. However, unfamiliar rats must live in separate home cages. In both cases, the pairs of rats must come from different litters but be as similar in age as possible. Regarding the observation cages, their holes should match those in the dividers, because this is the only way to guarantee olfactory contact between the rats. The dividers, on the other hand, should be clear enough to ensure visual contact or opaque enough to ensure no visual contact. The wooden partition between one pair of rats and the other must be sufficient to prevent the rats on one side from seeing those on the other side. Another crucial aspect is the appropriate design of the experiment. Whenever there is a suspicion that a process may be biased, a random procedure should be implemented[30].

The method should not present serious problems to users. Making the holes in the glass was the main technical problem faced, and because of that, acrylic was used instead. Nonetheless, glass may be used for making the entire observation cages, provided professional advice is obtained. It should be ensured that the edges of the holes are filed to avoid glass splinters that may injure the rats. However, modifying the main method is not recommended (e.g., making the holes larger). Additionally, use of a combined group of males and females may make it difficult to detect yawn contagion.

The dividers used here may be insufficient to prevent the rats from using auditory cues, because rats are able to produce and perceive sounds at frequencies at which the materials of the observation cages and dividers likely did not block. However, this situation itself makes it possible to infer that auditory cues caused yawn contagion[18] and that olfactory cues only facilitated the recognition of the partner's degree of familiarity. Therefore, the method proposed here still provides reasonable evidence to identify the senses involved in contagious yawning and their intensities.

Earlier methods have been designed to study contagious yawning in laboratory conditions mainly by presenting videos to the experimental individuals[12,13], but these were questionable approaches in terms of biological validity. The method presented here solves this concern by using socially interacting animals in conditions more similar to what occurs in the real world. Furthermore, it is possible to simultaneously explore the participation of several sensory modalities in a single experimental set-up. It is recognized that this method does not absolutely discriminate between the effects of auditory cues and other sensory cues. However, a well-designed further experiment may allow researchers to infer the most likely sensory modality involved[18]. One possible noninvasive solution is the use of white noise to mask sounds and eliminate auditory cues. Similarly, researchers may expose naïve rats to bedding from OC rats to determine the role of olfactory cues, which is a proven procedure in social facilitation studies[32].

The use of this method can be extended to study yawn contagion in other species. For example, this set-up can be used after simple modifications with animals such as mice and hamsters to compare yawn contagion curves. Comparisons among different species may reveal unexpected patterns. The basic experimental plan can work with larger animals such as guinea pigs, cats, and rabbits. Likewise, the method can be used to study other potentially contagious behaviors such as grooming and scratching. The R-based program can reduce the time spent

calculating yawn contagion for several time windows and can be used to measure yawn contagion in other vertebrate species, provided the user has previously collected relevant data.

In summary, the main advantages of this method are the leading to acquisition of yawn contagion curves and aiding in discrimination between the relative roles of sensory modalities involved. The acquisition of yawn contagion curves is, as far as we know, a novel approach that may be useful to measure the strength of a contagion and observe how this intensity varies among species. Accordingly, the method can also be used with some modifications in other animal species such as sheep[16], wolves[15], dogs[33], snakes[34], and fish[5]. In all these species except snakes, yawning has been previously documented. In fact, a method similar to the one presented here has been used successfully in budgerigars[27]. This method may also be used to study other types of behavior that are contagious. For example, behaviors such as emotional reactivity, grooming, and scratching in rodents may be contagious. In fact, the method proposed here showed contagious emotional reactivity in familiar rats[18].

Disclosures

The authors declare no conflicts of interest.

Acknowledgments

A. M. was partially funded by the Vicerrectoría de Docencia, Benemérita Universidad Autónoma de Puebla. We are especially indebted to the staff of the animal facility “Claude Bernard” for the use of the rats for the shoot. We thank anonymous referees for their comments on early versions of this manuscript. The presentation is less strident and more balanced because of their thoughtful comments

References

1. Cézilly, F. A. History of Behavioural Ecology. In *Behavioural Ecology.* Danchin, E., Giraldeau, L-A., Cézilly, F. Oxford University Press. Oxford. 3-27 (2008).
2. Medina, L. M., Garcia, C. M., Urbina, A. F., Manjarrez, J., Moyaho, A. Female vibration discourages male courtship behaviour in the Amarillo fish (Girardinichthys multiradiatus). *Behavioural Processes.* **100,** 163-168 (2013).
3. Mekdara, P. J., Schwalbe, M. A., Coughlin, L. L., Tytell, E. D. The effects of lateral line ablation and regeneration in schooling giant danios. *Journal of Experimental Biology.* **221** (8), 175166 (2018).
4. Walusinski, O. Contagious Yawning. In *Encyclopedia of Animal Cognition and Behavior.* Vonk, J., & Shackelford, T.K. Springer Nature. Switzerland AG. 1-5 (2018).
5. Baenninger, R. Some comparative aspects of yawning in *Bettasplendens*, *Homosapiens*, *Pantheraleo*, and *Papiosphinx. Journal of Comparative Psychology.* **101** (4), 349-354 (1987).
6. Gallup, A. C., Church, A. M., Pelegrino, A.J. Yawn duration predicts brain weight and cortical neuron number in mammals. *Biology Letters.* **12**, 20160545 (2016).
7. Baenninger, R. On yawning and its functions. *Psychonomic Bulletin and Review.* **4** (2), 198-207 (1997).
8. Anías, J., Holmgren, B., Urbá-Holmgren, R., Eguíbar, J. R. Circadian variation of yawning behavior. *Acta Neurobiologiae Experimentalis* . **44** (4), 179-186 (1984).
9. Holmgren, B. et al. Food anticipatory yawning rhythm in the rat. *Acta Neurobiologiae Experimentalis.* **51** (3-4), 97-105 (1991).
10. Byrne, R. W. The evolution of intelligence. In *Behaviour and Evolution,.* Slater, P. J. B., Halliday, T. R. Cambridge University Press. Cambridge. 223-265 (1994).
11. Provine, R. R. Yawning as a stereotyped action pattern and releasing stimulus. *Ethology.* **72** (2), 109-122 (1986).
12. Provine, R. R. Faces as releasers of contagious yawning: An approach to face detection using normal human subjects. *Bulletin of the Psychonomic Society.* **27** (3), 211-214 (1989).
13. Anderson, J. R., Myowa-Yamakoshi, M., Matsuzawa, T. Contagious yawning in chimpanzees. *Proceedings of the Royal Society B: Biological Sciences.* **271** (Suppl 6), S468-S470 (2004).
14. Palagi, E., Leone, A., Mancini, G., Ferrari, P. F. Contagious yawning in gelada baboons as a possible expression of empathy. *Proceedings of the National Academy of Sciences.* **106** (46), 19262-19267 (2009).
15. Romero, T., Ito, M., Saito, A., Hasegawa, T. Social modulation of contagious yawning in wolves. *PLoS One.* **9** (8), e105963 (2014).
16. Yonezawa, T., Sato, K., Uchida, M., Matsuki, N., Yamazaki, A. Presence of contagious yawning in sheep. *Animal Science Journal.* **88** (1), 195-200 (2017).
17. Miller, M. L., Gallup, A. C., Vogel, A. R., Vicario, S. M., Clark, A. B. Evidence for contagious behaviors in budgerigars (Melopsittacus undulatus): an observational study of yawning and stretching. *Behavioral Process.* **89** (3), 264-270 (2012).
18. Moyaho, A., Rivas-Zamudio, X., Ugarte, A., Eguíbar, J. R., Valencia, J. Smell facilitates auditory contagious yawning in stranger rats. *Animal Cognition.* **18** (1), 279-290 (2015).
19. Moyaho, A., Flores Urbina A., Monjaraz Guzmán E., Walusinski O. Yawning: a cue and a signal. *Heliyon.* **3** (1), e00437 (2017).
20. Gallup, A. C. Why do we yawn? Primitive versus derived features. *Neuroscience&BiobehavioralReviews.* **35**, 765-769 (2011).
21. Gallup, A. C., Clark, A. B. Commentary: Yawning, acute stressors, and arousal reduction in Nazca booby adults and nestlings. *Frontiers in Psychology.* **6**, 1654 (2015).
22. Fentress, J. C. Development of grooming in mice with amputated forelimbs. *Science.* **179** (4074), 704-705. (1973).
23. Nicoletto, P. F. The roles of vision and the chemical senses in predatory behavior of the skink, *Scincellalateralis. Journal of Herpetology.* **19** (4), 487-491 (1985).
24. Fehér, O., Wang, H., Saar, S., Mitra, P. P., Tchernichovski, O. De novo establishment of wild-type song culture in the zebra finch. *Nature.* **459** (7246), 564-568 (2009).
25. Kapitány, R., Nielsen, M. Are yawns really contagious? A critique and quantification of yawn contagion. *Adaptive Human Behavior Physiology.* **3** (2), 134-155 (2017).
26. Langford, D. J. et al. Social modulation of pain as evidence for empathy in mice. *Science.* **312** (5782), 1967-1970 (2006).

27. Gallup, A. C., Swartwood, L., Militello, J., Sackett, S. Experimental evidence of contagious yawning in budgerigars (*Melopsittacus undulatus*). *Animal Cognition.* **18**, 1051-1058 (2015).
28. Campbell, M. W., de Waal, F. B. Methodological Problems in the Study of Contagious Yawning. In *The mystery of yawning in physiology and disease.* Walusinski, O. Karger Publishers. Basel. 120-127 (2010).
29. R Development Core Team. *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, <http://www.R-project.org> (2009).
30. Moyaho, A., Beristain-Castillo, E. Experimental Design: Basic Concepts. In *Encyclopedia of Animal Behavior.*, Choe, J. C. Academic Press. Second Edition. **3**, 471-479 (2019).
31. Urbá-Holmgren, R. et al. Genotypic dependency of spontaneous yawning frequency in the rat. *Behavioral Brain Research.* **40** (1), 29-35 (1990).
32. Smith, M. L., Hostetler, C. M, Heinricher, M. M., Ryabinin, A. E. Social transfer of pain in mice. *Science Advances.* **2**, e1600855 (2016).
33. Joly-Mascheroni, R. M., Senju, A., Shepherd, A. J. Dogs catch human yawns. *Biology Letters.* **4** (5), 446-448 (2008).
34. Barthalmus, G. T., Zielinski, W. J. Xenopus skin mucus induces oral dyskinesias that promote escape from snakes. *Pharmacology Biochemistry and Behavior.* **30** (4), 957-959 (1988).

scientific reports

Check for updates

OPEN

Experimental evidence for yawn contagion in orangutans (*Pongo pygmaeus*)

Evy van Berlo[1,2]✉, Alejandra P. Díaz-Loyo[3], Oscar E. Juárez-Mora[3], Mariska E. Kret[1,2] & Jorg J. M. Massen[4]

Yawning is highly contagious, yet both its proximate mechanism(s) and its ultimate causation remain poorly understood. Scholars have suggested a link between contagious yawning (CY) and sociality due to its appearance in mostly social species. Nevertheless, as findings are inconsistent, CY's function and evolution remains heavily debated. One way to understand the evolution of CY is by studying it in hominids. Although CY has been found in chimpanzees and bonobos, but is absent in gorillas, data on orangutans are missing despite them being the least social hominid. Orangutans are thus interesting for understanding CY's phylogeny. Here, we experimentally tested whether orangutans yawn contagiously in response to videos of conspecifics yawning. Furthermore, we investigated whether CY was affected by familiarity with the yawning individual (i.e. a familiar or unfamiliar conspecific and a 3D orangutan avatar). In 700 trials across 8 individuals, we found that orangutans are more likely to yawn in response to yawn videos compared to control videos of conspecifics, but not to yawn videos of the avatar. Interestingly, CY occurred regardless of whether a conspecific was familiar or unfamiliar. We conclude that CY was likely already present in the last common ancestor of humans and great apes, though more converging evidence is needed.

Yawning is an evolutionarily old phenomenon as its associated motor features can be recognized in different groups of animals[1]. It follows a stereotyped pattern that, once started, is unstoppable[2]. Apart from its spontaneous form, it is also notoriously contagious, at least for some species; i.e. individuals yawn as an unconscious and automatic response to seeing or hearing other individuals yawn[3]. While a yawning-like pattern is observed in a wide range of vertebrates[1], contagious yawning (CY) is less wide-spread. To date, CY appears to be present in only a few, generally social species, including tonkean macaques[4] (and possibly stumptail macaques[5]), gelada baboons[6], chimpanzees[7–14], bonobos[15,16], dogs and wolves[17–19], sheep[20], elephant seals[21], budgerigars[22], and rats[23]. In contrast, studies failed to show CY in grey-cheeked mangabeys and long-tailed macaques[24], mandrills[1], common marmosets[25], lemurs[26], horses[27], lions[1], tortoises[28], and fish[1], even though some of these species are also very social. Despite growing interest in CY, both its proximate mechanisms (how it functions and develops) and ultimate causes (why and how it evolved) currently remain unclear.

Several hypotheses have been put forward, following a Tinbergian approach[29]. One view on the proximate mechanism underlying CY is that it is an automatic form of physiological or emotional state-matching between individuals. This synchrony of states between individuals may work via a perception–action mechanism (PAM), an adaptive mechanism that serves to create and maintain relationships in highly social species and that can give rise to higher-order cognitive phenomena such as empathy[30]. Some scholars argue that CY taps into the same PAM as emotion contagion (e.g.[6,7,31,32]), which is the tendency to automatically synchronize emotional states with another individual[33]. Following this line of thought, CY can thus potentially be a proxy for empathy (i.e. the CY-empathy hypothesis)[6,9,12,18,31,34,35]. Indeed, neuroimaging studies have shown increased brain activity during CY in areas involved in theory of mind and social cognition [36–38], corroborating the idea that CY is linked with emotional state-matching and perhaps even empathy. Furthermore, individuals who score low on empathy scales (e.g. individuals on the autism spectrum) are less likely to engage in CY[39], and females yawn more frequently in response to seeing others yawn than males do, reflecting the idea that females show higher levels of empathy than males because of their investment in offspring care[40]. Nevertheless, there are some studies that do not find such a clear link between CY and empathy. For instance, when people with autism spectrum disorder (ASD) are

[1]Institute of Psychology, Cognitive Psychology Unit, Leiden University, Leiden, The Netherlands. [2]Leiden Institute for Brain and Cognition (LIBC), Leiden, The Netherlands. [3]Laboratorio de Ecología de La Conducta, Instituto de Fisiología, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico. [4]Department of Biology, Animal Ecology Group, Utrecht University, Utrecht, The Netherlands. ✉email: evyvanberlo@gmail.com

instructed to pay attention to the eyes (avoidance of the eyes is one of the characteristics of ASD), they are just as likely to yawn contagiously as neurotypical individuals[41]. Furthermore, the gender bias is not consistently found (e.g. [10,42]) and heavily debated[43,44]. For instance, in chimpanzees, it appears that males yawn more frequently than females in response to seeing other males yawn[10]. Finally, while dogs do engage in CY, its presence is not affected by whether the yawner is prosocial versus the yawner being antisocial[45]. The mixed findings in the studies investigating the relationship between CY and a complex construct such as empathy show that the topic deserves more attention, and that it is still debated (see Massen & Gallup (2017) for a critical review).

The emotional bias hypothesis is a more detailed specification of how CY can be socially modulated through a shared PAM, namely via social closeness and familiarity. The hypothesis predicts that individuals who are socially, and thus emotionally close are also more likely to yawn contagiously in response to each other[15,16,18,19,34,46,47]. Additionally, individuals from a group (i.e. familiar others) are more likely to yawn in response to each other than to unfamiliar others[9,18]. A potential issue that has been raised is that these studies often fail to rule out simple alternative explanations for CY that do not require higher-order cognition[48]. For instance, effects of familiarity on CY may be explained by a general tendency to bias attention to familiar and socially close others[48]. Nevertheless, in a recent study investigating auditory yawn contagion in humans, yawns were most contagious between family and friends while controlling for the potential effects of increased attention to socially close others using a non-visual stimuli[34]. Still, in quite some social species, the linkage between CY and social closeness or familiarity is not found[10,45,49–51]. For example, a recent study analyzing a large dataset on CY in dogs shows CY is present in dogs, but is not affected by familiarity or other potential mediators such as sex or prosociality[45]. It therefore remains possible that mechanisms other than the same PAM that underlies emotion contagion or empathy are mediating CY. For instance, CY may result from stress induced by a common stressor in the environment[5,52]. Thus, rather than being mediated by seeing others yawn, yawning occurs as a response to the stressor. Individuals that are stressed are known to show higher rates of self-directed behaviors, of which yawning and scratching are examples[53], and indeed, in one study involving stumptail macaques, monkeys yawned more frequently in response to a video clip of yawns as compared to a control, but also scratched more[5]. The authors concluded that tension was most likely mediating the occurrence of yawning in the yawn condition. In short, while it is likely that CY is a social phenomenon, its exact mechanisms remain an active field of investigation.

Notwithstanding the debate on proximate mechanisms, little attention has been given to more ultimate explanations for CY. One of the few hypotheses out there is that CY is an adaptive mechanism that helps with social coordination[54]. Accumulating evidence suggests that yawning itself serves to cool the brain as to maintain homeostasis[55–60] and consequently may increase alertness and aid in vigilance. Within this social coordination hypothesis, CY, in turn, may help to spread vigilance within the group, for instance to remain alert for potential predators[54,57]. Specifically, it may be adaptive to match the state of a vigilant conspecific as it may have sensed a predator, which the individual itself did not yet sense. To date, however, the social coordination hypothesis remains untested, and the thermoregulatory function of yawning is still debated (e.g.[61,62], but see[58] for a response to the critique).

Another fruitful way to explore evolutionary hypotheses is through phylogenetic comparisons. Palagi et al. (2019) proposed the *common trait among hominids* hypothesis which states that, given the shared phylogeny between humans and great apes, CY may find its roots in a shared underlying socio-cognitive mechanism that was already present in at least the last common ancestor (LCA) of all hominids. Moreover, since CY is also present in some Old-World monkeys and non-primate species, its roots could be much older, or CY is an example of convergent evolution. To date, few data exist to perform comparisons and most interestingly, the picture among the great apes is not yet clear. There is convincing evidence for CY in chimpanzees[7,8,10,12,14]. In bonobos, two observational studies[15,16] and an experiment[63] show clear evidence for CY, while one experimental study did not[12]. However, the latter study only tested four individuals, thus making it very likely that CY is, indeed, present in bonobos. Finally, the first comprehensive study on gorillas combining an experimental and naturalistic approach found no evidence for CY[64]. Notoriously absent are data on CY in orangutans, which, considering their semi-solitary lifestyle[65] may be of comparative interest for a social phenomenon like CY. To date, the only existing study involving orangutans failed to find evidence for CY[12], yet the sample size was too small to be conclusive. In general, orangutans in the wild roam mostly solitarily: males travel alone, and mothers travel with their offspring[66]. Due to overlapping home ranges, occasional encounters and affiliation are possible, but generally do not occur frequently[66,67]. Consequently, finding out whether CY is present in orangutans will further help elucidate the hypotheses previously discussed.

The current study attempts to clear up the picture of CY in hominids in two ways. First, we aim to find a convincing answer to whether CY is present in orangutans or not via an experimental design involving the presentation of yawning and neutral stimuli of orangutans to 8 orangutans. Second, we also investigate whether this potential yawn contagion is affected by a familiarity bias, i.e. whether CY is stronger between individuals that know each other versus unfamiliar individuals. To this end, we exposed orangutans to videos showing either yawn or control clips of familiar (i.e. conspecifics living in close proximity) and unfamiliar orangutans, as well as a 3D avatar[68] and measured their response (yawns). Additionally, we also measured the occurrence of scratching to rule out potential effects of stress on the occurrence of yawning[53]. So far, CY appears to be exclusively present in highly social species, and because orangutans do not show high affiliative tendencies, we therefore expected that orangutans do not show CY.

Name	Sex	Date of Birth	Developmental Stage[1]	Relationship
Amos	Male	20-12-2000	Adult	Father of Kawan, Baju and Indah
Baju	Male	02-12-2015	Juvenile	Son of Amos and Wattana
Indah	Female	19-10-2017	Infant	Daughter of Amos and Samboja, granddaughter of Sandy
Kawan	Male	22-02-2010	Adolescent (unflanged)	Son of Amos and Wattana
Kevin	Male	~1982	Adult	Born in the wild, no kin in group
Sandy	Female	29-04-1982	Adult	Mother of Samboja, grandmother of Indah
Samboja	Female	09-01-2005	Adult	Daughter of Sandy, mother of Indah
Wattana	Female	17-11-1995	Adult	Mother of Kawan and Baju

Table 1. Overview of test subjects, their sex, age, and relationship. [1]At start of experiment (January 2019).

Materials and methods

Ethics. The care and housing of the orangutans was adherent to the guidelines of the EAZA Ex situ Program (EEP). As the study was non-invasive in nature, there was no need for the approval of the Ethics Committee of Apenheul primate park and the study complied with the requirements of the Dutch Animal Care and Use Committee.

Subjects. Eight orangutans (ages: 15 months-36 years, 4 males) housed at Apenheul (The Netherlands), were tested (see Table 1 for an overview on sex and age). Individuals were divided over four neighboring enclosures and group composition varied weekly (Figure S1). The two adult females that had dependent offspring were always housed with their offspring and sometimes with one adult male. Experiments took place in the visitor area but while the park was closed to visitors. We tested individuals using a movable 47″ TV (LG 47LH5000, 1920 × 1080 pixels) placed in front of the enclosures to which the orangutans were habituated before commencing testing. The screen was always directed at one of the four enclosures, which prevented orangutans in the other enclosures from seeing the videos. Food was provided four to six times a day and consisted of a variety of vegetables, and sometimes nuts, hay, and fruit, hidden in the enclosure for foraging purposes. Water was available ad libitum.

Stimuli. The experiment involved three categories of mute, full-screen videos, each consisting of both a yawn and control condition (see Fig. 1 for examples). We used mute videos as the enclosures were sealed with thick glass that dampened most of the sound both ways. Yawn videos showed clear yawns either filmed from the front or side, whereas control videos consisted of individuals with a neutral face and in a relaxed body position. Both types of videos involved movement, with yawn videos showing a wide gaping of the mouth followed by a relaxation of the mouth and jaw[69], including display of the teeth, and control videos showing an individual with a closed mouth with random movements of the lips. Both control and yawn videos were always of the same individual, and therefore the body position and face were identical. The *familiar* video category consisted of two adult males housed in the zoo (published under CC BY-NC-SA). For the *unfamiliar* video category, we used two adult males taken from clips on YouTube[70,71](published under the YouTube Standard License). Finally, in the *avatar* video category we used two mirrored videos of a computer-generated adult male. The 3D orangutan was created by Paul Kolbrink[68] from XYZ-Animation and designed in Autodesk 3ds Max (2017) using the Octane render engine (published under CC BY-NC-SA). Using these videos, we created video sequences starting with a primer video that depicted caretakers beckoning the orangutans towards the TV screen, which were created to grab the orangutans' attention right before the start of a trial. As we repeated the presentation of our video database four times during the course of the experiment, there were four different primers; one for every repetition.

Procedure. The experiment was carried out between 21–01-2019 and 13–03-2019. In this period, the park was closed for visitors. A test session involved the presentation of two different trials, each consisting of a specific video sequence, and each trial followed by an observation period. The video sequence consisted of a primer, followed by either a yawn or control video (lasting 14 s), which was repeated 4 times and with a colored screen (again to grab attention) for 1 s in between each video. The length of one video sequence was thus 90 s (cf. Massen et al., 2013): primer (30 s) – colored screen (1 s) – yawn/control video (14 s) – colored screen (1 s) – yawn/control video (14 s) – colored screen (1 s) – yawn/control video (14 s) – colored screen (1 s) – yawn/control video (14 s). The presentation of one video sequence (representing one trial) was then followed by a 3.5-min observation period, after which the second trial started. If the first trial involved yawn videos, the second trial involved control videos and vice versa. The second trial was also followed by a 3.5-min observation period, completing one test session. Within one test session we always showed the same stimulus individual.

We cycled through the entire video database four times (i.e. 4 blocks) over the course of the experiment to ensure sufficient data points. The order of control and yawn trials were counterbalanced per subject, and was further counterbalanced over the subjects per block. Within each block, *trigger* (i.e. familiar/unfamiliar/avatar) was also randomized per subject. We designed a testing schedule based on eight test subjects, but two of those subjects involved a mother-infant pair and a mother-juvenile pair in which the infant/juvenile never left the mother. As such, we created a test schedule for six individuals rather than eight. With these six test subjects, three types of triggers, two conditions (yawn and control), two orders of condition presentation (yawn-control,

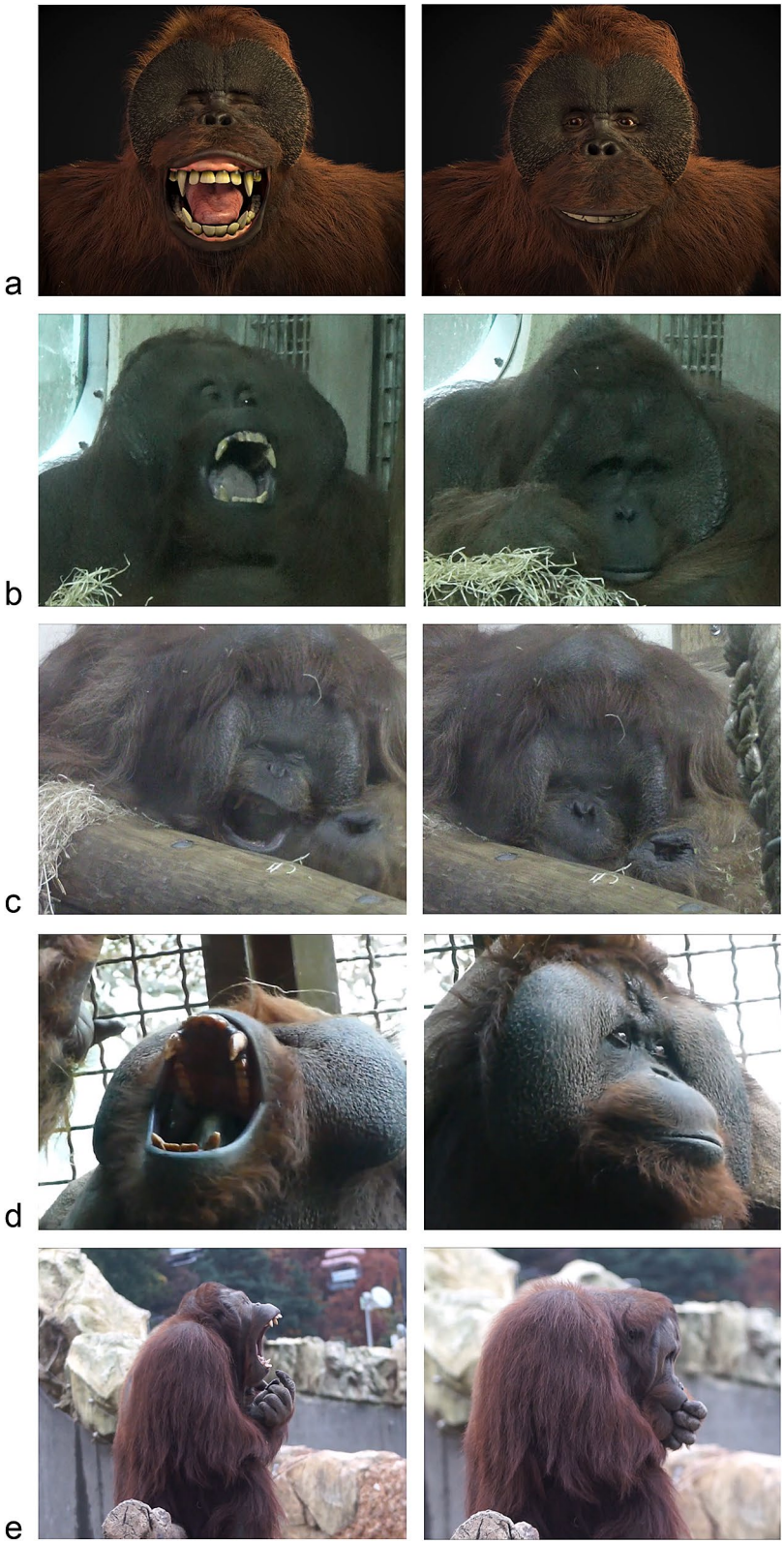


Figure 1. Stills of the videos used in the experiment with yawns on the left and controls on the right. A: Avatar*[68], B: Familiar adult 1, C: Familiar adult 2, D: Unfamiliar adult 1[70], E: Unfamiliar adult 2[71]. *To decrease the chances of pseudo-replication within this category we created horizontally mirrored copies of the yawn and control videos of the avatar such that – similar to the other triggers – we had two yawn and two control videos in total.

or control-yawn), and finally four repetitions, we had a total of 288 test sessions and 576 trials planned (see Table S1a-d and S2 for an overview). However, one video sequence was accidentally presented an extra time, resulting in 289 rather than the planned 288 sessions after data collection finished. On any given testing day, individuals participated in one or two sessions with 30 min breaks between video presentations to the tested subject. Furthermore, subjects never saw a video sequence more than once on any given day.

APDL and OEJM recorded all occurrences of yawning and scratching, and scratching was recorded as a measure for arousal and tension[53]. It was not possible to reliably quantify the amount of time spent looking at the screen due to the lack of continuous visibility of the gaze of the orangutans. To nonetheless ensure maximum attention to the screen, we presented primers before video sequences and colored videos in-between yawn and control clips, and we only started testing when orangutans had a direct line of sight towards the screen. Additionally, before each trial, we observed the orangutans for five minutes, and only started a trial if there were no yawns before the presentation so as to rule out that yawns within a trial were potentially caused by a previous yawn outside of the trial. Furthermore, yawns were scored in response to either the yawn or control video only if a subject looked at least once to the screen during presentation. If bystanders in the same enclosure attended to the screen, their behaviors were also scored. Data collection ended after 10 min, concluding one test session. Finally, EvB coded 15% of the videotapes for inter-rater reliability purposes. Results showed a good agreement on occurrences of yawning (ICC = 0.764, $p < 0.001$ Table S3) and scratching (ICC = 0.894, $p < 0.001$, Table S4). In subsequent analyses, only yawns on which the raters agreed were used.

Statistical analysis. The dependent variable was whether a subject yawned in response to a video or not. Because it is difficult to disentangle between whether multiple yawns occurring in succession are caused by another individual, or whether they are simply the result of an urge to yawn multiple times perhaps because of self-contagion (i.e. where your own yawns cause you to yawn again), we did not compare rates of yawning to establish CY[72]. Rather, we looked at the likelihood of yawning within the yawn and control condition to establish the presence or absence of CY in orangutans. Nevertheless, when contagion indeed occurred, yawning rate could inform about the *strength* of contagion[72]. As such, we analyzed our data using hurdle models in R (lme4 package). Hurdle models follow a two-step method that first deals with zero-inflated count data and subsequently with positive counts once the initial hurdle is crossed[73], which make them applicable to our dataset.

In the first hurdle model we focused on whether CY is present or absent in orangutans by comparing the likelihood of yawning in the yawn and control condition using a binomial GLMM, in which we added *condition* as a fixed effect and *subject* nested in *trial* as a random effect. In the second step of the model, we analyzed the rates of yawning using a negative binomial GLMM only in those cases where at least one yawn occurred. Again, we entered *condition* as a fixed effect and *subject* nested in *trial* as a random effect. In the second hurdle model, we tested for potential effects of both *condition* and *trigger* (i.e. familiar/unfamiliar/avatar) and their interaction on the likelihood of yawning using a binomial GLMM, entering *condition* and *trigger* and their interaction as fixed effects, and again *subject* nested in *trial* as random effect. In the second step of the model, we were interested in how the conditions and triggers affected yawning rates in those cases that at least one yawn occurred. To investigate this, we entered *condition* and *trigger* and their interaction as fixed effects and *subject* nested in *trial* as random effect using a negative binomial GLMM.

It is possible that the likelihood of yawning in the conditions is due to the stimuli somehow being arousing to the observers, complicating the interpretation of the underpinnings of CY (see e.g.[5]). For instance, yawning often involves display of the canines, which may be arousing for the orangutans[74]. Therefore, as a control analysis, we looked at self-scratching behavior as this is indicative of arousal in primates[53]. In a third hurdle model, we checked whether the likelihood of scratching is affected by *condition* (fixed factor), with *subject* nested in *trial* as random factor and using a binomial GLMM. In the second step of the model using a negative binomial GLMM with *subject* nested in *trial* as random factor, we investigated whether scratching rate was affected by *condition, trigger*, and their interaction as fixed factors only in those cases when scratching occurred.

In all analyses, we compared the models to their respective null-models (i.e. including only the random effects) and only report on significant values if the models and null-models differ significantly from each other[75]. For post-hoc contrasts of interaction effects we report corrected p-values using Tukey-adjustments. Alpha was set to 0.05.

Results

In total, we witnessed 83 yawns across 8 individuals and 289 sessions. First, we investigated the likelihood of yawning in the two conditions. We found a significant effect of *condition*; yawning was more likely to occur in the yawn versus the control condition ($\beta = 3.45$, $SE = 1.06$, $p = 0.001$). Next, we compared the yawning rate between the two conditions in those cases that at least one yawn occurred, but this alternative model did not deviate significantly ($\chi 2(1) = 3.09$, $p = 0.079$) from its respective null-model.

Assessing whether familiarity affects the occurrence of CY, we found a significant interaction effect of *trigger* (familiar, unfamiliar, avatar) with *condition*. Specifically, we found a significant contrast of yawns between the yawn and control condition in the familiar ($\beta = 6.62$, $SE = 1.59$, $p < 0.001$) and unfamiliar trigger ($\beta = 3.45$, $SE = 1.52$, $p = 0.023$), but not in the avatar trigger ($\beta = 0.09$, $SE = 1.58$, $p = 0.950$) (Fig. 2). Hence, orangutans are more likely to yawn in response to yawning videos rather than to control videos, but only when the yawning individual is a 'real' orangutan (i.e. a familiar or unfamiliar conspecific), and are less likely to yawn in response to the avatar. To investigate whether the likelihood of CY differed with regard to familiarity with the 'real' orangutan stimuli, we also ran an additional binomial model on a reduced dataset that excluded all trials with the avatar (see supplemental materials). Whereas this model confirmed the previously found effect of *condition*, here we did not find a significant interaction between *condition* and *familiarity*, suggesting that the likelihood

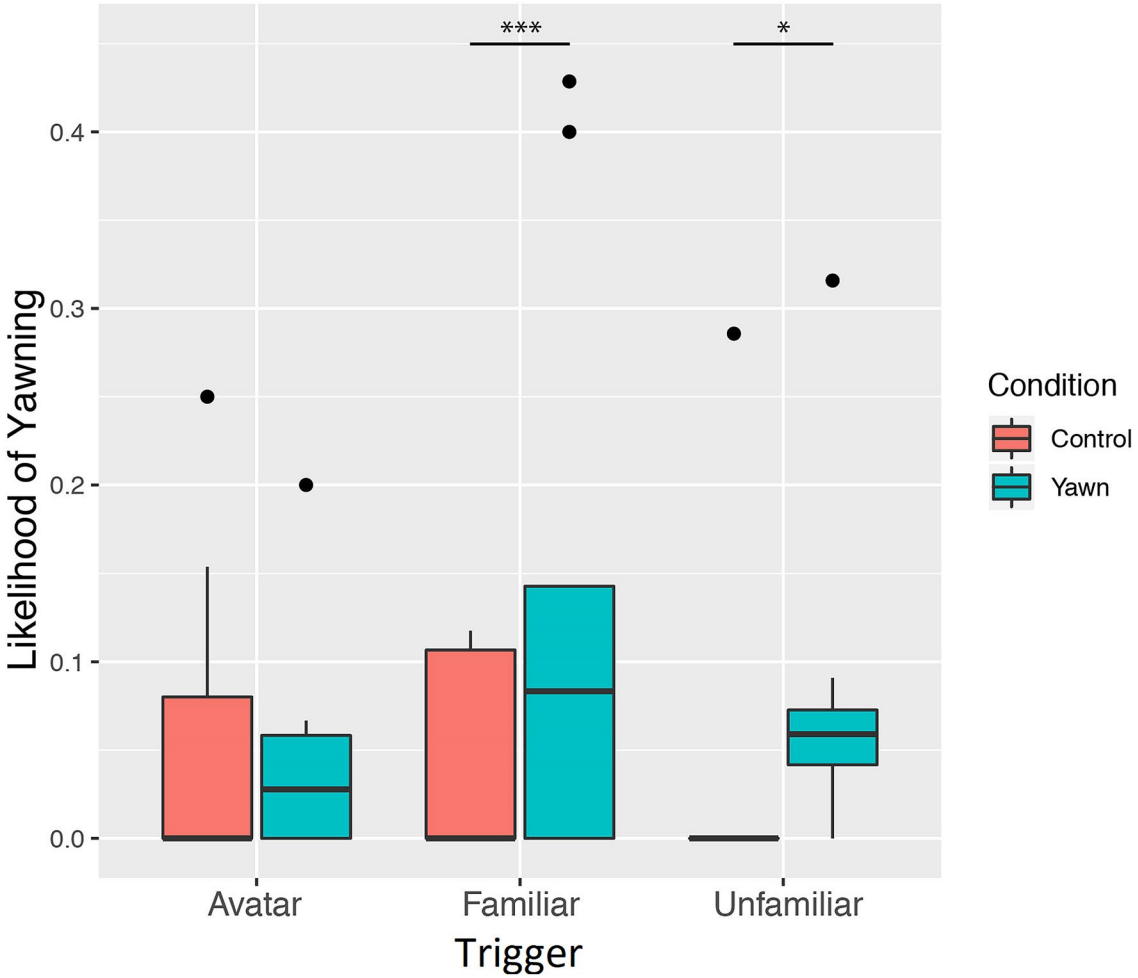


Figure 2. Likelihood of yawning across conditions and triggers. Boxplots show the median (solid line), 25th-75th percentile (box) and the largest and smallest value within 1.5 times the interquartile ranges respectively (whiskers). Dots reflect outliers.

of CY was not being modulated by the familiarity with the 'real' orangutan. We also investigated the effect of *familiarity* on yawning rate using the same reduced dataset, but the model including the interaction between *condition* and *trigger* did not significantly improve the null model ($\chi 2(3) = 3.50$, $p = 0.321$). As such, while we can establish that orangutans do show CY in response to yawn videos of familiar and unfamiliar conspecifics, this likelihood of CY is not modulated by familiarity and we cannot draw any conclusions regarding the *strength* of CY in relation to familiarity.

Looking at scratching, we first investigated the likelihood of scratching when viewing yawning and control videos, and found that the *occurrence* of scratching did not differ between conditions ($\beta = -0.17$, $SE = 0.17$, $p = 0.319$). Similarly, scratching *rates* were not significantly higher in the yawn versus control condition ($\beta = 0.10$, $SE = 0.09$, $p = 0.301$). Moreover, both models did not deviate significantly from their null-model ($\chi 2(1) = 1.06$, $p = 0.303$). Hence, it is unlikely that orangutans were more aroused viewing yawn videos compared to viewing control videos, at least when measured via scratching Additionally, we also included scratching in our original models on yawning as a covariate, and found it to not significantly explain the likelihood of yawning, nor to influence the found effects of condition and the lack thereof in the avatar treatment (see Supplements).

Discussion

Here we find that orangutans yawn contagiously in response to conspecifics yawning, independent of whether the conspecific is a familiar or unfamiliar individual. Furthermore, orangutans were not susceptible to yawns of an avatar. Additionally, the videos used in our experiment appeared to be similarly arousing. That is, there was no difference in scratching (an indicator of stress) between the conditions. We here discuss the consequences of our findings for the different proximate and ultimate hypotheses that currently exist.

CY has thus far been observed in highly social species[6,7,15,17,19–22] (but see:[1,24–27]). Orangutans have meaningful social interactions that occur more often than is expected by chance alone[76], but these interactions occur at a much lower frequency compared to bonobos and chimpanzees[66,67]. Interestingly, our results show that orangutans exhibit CY, suggesting that a high degree of affiliation within a species is not necessary for CY to occur. This also indicates that more studies are needed that investigate the presence or, importantly, absence of CY in a

variety of species that differ on their social organization and affiliative tendencies. At the same time, it has to be noted that our sample consists of zoo-housed orangutans that were also born in captivity. In captivity, frequencies of affiliation can exceed those observed in the wild[77], thus potentially increasing the likelihood of CY to occur. Nevertheless, our results do show the presence of CY in orangutans and the few generations of zoo-living individuals cannot inform us about any selection pressures that have resulted in this tendency in orangutans. Our results must therefore be discussed in light of the orangutans' natural behavior and social environment.

In our study, we did not find an effect of familiarity on CY, suggesting that at least in orangutans, social modulation of CY may not be present. While presence of social modulation of CY is often used as confirmation of CY and emotion contagion sharing the same underlying perception–action mechanism[9,15,16,18,35], its absence in our data makes it more difficult to interpret the emotional bias hypothesis. Orangutans do have some preferences when it comes to their interaction partners, thus one could expect social modulation of CY under the emotional bias hypothesis. For instance, related female orangutans are known to associate more often than unrelated females[78], and prefer the long-calls of dominant males[79]. Additionally, in a recent study, orangutans were shown to scratch contagiously in response to conspecifics scratching, suggesting a potential case of emotion contagion[80]. Interestingly, scratch contagion was stronger between weakly bonded individuals during tense situations, which shows a social closeness bias in the opposite direction. This suggests that a familiarity bias may be more flexible depending on the situation individuals are in (e.g. relaxed versus stressful contexts) and the nature of the behavior that is copied (e.g. scratching as an expression of tension). At the same time, there are other studies on highly social species that do not show a familiarity bias (e.g. chimpanzees[10], dogs[45], macaques[24], and marmosets[25]). As such, there may be (currently unknown) species-specific traits that determine whether a familiarity bias occurs or not. The exact (social) function of CY remains unclear and thus alternative explanations that do not involve the PAM that is underlying empathy may still be possible (e.g. spreading of vigilance). As has been pointed out by others, solving this issue requires a more systematic study of CY that includes a bigger variety of animals, including solitary animals such as reptiles and amphibians[48].

From an evolutionary perspective, our results pose an interesting conundrum: while we found CY in orangutans, it is not present in gorillas, even though the split between orangutans and other hominids is evolutionarily older than the split between gorillas and other hominids[81]. It is possible that the number of trials in the study by Palagi et al. (2019) were not sufficient to detect CY, as in our study, even with a large number of trials, we only detected yawns in 11.9% of all cases. Nevertheless, studies with chimpanzees that have few trials were able to establish CY in the past, albeit with a relatively large number of subjects[8,10,12], and there was also no evidence for CY in naturalistic observations in gorillas[64]. Interestingly, it has been argued that in the past, orangutans may have been more social, but that due to long periods of low food availability, orangutan gregariousness was no longer viable[82]. This may suggest that the ancestor of all apes already possessed the mechanism underlying CY. However, based on observational and relatedness data, it has been suggested that this hominid lived in a group with gorilla-like structure in which one male could monopolize multiple females[82]. In this sense, it is difficult to explain why, given a similar social structure, gorillas do not show CY and orangutans do. It is possible that CY was somehow lost in the gorilla lineage, or that CY developed multiple times over the course of evolution. The loss of CY is theoretically possible, given that CY has been found in some, but not all primates[1,64]. Here, there is a role for the type of social system that characterizes a species in the loss (or occurrence) of CY[64]. There is, however, not yet enough variation in data on CY in different species of primates to draw clear conclusions. Furthermore, it is possible that the measures to detect CY in certain species are simply not sensitive enough. All these explanations can be true, given that the occurrence of CY is highly variable in primates in general. It is clear that more studies are needed in order to draw robust conclusions about the evolution of CY.

In our study orangutans did not significantly respond to the avatar, which contrasts with findings in chimpanzees[8]. Potentially, orangutans experienced the uncanny valley phenomenon in which the avatar looks very realistic, yet fails to behave like a real orangutan, therefore violating natural expectations of orangutan behavior. Indeed, previous research on monkeys showed that they preferentially looked at real or completely unrealistic 3D model monkeys compared to very realistic 3D models[83]. Nevertheless, this would likely have increased scratching when viewing the avatar, which was not evident in our study. Furthermore, a recent study investigating the uncanny effect in macaques showed that looking times did not differ between the Primatar (3D monkey head) and real or unrealistic images, indicating that the use of virtual stimuli can still be a promising way to study social cognition[84]. Future studies will have to verify whether the lack of evidence for CY using an avatar in our study is because the effect is truly absent, for instance by looking specifically at how similarity with another individual (on a physical level) affects CY. In humans, there is ample evidence that the more similar that individuals are in terms of physical characteristics, but also personal convictions and views, the more likely they are to automatically mimic behavior[85].

Future studies can improve on the current study design in several ways. First, we only used orangutan males as stimuli. In previous studies with chimpanzees[10] and bonobos[15], the sex of the triggering yawner affected the occurrence of CY; i.e. in chimpanzees, male yawns were more contagious whereas in bonobos, female yawns were more contagious. In gelada baboons, CY is more prevalent among females, especially when they are closely bonded[6]. It is possible that these results can be explained by emotional closeness between individuals, as in chimpanzees males typically form strong social relationships[86], and in bonobos and gelada baboons it is mostly females that bond[87,88]. Alternatively, results could be explained by the differences in hierarchy with chimpanzees being male dominant[89] and bonobos female dominant[87], and by the strong matrilineal bonds between gelada baboons[90]. Investigating whether there is an interaction between sex of the stimulus and of the responder in orangutans could help elucidate the roots of the observed sex effects in CY in some species. The restricted selection of stimuli and the low sample size did unfortunately not allow us to perform such analyses. It is noteworthy, however, that the males in our study yawned more frequently than the females (i.e. the total yawning rate of males was 74, whereas females yawned only 9 times. See Table S1a). Yawns occur more frequently in males of

species with canine polymorphism, and also during aggressive contexts[91]. Given that all our stimuli were male, perhaps there is a role for dominance or rivalry in the occurrence of CY in orangutans[23]. Nevertheless, one could argue that this leads to tense situations, thus leading to more scratching when observing yawns of others, which is not what we found.

Additionally, all of our videos contained flanged males. Flanged adult males are often preferred over unflanged males by receptive female orangutans[92], and can be viewed as threatening by unflanged males[93]. As such, in addition to interactions between the different sexes and CY, it may also be interesting to study potential effects of the two different morphs of orangutan males on CY.

Furthermore, due to power issues, we could not reliably test effects of age on CY. In humans, while spontaneous yawns can occur already before birth[94], CY does not seem to appear until the age of four to five[95,96], although when children of 3 years old are specifically told to look at the eyes of the stimulus they show CY as well[97]. Similar developmental trajectories of CY have been reported in other animals[6,7,11,50]. In our study, there were only two individuals younger than 5; one 15 months (Indah) and one three-year old (Baju). We observed one yawn occurrence in Indah (in the yawn condition), in Baju we observed six events (four in the yawn and two in the control condition). We decided to include these individuals in our study because while it is true that CY shows a relatively slow developmental pattern in humans, orangutans are born more precocial, and developmental rates in nonhuman primates are much faster compared to humans[98]. Therefore, CY may possibly also occur earlier in development in orangutans, but with only anecdotal evidence we cannot verify this in our study.

Third, while we tested effects of familiarity in our study by including both familiar and unfamiliar yawners, the fact that we only had yawns from the two adult males to use as stimuli restricted any potential investigation of the potential link between social closeness of the responders and the familiar individuals on the stimuli. The positive effect of social closeness on the occurrence of CY is well established in humans[99], chimpanzees (but see[10]), and bonobos[15], but is strongly debated in other species such as dogs[45] and budgerigars[51]. For dogs, it should be noted that CY is interspecific, and that domestication might have had influential effects on how CY is modulated. Inverse effects have also been reported. For instance, a large study in rats has shown a familiarity bias in the opposite direction with rats being more likely to yawn in response to unfamiliar yawns[23]. Similarly, a recent study investigating scratch contagion in orangutans found that during tense situations, orangutans are more likely to take over scratching from individuals with whom they have a weak bond[80], indicating a (negative) correlation between social closeness and the contagiousness of a behavior or motor pattern. Thus, it remains possible that social modulation of CY is present in orangutans, at least in those living with conspecifics in captivity, although its presence was not shown in our sample. Yet, given our small sample size, replications that test for the presence and subsequent direction of social modulation of CY in orangutans are needed.

Finally, we could not quantify attention to the screen, which is one of the common methodological issues raised by Massen et al. (2017). We tried to maximize attention to the screen by using attention-grabbing videos of caretakers at the start of every video sequence, and by adding colored screens in-between stimulus presentations. Furthermore, we made sure that orangutans had a direct line of sight towards the screen at the start of the experiment, and only recorded yawns when they directed their attention to the screen at least once during stimulus presentation. Nevertheless, quantification of attention to the stimuli (either measured as a continuous variable or a frequency of gazes) remains the most robust way to control for potential effects of attentional bias.

To summarize, our findings contribute to understanding the evolutionary basis of CY in hominids by showing that orangutans, like humans, chimpanzees and bonobos, yawn contagiously.

Data availability

All data, code, and materials that are associated with this paper and used to conduct the analyses are uploaded and made openly accessible on the archiving platform DataverseNL: https://doi.org/10.34894/JIWWCN.

Received: 6 March 2020; Accepted: 3 December 2020
Published online: 17 December 2020

References

1. Baenninger, R. Some comparative aspects of yawning in Betta splendens, Homo sapiens, Panthera leo, and Papio sphinx. *J. Comp. Psychol.* **101**, 349–354 (1987).
2. Provine, R. R. Yawning as a stereotyped action pattern and releasing stimulus. *Ethology* **72**, 109–122 (1986).
3. Massen, J. J. M., Church, A. M. & Gallup, A. C. Auditory contagious yawning in humans: An investigation into affiliation and status effects. *Front. Psychol.* **6**, 1–8 (2015).
4. Palagi, E. & Norscia, I. The ethology of animal emotions: Investigation and interpretation. *Sist. Intelligenti* **31**, 11–32 (2019).
5. Paukner, A. & Anderson, J. R. Video-induced yawning in stumptail macaques (Macaca arctoides). *Biol. Lett.* **2**, 36–38 (2006).
6. Palagi, E., Leone, A., Mancini, G. & Ferrari, P. F. Contagious yawning in gelada baboons as a possible expression of empathy. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 19262–19267 (2009).
7. Anderson, J. R., Myowa-Yamakoshi, M. & Matsuzawa, T. Contagious yawning in chimpanzees. *Proc. R. Soc. B Biol. Sci.* **271**, 468–470 (2004).
8. Campbell, M. W., Carter, J. D., Proctor, D., Eisenberg, M. L. & De Waal, F. B. M. Computer animations stimulate contagious yawning in chimpanzees. *Proc. R. Soc. B Biol. Sci.* **276**, 4255–4259 (2009).
9. Campbell, M. W. & de Waal, F. B. M. Ingroup-outgroup bias in contagious yawning by chimpanzees supports link to empathy. *PLoS ONE* **6**, 19–22 (2011).
10. Massen, J. J. M., Vermunt, D. A. & Sterck, E. H. M. Male yawning is more contagious than female yawning among chimpanzees (Pan troglodytes). *PLoS ONE* **7**, 1–5 (2012).
11. Madsen, E. A., Persson, T., Sayehli, S., Lenninger, S. & Sonesson, G. Chimpanzees show a developmental increase in susceptibility to contagious yawning: a test of the effect of ontogeny and emotional closeness on yawn contagion. *PLoS One* **8**, (2013).
12. Amici, F., Aureli, F. & Call, J. Response facilitation in the four great apes: Is there a role for empathy? *Primates* **55**, 113–118 (2014).
13. Campbell, M. W. & de Waal, F. B. M. Chimpanzees empathize with group mates and humans, but not with baboons or unfamiliar chimpanzees. *Proc. R. Soc. B Biol. Sci.* **281**, (2014).

14. Campbell, M. W. & Cox, C. R. Observational data reveal evidence and parameters of contagious yawning in the behavioral repertoire of captive-reared chimpanzees (Pan troglodytes). *Sci. Rep.* **9**, 1–13 (2019).
15. Demuru, E. & Palagi, E. In bonobos yawn contagion is higher among kin and friends. *PLoS One* **7**, (2012).
16. Palagi, E., Norscia, I. & Demuru, E. Yawn contagion in humans and bonobos: emotional affinity matters more than species. *PeerJ* **2014**, 1–17 (2014).
17. Joly-Mascheroni, R. M., Senju, A. & Shepherd, A. J. Dogs catch human yawns. *Biol. Lett.* **4**, 446–448 (2008).
18. Romero, T., Konno, A. & Hasegawa, T. Familiarity Bias and Physiological Responses in Contagious Yawning by Dogs Support Link to Empathy. *PLoS One* **8**, (2013).
19. Romero, T., Ito, M., Saito, A. & Hasegawa, T. Social modulation of contagious yawning in wolves. *PLoS One* **9**, (2014).
20. Yonezawa, T., Sato, K., Uchida, M., Matsuki, N. & Yamazaki, A. Presence of contagious yawning in sheep. *Anim. Sci. J.* **88**, 195–200 (2017).
21. Wojczulanis-Jakubas, K., Plenzler, J. & Jakubas, D. Indications of contagious behaviours in the southern elephant seal: an observational study. *Behaviour* **156**, 59–77 (2019).
22. Gallup, A. C., Swartwood, L., Militello, J. & Sackett, S. Experimental evidence of contagious yawning in budgerigars (Melopsittacus undulatus). *Anim. Cogn.* **18**, 1051–1058 (2015).
23. Moyaho, A., Rivas-Zamudio, X., Ugarte, A., Eguibar, J. R. & Valencia, J. Smell facilitates auditory contagious yawning in stranger rats. *Anim. Cogn.* **18**, 279–290 (2015).
24. Deputte, B. L. Study of Yawning in Two Species of Cercopithecidae; Cercocebus Albigena Gray and Macaca Fascicularis Raffles: Research on Causal and Functional Factors; a Consideration of Socio-bioenergetic Factors Master Dissertation. (University of Rennes, 1978).
25. Massen, J. J. M., Šlipogor, V. & Gallup, A. C. An observational investigation of behavioral contagion in common marmosets (Callithrix jacchus): Indications for contagious scent-marking. *Front. Psychol.* **7**, 1190 (2016).
26. Reddy, R. B., Krupenye, C., MacLean, E. L. & Hare, B. No evidence for contagious yawning in lemurs. *Anim. Cogn.* **19**, 889–898 (2016).
27. Malavasi, R. Social modulation of yawning behavior in the domestic horse–an exploratory analysis. in *Conference Paper: 48th International Conference of the International Society for Applied Ethology.* (2014).
28. Wilkinson, A., Sebanz, N., Mandl, I. & Huber, L. No evidence of contagious yawning in the red-footed tortoise Geochelone carbonaria. *Curr. Zool.* **57**, 477–484 (2011).
29. Tinbergen, N. On aims and methods of Ethology. *Z. Tierpsychol.* **20**, 410–433 (1963).
30. Preston, S. D. & de Waal, F. B. M. Empathy: Its ultimate and proximate bases. *Behav. Brain Sci.* **25**, 1–20 (2002).
31. Platek, S. M., Critton, S. R., Myers, T. E. & Gallup, G. G. Contagious yawning: The role of self-awareness and mental state attribution. *Cogn. Brain Res.* https://doi.org/10.1016/S0926-6410(03)00109-5 (2003).
32. Lehmann, H. E. Yawning. A homeostatic reflex and its psychological significance. *Bull. Menninger Clin.* **43**, 123 (1979).
33. Hatfield, E., Cacioppo, J. T. & Rapson, R. L. Emotional contagion. *Curr. Dir. Psychol. Sci.* https://doi.org/10.1111/1467-8721.ep10770953 (1993).
34. Norscia, I., Zanoli, A., Gamba, M. & Palagi, E. Auditory contagious yawning is highest between friends and family members: support to the emotional bias hypothesis. *Front. Psychol.* **11**, 442 (2020).
35. Norscia, I. & Palagi, E. Yawn contagion and empathy in homo sapiens. *PLoS ONE* **6**, 1–5 (2011).
36. Cooper, N. R. *et al.* Bridging a yawning chasm: EEG investigations into the debate concerning the role of the human mirror neuron system in contagious yawning. *Cogn. Affect. Behav. Neurosci.* **12**, 393–405 (2012).
37. Haker, H., Kawohl, W., Herwig, U. & Rössler, W. Mirror neuron activity during contagious yawning-an fMRI study. *Brain Imaging Behav.* **7**, 28–34 (2013).
38. Platek, S. M., Mohamed, F. B. & Gallup, G. G. Contagious yawning and the brain. *Cogn. Brain Res.* **23**, 448–452 (2005).
39. Senju, A. *et al.* Absence of contagious yawning in children with autism spectrum disorder. *Biol. Lett.* https://doi.org/10.1098/rsbl.2007.0337 (2007).
40. Norscia, I., Demuru, E. & Palagi, E. She more than he: Gender bias supports the empathic nature of yawn contagion in Homo sapiens. *R. Soc. Open Sci.* https://doi.org/10.1098/rsos.150459 (2016).
41. Usui, S. *et al.* Presence of contagious yawning in children with autism spectrum disorder. *Autism Res. Treat.* https://doi.org/10.1155/2013/971686 (2013).
42. Bartholomew, A. J. & Cirulli, E. T. Individual variation in contagious yawning susceptibility is highly stable and largely unexplained by empathy or other known factors. *PLoS ONE* https://doi.org/10.1371/journal.pone.0091773 (2014).
43. Gallup, A. C. & Massen, J. J. M. There is no difference in contagious yawning between men and women. *R. Soc. Open Sci.* https://doi.org/10.1098/rsos.160174 (2016).
44. Norscia, I., Demuru, E. & Palagi, E. Difference in contagious yawning between Susceptible men and women: why not?. *R. Soc. Open Sci.* https://doi.org/10.1098/rsos.160477 (2016).
45. Neilands, P. *et al.* Contagious yawning is not a signal of empathy: No evidence of familiarity, gender or prosociality biases in dogs. *Proc. R. Soc. B Biol. Sci.* **287**, 20192236 (2020).
46. Palagi, E., Paoli, T. & Tarli, S. B. Reconciliation and consolation in captive bonobos (Pan paniscus). *Am. J. Primatol.* **62**, 15–30 (2004).
47. Silva, K., Bessa, J. & de Sousa, L. Auditory contagious yawning in domestic dogs (Canis familiaris): first evidence for social modulation. *Anim. Cogn.* **15**, 721–724 (2012).
48. Massen, J. J. M. & Gallup, A. C. Why contagious yawning does not (yet) equate to empathy. *Neurosci. Biobehav. Rev.* **80**, 573–585 (2017).
49. O'Hara, S. J. & Reeve, A. V. A test of the yawning contagion and emotional connectedness hypothesis in dogs Canis familiaris. *Anim. Behav.* **81**, 335–340 (2011).
50. Madsen, E. A. & Persson, T. Contagious yawning in domestic dog puppies (Canis lupus familiaris): the effect of ontogeny and emotional closeness on low-level imitation in dogs. *Anim. Cogn.* **16**, 233–240 (2013).
51. Miller, M. L., Gallup, A. C., Vogel, A. R., Vicario, S. M. & Clark, A. B. Evidence for contagious behaviors in budgerigars (Melopsittacus undulatus): an observational study of yawning and stretching. *Behav. Processes* **89**, 264–270 (2012).
52. Buttner, A. P. & Strasser, R. Contagious yawning, social cognition, and arousal: an investigation of the processes underlying shelter dogs' responses to human yawns. *Anim. Cogn.* **17**, 95–104 (2014).
53. Troisi, A. Displacement activities as a behavioral measure of stress in nonhuman primates and human subjects. *Stress* **5**, 47–54 (2002).
54. Miller, M. L., Gallup, A. C., Vogel, A. R. & Clark, A. B. Auditory disturbances promote temporal clustering of yawning and stretching in small groups of budgerigars (Melopsittacus undulatus). *J. Comp. Psychol.* **126**, 324 (2012).
55. Eguibar, J. R., Uribe, C. A., Cortes, C., Bautista, A. & Gallup, A. C. Yawning reduces facial temperature in the high-yawning subline of Sprague-Dawley rats. *BMC Neurosci.* **18**, 1–8 (2017).
56. Gallup, G. G. & Gallup, A. C. Excessive yawning and thermoregulation: two case histories of chronic, debilitating bouts of yawning. *Sleep Breath.* **14**, 157–159 (2010).
57. Gallup, A. C. & Gallup, G. G. Yawning as a Brain Cooling Mechanism: Nasal Breathing and Forehead Cooling Diminish the Incidence of Contagious Yawning. *Evol. Psychol.* **5**, (2007).

58. Gallup, A. C. & Eldakar, O. T. The thermoregulatory theory of yawning: What we know from over 5 years of research. *Front. Neurosci.* **6**, 188 (2012).
59. Gallup, A. C. & Gallup, G. G. Yawning and thermoregulation. *Physiol. Behav.* **95**, 10–16 (2008).
60. Massen, J. J. M., Dusch, K., Eldakar, O. T. & Gallup, A. C. A thermal window for yawning in humans: YAWNING as a brain cooling mechanism. *Physiol. Behav.* **130**, 145–148 (2014).
61. Elo, H. Yawning cannot cause significant temperature decreases in humans. *Sleep Med.* https://doi.org/10.1016/j.sleep.2010.06.005 (2011).
62. Guggisberg, A. G., Mathis, J., Schnider, A. & Hess, C. W. Why do we yawn? The importance of evidence for specific yawn-induced effects. *Neurosci. Biobehav. Rev.* https://doi.org/10.1016/j.neubiorev.2010.12.004 (2011).
63. Tan, J., Ariely, D. & Hare, B. Bonobos respond prosocially toward members of other groups. *Sci. Rep.* **7**, 1–11 (2017).
64. Palagi, E., Norscia, I. & Cordoni, G. Lowland gorillas (gorilla gorilla gorilla) failed to respond to others' yawn: experimental and naturalistic evidence. *J. Comp. Psychol.* **133**, 406 (2019).
65. Van Schaik, C. P. The socioecology of fission-fusion sociality in orangutans. *Primates* **40**, 69–86 (1999).
66. Te Broekhorst, I. J. A., Schürmann, C. L. & Sugardjito, J. Residential status and seasonal movements of wild orang-utans in the Gunung Leuser Reserve (Sumatera, Indonesia). *Anim. Behav.* **39**, 1098–1109 (1990).
67. Singleton, I. & Van Schaik, C. P. The social organisation of a population of Sumatran orang-utans. *Folia Primatol.* **73**, 1–20 (2002).
68. Kolbrink, P. Waldo by XYZ-animation. http://animatio.com/waldo-page.html (2017).
69. Barbizet, J. Yawning. *J. Neurol. Neurosurg. Psychiatry* **21**, 203–209 (1958).
70. Bartlebydc. Orangutan yawns. https://www.youtube.com/watch?v=Ob2L_P80_oo (2008).
71. ⅄|L|tv. Yawning Orangutan. https://www.youtube.com/watch?v=bMhreSjl5sE (2013).
72. Kapitány, R. & Nielsen, M. Are yawns really contagious? a critique and quantification of yawn contagion. *Adapt. Hum. Behav. Physiol.* **3**, 134–155 (2017).
73. Cameron, A. C. & Trivedi, P. K. *Regression analysis of count data (Vol. 53)*. (Cambridge University Press, Cambridge, 2013).
74. Plavcan, J. M. Sexual dimorphism in primate evolution. *Yearb. Phys. Anthropol.* **116**, 25–53 (2001).
75. Forstmeier, W. & Schielzeth, H. Cryptic multiple hypotheses testing in linear models: overestimated effect sizes and the winner's curse. *Behav. Ecol. Sociobiol.* **65**, 47–55 (2011).
76. Roth, T. S., Rianti, P., Fredriksson, G. M., Wich, S. A. & Nowak, M. G. Grouping behavior of Sumatran orangutans (Pongo abelii) and Tapanuli orangutans (Pongo tapanuliensis) living in forest with low fruit abundance. *Am. J. Primatol.* https://doi.org/10.1002/ajp.23123 (2020).
77. Edwards, S. D. & Snowdon, C. T. Social behavior of captive, group-living Orangutans. *Int. J. Primatol.* **1**, 39–62 (1980).
78. van Noordwijk, M. A. *et al.* Female philopatry and its social benefits among Bornean orangutans. *Behav. Ecol. Sociobiol.* https://doi.org/10.1007/s00265-012-1330-7 (2012).
79. Mitra Setia, T. & Van Schaik, C. P. The response of adult orang-utans to flanged male long calls: Inferences about their function. in *Folia Primatologica* (2007). doi:https://doi.org/10.1159/000102317.
80. Laméris, D. W., van Berlo, E., Sterck, E. H. M., Bionda, T. & Kret, M. E. Low relationship quality predicts scratch contagion during tense situations in orangutans (Pongo pygmaeus). *Am. J. Primatol.* **82**, 1–9 (2020).
81. Schwartz, J. H. *The red ape* (Elm Tree Books, London, 1987).
82. Harrison, M. E. & Chivers, D. J. The orang-utan mating system and the unflanged male: a product of increased food stress during the late Miocene and Pliocene?. *J. Hum. Evol.* **52**, 275–293 (2007).
83. Steckenfinger, S. A. & Ghazanfar, A. A. Monkey visual behavior falls into the uncanny valley. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 18362–18366 (2009).
84. Wilson, V. A. D. *et al.* Macaque gaze responses to the primatar: a virtual macaque head for social cognition research. *Front. Psychol.* https://doi.org/10.3389/fpsyg.2020.01645 (2020).
85. Chartrand, T. L. & Bargh, J. A. The chameleon effect: The perception-behavior link and social interaction. *J. Pers. Soc. Psychol.* **76**, 893 (1999).
86. Mitani, J. C., Merriwether, D. A. & Zhang, C. Male affiliation, cooperation and kinship in wild chimpanzees. *Anim. Behav.* **59**, 885–893 (2000).
87. Furuichi, T. Social interactions and the life history of female Pan paniscus in Wamba Zaire. *Int. J. Primatol.* **10**, 173–197 (1989).
88. Dunbar, R. & Dunbar, P. Social dynamics of gelada baboons. *Contrib. Primatol.* **6**, 1–157 (1975).
89. Goldberg, T. L. & Wrangham, R. W. Genetic correlates of social behaviour in wild chimpanzees: evidence from mitochondrial DNA. *Anim. Behav.* **54**, 559–570 (1997).
90. Silk, J. B., Alberts, S. C. & Altmann, J. Patterns of coalition formation by adult female baboons in Amboseli Kenya. *Anim. Behav.* **67**, 573–582 (2004).
91. Leone, A., Ferrari, P. F. & Palagi, E. Different yawns, different functions? Testing social hypotheses on spontaneous yawning in Theropithecus gelada. *Sci. Rep.* https://doi.org/10.1038/srep04010 (2014).
92. Knott, C. D., Thompson, M. E., Stumpf, R. M. & McIntyre, M. H. Female reproductive strategies in orangutans, evidence for female choice and counterstrategies to infanticide in a species with frequent sexual coercion. *Proc. R. Soc. B Biol. Sci.* **277**, 105–113 (2010).
93. Delgado, R. A. & Van Schaik, C. P. The behavioral ecology and conservation of the orangutan (Pongo pygmaeus): a tale of two islands. *Evol. Anthropol.* **9**, 201–218 (2000).
94. De Vries, J. I., Visser, G. H. & Prechtl, H. F. The emergence of fetal behaviour. I. Qualitative aspects. *Early Hum. Dev.* **7**, (1982).
95. Anderson, J. & Meno, P. Psychological Influences on Yawning in Children. *Curr. Psychol. Lett. Behav. brain Cogn.* **2**, (2003).
96. Millen, A. & Anderson, J. R. Neither infants nor toddlers catch yawns from their mothers. *Biol. Lett.* **7**, 440–442 (2011).
97. Hoogenhout, M. Young children display contagious yawning when looking at the eyes. *J. Child Adolesc. Behav.* **01**, 1–4 (2013).
98. Clancy, B., Finlay, B. L., Darlington, R. B. & Anand, K. J. S. Extrapolating brain development from experimental species to humans. *Neurotoxicology* https://doi.org/10.1016/j.neuro.2007.01.014 (2007).
99. Provine, R. R. & Hamernik, H. B. Yawning: Effects of stimulus interest. *Bull. Psychon. Soc.* **24**, 437–438 (1986).

Acknowledgements

We thank Paul Kolbrink for creating the 3D orangutan, and Apenheul and the orangutan caretakers for facilitating the study. In addition, we thank Linde Oostinga and Vinne van Buuren for their valuable assistance with piloting the study. Furthermore, we thank Tom Roth and Jingzhi Tan for their valuable feedback on the discussion and analyses. Finally, we thank Patrick Neilands and three anonymous reviewers for their constructive feedback on the manuscript.

Author contributions

Conceptualization: E.v.B., J.J.M.M. Design: J.J.M.M, E.v.B., A.P.D.L., O.E.J.M., M.E.K. Data acquisition; A.P.D.L., O.E.J.M. Analyses: J.J.M.M., E.v.B. Manuscript: E.v.B., J.J.M.M. Review: J.J.M.M., A.P.D.L., O.E.J.M., M.E.K.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-020-79160-x.

Correspondence and requests for materials should be addressed to E.B.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

RESEARCH ARTICLE

HYBRID GENOMICS

Natural hybridization reveals incompatible alleles that cause melanoma in swordtail fish

Daniel L. Powell[1,2,3]*, Mateo García-Olazábal[2,3], Mackenzie Keegan[4], Patrick Reilly[5], Kang Du[6], Alejandra P. Díaz-Loyo[7], Shreya Banerjee[1], Danielle Blakkan[1], David Reich[8,9], Peter Andolfatto[10], Gil G. Rosenthal[2,3], Manfred Schartl[2,3,6,11,12], Molly Schumer[1,2]*

The establishment of reproductive barriers between populations can fuel the evolution of new species. A genetic framework for this process posits that "incompatible" interactions between genes can evolve that result in reduced survival or reproduction in hybrids. However, progress has been slow in identifying individual genes that underlie hybrid incompatibilities. We used a combination of approaches to map the genes that drive the development of an incompatibility that causes melanoma in swordtail fish hybrids. One of the genes involved in this incompatibility also causes melanoma in hybrids between distantly related species. Moreover, this melanoma reduces survival in the wild, likely because of progressive degradation of the fin. This work identifies genes underlying a vertebrate hybrid incompatibility and provides a glimpse into the action of these genes in natural hybrid populations.

The emergence of reproductive barriers between populations is the first step in the process of speciation and drives Earth's biological diversity, yet surprisingly little is known about how it occurs at the genetic level. The Dobzhansky-Muller model of hybrid incompatibility (*1*–*3*) posits that new mutations arising in diverging species can interact negatively in hybrids, generating lower hybrid viability or causing hybrid sterility. Although empirical work provides support for the general predictions of this model (*4*), progress in this area has been limited by a lack of knowledge about which genes interact to generate hybrid incompatibilities. Despite the effort devoted to this problem, only a dozen incompatible interactions have been mapped to the single-gene level [reviewed in (*5*)]. With so few known cases, it has been difficult to evaluate whether common genetic and evolutionary mechanisms underlie the emergence of incompatibilities (*6*–*8*).

[1]Department of Biology, Stanford University and Howard Hughes Medical Institute, Stanford, CA, USA. [2]Centro de Investigaciones Científicas de las Huastecas "Aguazarca", A.C., Calnali, Hidalgo, Mexico. [3]Department of Biology, Texas A&M University, College Station, TX, USA. [4]Department of Biology, Northeastern University, Boston, MA, USA. [5]Lewis Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ, USA. [6]Developmental Biochemistry, Biocenter, University of Würzburg, Würzburg, Bavaria, Germany. [7]Laboratorio de Ecología de la Conducta, Instituto de Fisiología, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico. [8]Department of Genetics, Harvard Medical School, Howard Hughes Medical Institute, and the Broad Institute of Harvard and MIT, Cambridge, MA, USA. [9]Department of Human Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA. [10]Department of Biological Sciences, Columbia University, New York, NY, USA. [11]Hagler Institute for Advanced Study, Texas A&M University, College Station, TX, USA. [12]*Xiphophorus* Genetic Stock Center, Texas State University San Marcos, San Marcos, TX, USA.
***Corresponding author. Email: dpowell8@stanford.edu (D.L.P.); schumer@stanford.edu (M.Schu.)**

With an increasing appreciation that hybridization is common across the tree of life (*9*–*13*), there has been renewed interest in identifying hybrid incompatibilities and understanding how these genes act as barriers in nature. Of hybrid incompatibilities that have been mapped to the single-gene level, most have been identified with crosses between model species that no longer naturally hybridize (*4*, *5*). As a result, it is unclear whether these mapped incompatibilities were important in the initial divergence between species or arose after these lineages had stopped exchanging genes.

One such example is the melanoma receptor tyrosine-protein kinase (*xmrk*) gene in swordtail fish (genus *Xiphophorus*). *xmrk* is one of two identified genes in vertebrates that drive hybrid incompatibility (the other being the regulator of mammalian recombination hotspots, *prdm9*) (*14*) and was one of the earliest described hybrid incompatibilities (*15*). In crosses between *Xiphophorus maculatus* and *Xiphophorus hellerii*, a malignant melanoma develops in a subset of F_2 hybrids, emanating from natural pigmentation spots on the body and fins. This hybrid incompatibility is the result of an interaction between the *xmrk* gene derived from *X. maculatus* and an unknown locus derived from *X. hellerii* (*16*).

Despite work that demonstrated the role of *xmrk* in the development of hybrid melanomas, its importance as a barrier between species has been debated (*17*). This is because *X. maculatus* and *X. hellerii* diverged ~3 million years ago and do not naturally hybridize (*18*). Moreover, because melanoma in *X. maculatus* x *X. hellerii* laboratory hybrids develops later in life, it is unclear whether it affects survival and reproduction (*17*).

Melanoma occurs in hybrids between recently diverged swordtail species

We identified a phenotypically similar melanoma in natural hybrids formed between the swordtail fish species *Xiphophorus birchmanni* and *Xiphophorus malinche*. *X. birchmanni* and *X. malinche* are closely related and hybridize in the wild (~0.5% differences per base pair and ~250,000 generations diverged) (*19*). Although a subset of hybrids are viable and fertile, there is evidence of selection against hybrid incompatibilities (*20*–*22*). In some populations, hybrids develop melanoma early in life (13 ± 4% of males develop melanoma before sexual maturity) (fig. S1).

Melanoma in *X. birchmanni* x *X. malinche* hybrids develops from a phenotype derived from *X. birchmanni* called the "spotted caudal," which is a dark blotch on the caudal fin generated by clusters of macromelanocyte cells (Fig. 1A and fig. S2) (*23*). The spotted caudal trait occurs at intermediate frequencies in *X. birchmanni* but is absent from *X. malinche* populations (Fig. 1B). Some hybrid populations have a high frequency of the trait and exhibit phenotypes that extend beyond the range of those observed in *X. birchmanni* (Fig. 1, B and C, and figs. S3 and S4), including invasion of macromelanocyte cells into the body, where they are normally absent. Tracking of hybrids in the laboratory documents the progression of the trait from a phenotype typical of the *X. birchmanni* spot to the expanded trait found in some hybrids (Fig. 1D and fig. S1) (*24*). Histological sections from hybrid individuals revealed penetration of melanocytes into the musculature and invasion of surrounding tissues, which is indicative of a malignant melanoma (Fig. 1E and fig. S5).

We performed mRNA-sequencing of hybrid individuals that varied in the degree of expansion of their spot (figs. S2 and S6). Functional enrichment analysis indicated changes in the regulation of a number of melanoma-associated gene categories, such as pigment cell differentiation and regulation of cytoskeletal organization, including several implicated in melanoma in other fish species (Fig. 1F and table S1) (*24*, *25*).

Melanoma is extremely rare in nonhybrid individuals (Fig. 1C) (*6*, *26*), and we have not identified a single wild-caught *X. birchmanni* male with melanoma (1296 males collected from 2017 to 2019, 0 with melanoma). Laboratory-reared individuals indicate that environmental levels of ultraviolet irradiance or other natural carcinogens do not underlie differences in the frequency of melanoma between hybrid and parental populations (*24*). The presence of melanoma in hybrids, but not the parental species, suggests that this melanoma is a hybrid incompatibility generated by interactions between alleles in the *X. birchmanni* and *X. malinche* genomes.

Loci associated with the spotting phenotype in *X. birchmanni*

We mapped the genetic basis of the spotted caudal in *X. birchmanni* and the genetic basis of melanoma in interspecific hybrids. We generated de novo assemblies for both species using a 10X-based linked read approach followed by assembly into chromosomes with Hi-C data and annotated the resulting assemblies with RNA-sequencing (RNA-seq) data (*24*).

We collected low-coverage whole-genome sequence data for 392 adult male *X. birchmanni*

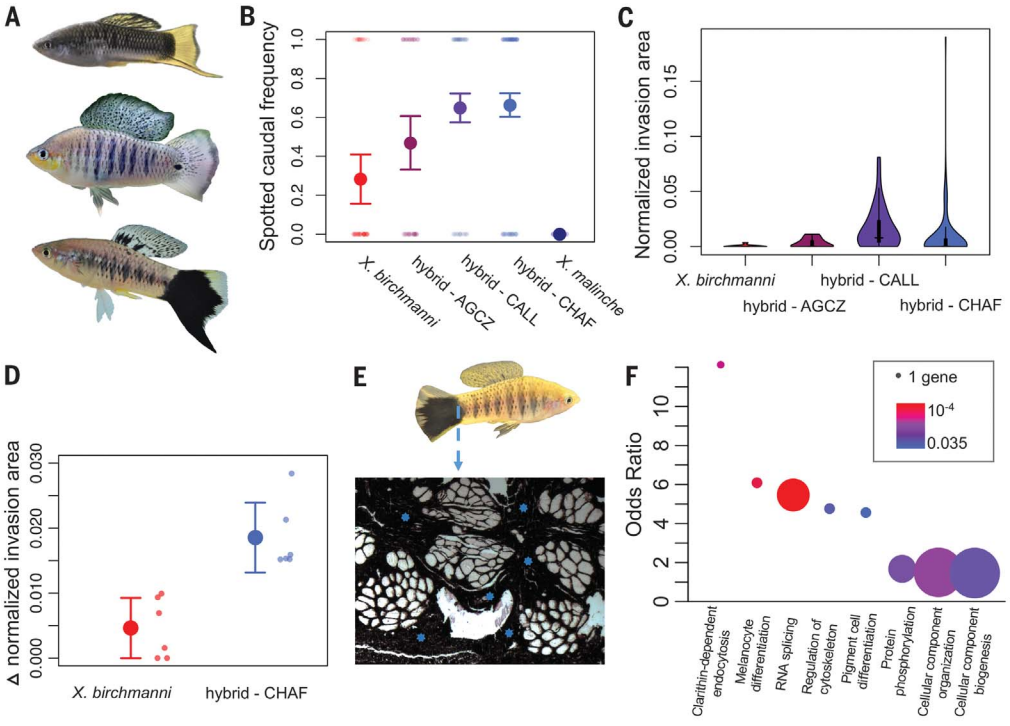


Fig. 1. Hybridization generates a high incidence of melanoma. (**A**) Naturally hybridizing species *X. malinche* (top) and *X. birchmanni* (middle) differ in morphological traits, including the presence of a melanin pigment spot that is polymorphic in *X. birchmanni*. In hybrids, this spotting phenotype can transform into a melanoma (bottom). (**B**) Whereas *X. birchmanni* populations segregate for the presence of this spot, the trait is absent in *X. malinche* populations; hybrid populations have high frequencies of this trait. (**C**) The trait is at higher frequencies in hybrid populations and covers more of the body. Shown here is invasion area, or the melanized body surface area outside of the caudal fin (normalized for body size). Hybrid phenotypes are shown from three populations on the Río Calnali (fig. S3). AGCZ, Aguazarca; CALL, Calnali low; CHAF, Chahuaco falls. (**D**) Spots expand more over a 6-month period in hybrids than in *X. birchmanni* individuals. (**E**) A cross section of the caudal peduncle from a Chahuaco falls hybrid (10× magnification). Melanoma cells invading the body and muscle bundles are visually evident (indicated with blue stars). (**F**) Gene ontology categories enriched in melanoma tissue compared with normal caudal tissue (*24*, *45*). The size of the dots reflects the number of genes identified, and the color corresponds to the *P* value. Categories with undefined odds ratios (not plotted) are listed in table S1. In (B) and (D), the plot shows the mean, and whiskers indicate two standard errors of the mean. Individual points show the raw data.

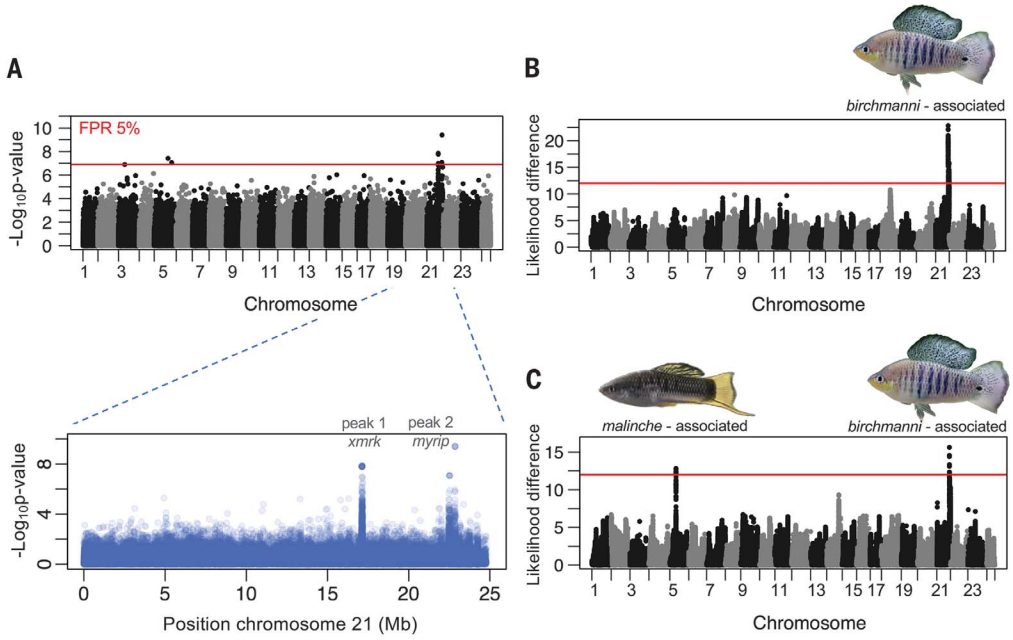


Fig. 2. Combined genome-wide association and admixture mapping approaches identify the genetic basis of the melanoma hybrid incompatibility. (**A**) Results of genome-wide association scan for allele frequency differences between spotted cases and unspotted controls. (Top) Results can be seen for all chromosomes, and the red line indicates the genome-wide significance threshold, determined by permutation (*24*). (Bottom) Results from chromosome 21, where two distinct regions are strongly associated with spotting. (**B**) Admixture mapping in hybrids identifies associations between *X. birchmanni* ancestry on chromosome 21 and spot presence. Plotted here are log likelihood differences between models with and without ancestry at the focal site included as a covariate. The red line indicates the genome-wide significance threshold, determined by permutation (*24*). (**C**) When we treated melanocyte invasion as the focal trait and mapped associations with ancestry, we again identified associations with *X. birchmanni* ancestry on chromosome 21 but also identified a second region on chromosome 5 associated with *X. malinche* ancestry.

individuals from a single collection site and performed a genome-wide association study (GWAS), scanning for allele frequency differences between spotted cases (n = 159) and unspotted controls (n = 233), evaluating the impact of population structure and low-coverage data (*24*). We identified a strong association between the spotting pattern and allele frequency differences on chromosome 21 at an estimated false positive rate of 5% (by permutation) (Fig. 2A and figs. S7 and S8) (*24*). Two distinct signals are evident on this chromosome, ~5 Mb apart (Fig. 2A) (*24*). The first peak is centered on the *xmrk* gene (fig. S9) (*27*), which arose through duplication of a gene homologous to the mammalian epidermal growth factor receptor ~3 million years ago (*11*, *28*, *29*). *xmrk* controls pigmentation patterns and drives hybrid melanomas in relatives of *X. birchmanni* and *X. malinche* (*16*). The signal at the second peak on chromosome 21 contains a single gene, the melanosome transporter gene myosin VIIA and Rab interacting protein (*myrip*) (Fig. 2A) (*24*, *30*).

Genetic architecture of the melanoma incompatibility

For the melanoma phenotype, we used an admixture mapping approach, focusing our efforts on a hybrid population with high incidence of melanoma (19 ± 3% of adult males, the Chahuaco falls population). To infer local ancestry, we generated ~1X low-coverage whole-genome sequence data for 209 adult males from this population and applied a hidden Markov model to 680,291 ancestry informative sites genome-wide (*20*, *22*, *31*) [approximately one ancestry informative site per kilobase, (*24*)]. Simulations and analyses of laboratory-generated crosses indicate that we should have high accuracy in local ancestry inference (figs. S10 to S13) (*24*).

Using these data, we performed admixture mapping for spot presence and melanocyte invasion of the body (59% of individuals had spots, and 19% of individuals had melanoma). Admixture mapping for the presence of the spotted caudal revealed one strongly associated region on chromosome 21 (log likelihood difference of linear models, 23) (*24*) where spotting correlated with *X. birchmanni* ancestry (Fig. 2B). Because of the lower resolution of admixture, mapping this peak is broad, but the signal occurs in the same regions identified by our GWAS scan (*24*), confirming that the genetic basis of the spot is the same in *X. birchmanni* and hybrids. Simulations accounting for effect size inflations owing to the

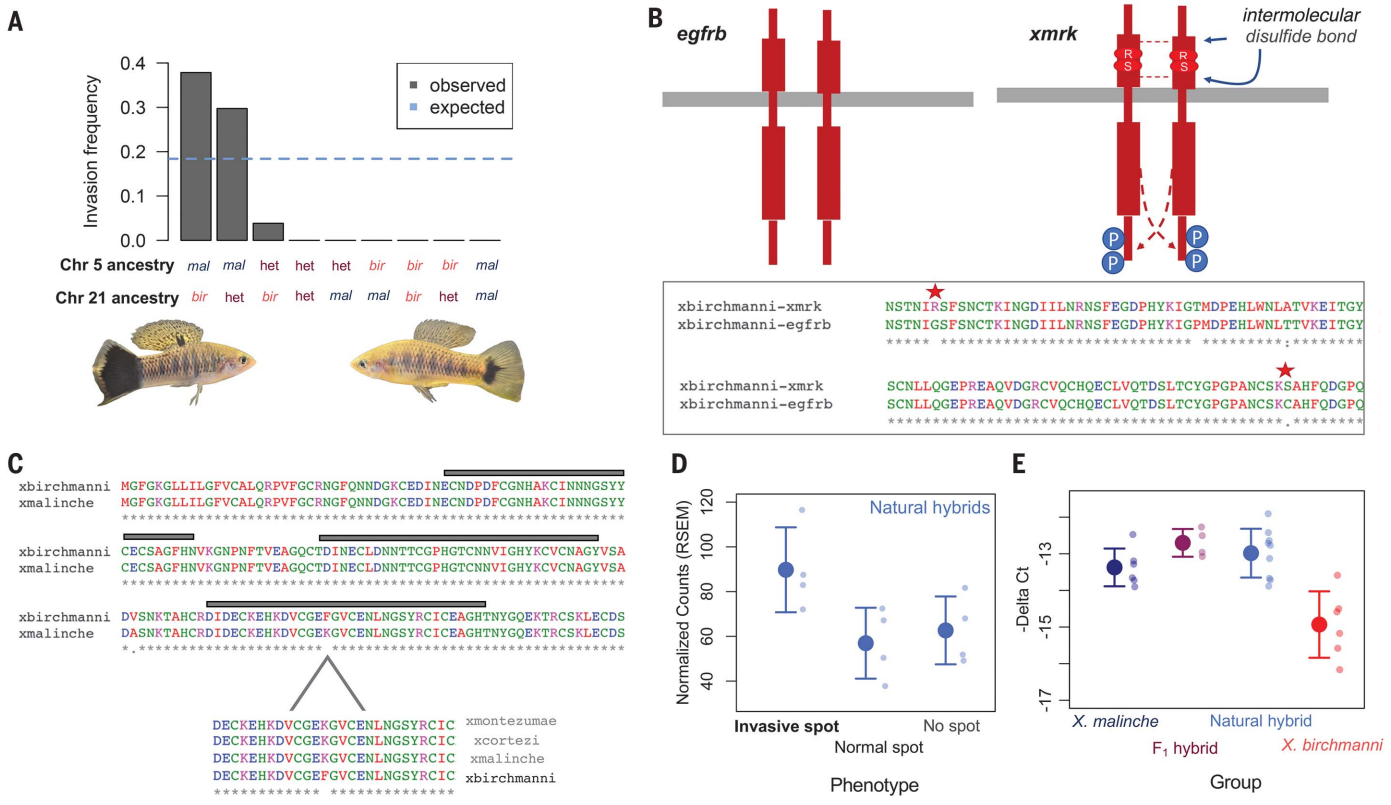


Fig. 3. Interactions between chromosomes 5 and 21 are associated with melanoma in hybrids. (**A**) Proportion of individuals with melanoma as a function of ancestry at the associated regions on chromosome 5 and chromosome 21. The blue dashed line indicates the expected proportion of cases if melanoma risk were equally distributed among individuals with at least one *birchmanni* allele at chromosome 21. We only had one observation for the *bir-bir* and *bir-het* genotypes. (**B**) The *xmrk* sequence in *X. birchmanni* harbors two mutations (G364R and C582S) that transform *xmrk* to a constitutively active state (*33*, *46*). The schematic compares the ancestral form of the protein (*egfrb*) to the predicted structure of *xmrk* in *X. birchmanni*. Proteins are shown in red, and the cell membrane is shown in gray. In *xmrk*, residues R364 and S582 promote intramolecular disulfide bonds that cause protein dimerization and phosphorylation (blue circles) (*33*, *46*). (Single-letter abbreviations for the amino acid residues are as follows: C, Cys; G, Gly; R, Arg; and S, Ser. In *xmrk*, amino acids were substituted at certain locations; for example, G364R indicates that glycine at position 364 was replaced by arginine.) (Inset) A partial clustal alignment of *X. birchmanni egfrb* and *xmrk* with these substitutions highlighted. Colors indicate properties of the amino acid, and asterisks indicate locations where the amino acid sequences are identical. (**C**) Clustal alignment showing the N terminus of *cd97* in *X. birchmanni* and *X. malinche*. We observed a substitution in a conserved epidermal growth factor–binding domain (gray rectangles). (Inset) The substitution found in *X. birchmanni* is not present in closely related species. (**D**) Expression of *cd97* based on RNA-seq data in melanoma, spotted, and unspotted tissue from Chahuaco falls hybrids (four biological replicates per group). (**E**) Real-time quantitative PCR of *cd97* from caudal fin tissue from *X. malinche*, *X. birchmanni*, and natural and F_1 hybrids (four to nine biological replicates per group). In (D) and (E), large solid dots indicate the mean, and whiskers indicate two standard errors of the mean. Individual points show the raw data.

"winner's curse" (*32*) suggest that *X. birchmanni* ancestry in this region explains ~75% of the variation in spot presence or absence (*24*).

Admixture mapping for melanoma identified an additional significant region on chromosome 5. In this case, melanocyte invasion was associated with *X. malinche* ancestry (Fig. 2C). A contingency test indicated a nonrandom association between *X. birchmanni* ancestry at the chromosome 21 peak and *X. malinche* ancestry at the chromosome 5 peak, with the prevalence of melanoma (Fisher's exact test, $P = 0.0005$) (Fig. 3A). Individuals heterozygous for *X. malinche* ancestry at this region on chromosome 5 appear to have a lower risk of melanoma (Fig. 3A). Moreover, regardless of melanoma phenotype, spotted individuals that were heterozygous at the chromosome 5 peak had smaller spots than individuals that were homozygous (Student's *t* test on log-normalized area $P = 0.007$) (fig. S14).

Linking molecular changes to hybrid melanoma

Our GWAS identified associations between the spotted caudal and both *xmrk* and *myrip* (Fig. 2A), making it initially unclear whether either or both of these genes interacts with *malinche* ancestry on chromosome 5 to produce melanoma. Although both are associated with the spotting pattern that precedes melanoma, *myrip* is not expressed in an RNA-seq dataset of adult caudal tissue, nor is it expressed in the melanoma itself (fig. S15). By contrast, *xmrk* is expressed in caudal tissue and has higher expression in spotted than unspotted tissues (fig. S15). In addition, functional studies have linked *xmrk* to the development of melanoma. We identified two amino acid substitutions in *X. birchmanni* that fall within the extracellular domain of *xmrk* known to drive the oncogenic properties of *xmrk* in vitro (Fig. 3B) (*33*), and transgenic studies have demonstrated that overexpression of *xmrk* causes the formation of tumors (*25*, *34*, *35*). Although *myrip* may not be directly involved in the development of melanoma, past work in other swordtail species has suggested the presence of "patterning" loci linked to *xmrk* [reviewed in (*23*)]. Given *myrip*'s role in melanosome transport, we speculate that it could play a role in pigmentation patterning, which occurs in the first several weeks of life.

The region on chromosome 5 associated with *X. malinche* ancestry and melanoma contains only two genes, a gene called *cd97* and a fatty acid transporter gene (Figs. 2C and 3C). Although this is unusually high resolution given our admixture-mapping approach, subsampling the data indicated that this scale of resolution is likely the result of high recombination rates in this region (*24*). We therefore sought to better characterize the two genes in this region.

The ortholog of *cd97* in mammals plays a role in epithelial metastasis and is associated with tumor invasiveness in cancers (*36*–*38*). Accordingly, we found that *cd97* is up-regulated in RNA-seq data from melanotic tissue in hybrids, whereas the fatty acid transporter gene is not (Fig. 3D); nor is this pattern of up-regulation observed in any other gene within 100 kb of this region (*24*). In addition, of five amino acid changes between *X. birchmanni* and *X. malinche* in *cd97*, one occurs in a conserved epidermal growth factor–like calcium-binding domain (Fig. 3C).

We further investigated differences in expression of *cd97* using a targeted quantitative polymerase chain reaction (qPCR) approach. We found that *cd97* was expressed at low levels in the caudal fin tissue of *X. birchmanni*, regardless of spotting phenotype, but at similarly high levels in *X. malinche* and in natural and artificial hybrids [analysis of variance (ANOVA) $P = 1^{-5}$, Tukey post hoc; all groups different from *X. birchmanni* at $P < 0.005$) (Fig. 3E). Higher expression of *cd97* in *X. malinche* and hybrids is not tissue-specific and surprisingly does not appear to be driven by cis-regulatory differences (fig. S16) (*24*). We do not know whether the link between *X. malinche* ancestry at *cd97* and melanoma is driven by coding or regulatory differences (*24*). However, in mammals, overexpression of *cd97* has been linked to tumor metastasis; a similar mechanism could be involved here, given that high expression of *cd97* coincides with invasion of other tissues with melanoma cells.

Independent evolution of a melanoma incompatibility

Although the role of *xmrk* in the *maculatus-hellerii* hybrid incompatibility has been known for 30 years (*39*), the identity of the interacting gene is not known. Laboratory crosses have

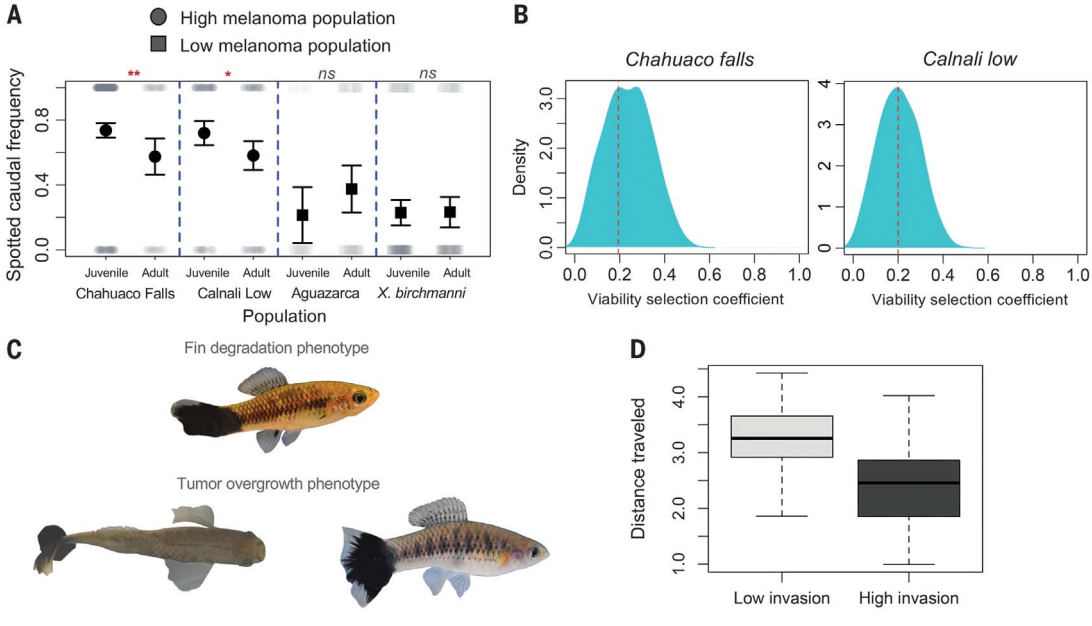


Fig. 4. Impact of the spotted caudal melanoma in natural hybrid populations. (**A**) Frequency of spotting in juvenile and adult males across populations with high (circles, Calnali low and Chahuaco falls) or low (squares, Aguazarca and *X. birchmanni*) melanoma incidence. Asterisks indicate significant differences by age class (*$P < 0.05$, **$P < 0.01$; ns indicates nonsignificant differences in a two-sample *z* test). Gray points indicate the raw data, black points indicate the mean, and error bars indicate one standard error of the mean. (**B**) Results of approximate Bayesian computation simulations indicate that the change in frequency of the spotting phenotype between juvenile and adult males is consistent with strong viability selection (*24*). Shown here are posterior distributions of viability selection coefficients consistent with the observed frequency change data in (left) Chahuaco falls and (right) Calnali low. (**C**) Because of where the melanoma develops, it can cause (top) the degradation of a fin essential in swimming or (bottom) the growth of tumors on the fin (overhead and side view of the same individual). (**D**) Visualization of the difference in fast-start response between individuals with low and high melanoma invasion (upper and lower 25% quantiles shown here). This representation is for visualization only; the statistical analysis comes from a linear model.

narrowed to a ~5 Mb region on chromosome 5 but have not yet identified the underlying gene, although candidates have been proposed (*16*). We identified a distinct region on chromosome 5 (Fig. 2C), more than 7 Mb away from the region identified in the *hellerii-maculatus* cross. Alignments of chromosome 5 confirm that *cd97* is at the same location in all four species (fig. S17), and linkage disequilibrium between *cd97* and the region identified in *hellerii-maculatus* decays to background levels in hybrids (*24*). Using simulations, we ruled out a lack of power to detect an association between this region and melanoma, assuming a similar effect size to that seen in the *hellerii-maculatus* cross (fig. S18). These results indicate that the incompatibility has a partially distinct genetic basis in the two crosses generating hybrid melanoma. However, we may not have mapped all components of the melanoma incompatibility, particularly if other genes have subtle impacts on melanoma risk (*24*).

These mapping results are surprising because they suggest that a melanoma incompatibility involving *xmrk* emerged independently in two distinct lineages. Despite the evolutionary distance between these species (fig. S19), it is possible that the melanoma incompatibility arose through similar evolutionary paths in both cases. *X. hellerii* and its relatives lack *xmrk* (*39*), either because the lineage leading to this clade diverged before *xmrk* arose (fig. S19) or because of an ancient loss of *xmrk*. By contrast, many species in the lineage leading to *X. birchmanni* and *X. malinche* retained *xmrk* (fig. S19) (*24*), but we found that *xmrk* has been deleted in *X. malinche* since its divergence from *X. birchmanni* (fig. S20) (*24*). We speculate that the loss of *xmrk* in *X. malinche* could have changed the level of constraint on interacting genes in this lineage, and if so, similar evolutionary mechanisms could be at play in *X. hellerii*.

Selection on melanoma in natural populations

Although the melanoma that forms in *birchmanni* x *malinche* hybrids appears to be deleterious from its development early in life (fig. S1) and its malignancy (Fig. 1E and fig. S5), we wanted to evaluate its impact in natural hybrid populations. Over several years, we observed shifts in the frequency of the spotted caudal trait between juvenile and adult males (*24*). Specifically, in hybrid populations with high incidences of melanoma, juvenile males had a significantly higher frequency of the spotted caudal trait than that of adult males (two-sample z test, both $P < 0.02$) (Fig. 4A). By contrast, this pattern was not observed in the *X. birchmanni* parental population or in a hybrid population with a low incidence of melanoma (Fig. 4A). Phenotype tracking of laboratory-raised individuals shows that once it appears, the spotted area always expands over time, indicating that we do not expect reversal of spotting due to some form of phenotypic plasticity (Fig. 1D) (*24*). We also did not find evidence for systematic shifts in ancestry genome-wide between the juvenile and adult male life stages that could explain this pattern (*24*). However, we do see a shift toward *X. birchmanni* ancestry at the melanoma risk locus (in the top 1% of changes genome-wide) (fig. S21) (*24*).

The observed shifts in spotting phenotype and ancestry at the melanoma risk locus, combined with an absence of substantial genome-wide shifts in ancestry, suggest that viability selection acts against spotted hybrids during maturation (*24*). Using an approximate Bayesian approach, we inferred that the strength of viability selection required to generate observed phenotypic shifts was extremely high and consistent across the two hybrid populations where melanoma is common (maximum a posteriori estimate of $s \sim 0.2$; 95% confidence intervals 0.05 to 0.44 and 0.04 to 0.38) (Fig. 4B).

Histology showed degradation of the muscle tissue that connects to the caudal fin in advanced melanomas (Figs. 1E and 4C, fig. S5, and movie S1). We thus measured its impact on swimming performance using two approaches. We did not find differences between phenotypes in ability to swim against a current (*24*). However, individuals with three-dimensional melanoma had slower escape responses when startled (linear model, $t = -2.6$, $p = 0.014$) (Fig. 4D) (*24*). This result is intriguing because fish with expanded spotting are likely more visible (movie S2), which could affect detection by avian and piscine predators.

Given the evidence for reduced survival of spotted individuals in populations with high rates of melanoma, it is surprising that this trait is still segregating in some hybrid populations (Fig. 4, A and B, and fig. S22) (*24*). Simulations suggest that high levels of gene flow from *X. birchmanni* would be required to maintain spotting at observed frequencies in hybrid populations (fig. S22) (*24*). However, because our inferences are based on viability rather than direct measures of fitness, we stress that there may be weaker effects of melanoma on overall fitness. Alternatively, other factors, such as mating advantages in individuals with large spots (*40*, *41*), may explain its maintenance.

Implications

The involvement of *xmrk* in a melanoma hybrid incompatibility in two distantly related swordtail species pairs raises the question of whether certain genetic interactions are particularly prone to breakdown in hybrids. Genes that interact with many other genes or those that are involved in evolutionary arms races may be especially likely to generate hybrid incompatibilities (such as observed in *Arabidopsis*) (*42*, *43*). Indeed, the only other known hybrid incompatibility in vertebrates, the recombination hotspot regulator *prdm9*, causes hybrid sterility in multiple crosses in mice (*44*). Whether unifying molecular or evolutionary forces drive the evolution of hybrid incompatibilities will become clearer as more incompatibilities are mapped to the single-gene level.

REFERENCES AND NOTES

1. T. Dobzhansky, *Zellforsch.* **21**, 169–223 (1934).
2. N. A. Johnson, *Genetics* **161**, 939–944 (2002).
3. T. Dobzhansky, *Biol. Rev. Camb. Philos. Soc.* **11**, 364–384 (1936).
4. J. A. Coyne, H. A. Orr, *Speciation* (Sinaeur Associates, 2004).
5. D. C. Presgraves, *Nat. Rev. Genet.* **11**, 175–180 (2010).
6. S. Maheshwari, D. A. Barbash, *Annu. Rev. Genet.* **45**, 331–355 (2011).
7. D. A. Barbash, P. Awadalla, A. M. Tarone, *PLOS Biol.* **2**, e142 (2004).
8. N. A. Johnson, *Trends Genet.* **26**, 317–325 (2010).
9. E. H. Stukenbrock, F. B. Christiansen, T. T. Hansen, J. Y. Dutheil, M. H. Schierup, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 10954–10959 (2012).
10. Y. Brandvain, A. M. Kenney, L. Flagel, G. Coop, A. L. Sweigart, *PLOS Genet.* **10**, e1004410 (2014).
11. R. Cui *et al.*, *Evolution* **67**, 2166–2179 (2013).
12. S. Sankararaman *et al.*, *Nature* **507**, 354–357 (2014).
13. D. A. Turissini, D. R. Matute, *PLOS Genet.* **13**, e1006971 (2017).
14. O. Mihola, Z. Trachtulec, C. Vlcek, J. C. Schimenti, J. Forejt, *Science* **323**, 373–375 (2009).
15. M. Gordon, *J. Hered.* **28**, 223–230 (1937).
16. S. Meierjohann, M. Schartl, *Trends Genet.* **22**, 654–661 (2006).
17. M. Schartl, *BioEssays* **30**, 822–832 (2008).
18. E. Clark, L. R. Aronson, M. Gordon, *Bull. Am. Mus. Nat. Hist.* **103**, 135–226 (1954).
19. Z. W. Culumber *et al.*, *Mol. Ecol.* **20**, 342–356 (2011).
20. M. Schumer *et al.*, *eLife* **3**, e02535 (2014).
21. M. Schumer, Y. Brandvain, *Mol. Ecol.* **25**, 2577–2591 (2016).
22. M. Schumer *et al.*, *Science* **360**, 656–660 (2018).
23. Z. W. Culumber, *Zebrafish* **11**, 57–70 (2014).
24. Materials and methods are available as supplementary materials.
25. B. Klotz *et al.*, *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **208**, 20–28 (2018).
26. A. Schartl, B. Malitschek, S. Kazianis, R. Borowsky, M. Schartl, *Cancer Res.* **55**, 159–165 (1995).
27. A. Gómez, J. N. Volff, U. Hornung, M. Schartl, C. Wellbrock, *Mol. Biol. Evol.* **21**, 266–275 (2004).
28. J. N. Volff, M. Schartl, *J. Struct. Funct. Genomics* **3**, 139–150 (2003).
29. J. C. Jones, J.-A. Perez-Sato, A. Meyer, *Mol. Ecol.* **21**, 2692–2712 (2012).
30. J. S. Ramalho, V. S. Lopes, A. K. Tarafder, M. C. Seabra, A. N. Hume, *Pigment Cell Melanoma Res.* **22**, 461–473 (2009).
31. R. Corbett-Detig, R. Nielsen, *PLOS Genet.* **13**, e1006529 (2017).
32. S. Xu, *Genetics* **165**, 2259–2268 (2003).
33. I. Gomez-Mestre, D. R. Buchholz, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 19021–19026 (2006).
34. M. Schartl *et al.*, *J. Invest. Dermatol.* **130**, 249–258 (2010).
35. J. Regneri *et al.*, *Pigment Cell Melanoma Res.* **32**, 248–258 (2019).
36. M. Safaee *et al.*, *Int. J. Oncol.* **43**, 1343–1350 (2013).
37. D. Liu *et al.*, *PLOS ONE* **7**, e39989 (2012).
38. Y. Ward *et al.*, *Cell Rep.* **23**, 808–822 (2018).
39. J. Wittbrodt *et al.*, *Nature* **341**, 415–421 (1989).
40. A. A. Fernandez, M. R. Morris, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 13503–13507 (2008).
41. Z. W. Culumber, G. G. Rosenthal, *Naturwissenschaften* **100**, 801–804 (2013).
42. E. Chae *et al.*, *Cell* **159**, 1341–1351 (2014).
43. R. Alcázar *et al.*, *PLOS Genet.* **10**, e1004848 (2014).
44. B. Davies *et al.*, *Nature* **530**, 171–176 (2016).
45. F. Supek, M. Bošnjak, N. Škunca, T. Šmuc, *PLOS ONE* **6**, e21800 (2011).
46. S. Meierjohann, T. Mueller, M. Schartl, M. Buehner, *Zebrafish* **3**, 359–369 (2006).
47. D. Powell, Natural hybridization reveals incompatible alleles that cause melanoma in swordtail fish. Dryad (2020).
48. M. Schumer, schumerm/Powell_etal_hybrid_melanoma_manuscript: Spotted caudal hybrid incompatibility scripts release. Zenodo (2020).

49. M. Schumer, Schumerlab/mixnmatch: Mixnmatch version used in Powell et al 2020. Zenodo (2020).
50. M. Schumer, Schumerlab/ncASE_pipeline: ncASE pipeline version used in Powell et al 2020. Zenodo (2020).

ACKNOWLEDGMENTS

We thank E. Calfee; H. Fraser; B. Kim; M. Lipson; P. Moorjani; M. Przeworski; and members of the Reich, Rosenthal, and Schumer laboratories for feedback on this work. We appreciate the help of A. Moyaho-Martinez in the design of swim trials, J. Lim and members of the Rosenthal laboratory in collecting samples, and O. Juárez-Mora for help conducting swim trials. We thank the federal government of Mexico for permission to collect fish. Stanford University and the Stanford Research Computing Center provided computational support for this project. **Funding:** This work was supported by NSF LTREB 1354172 to G.G.R.; funding from the Hagler Institute for advanced study to M.Scha.; and a Hanna H. Gray fellowship, L'Oreal for Women in Science grant, and NIH 1R35GM133774 grant to M.Schu. **Author contributions:** D.L.P., M.G.-O., and M.Schu. designed the project; D.L.P., M.G.-O., M.K., A.P.D.-L., S.B., D.B., M.Schu., and M.Scha. collected data; D.L.P., M.G.-O., M.K., K.D., P.R., and M.Schu. performed analyses; D.R., P.A., M.Scha., and G.G.R. provided expertise and technical support. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** Data are available through NCBI (BioProject PRJNA610049; SRA accessions: SRX7847847-SRX7847871, SRX7866838-SRX7867011, SRX7861514-SRX7861761, and SRX7860174-SRX7860180) and Dryad (*47*). Code is available on github (https://github.com/Schumerlab) and archived at Zenodo (*48–50*).

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/368/6492/731/suppl/DC1
Materials and Methods
Figs. S1 to S41
Tables S1 to S3
Appendix S1
References (*51–108*)
Movies S1 and S2

12 December 2019; accepted 27 March 2020
10.1126/science.aba5216

Science

Natural hybridization reveals incompatible alleles that cause melanoma in swordtail fish

Daniel L. Powell, Mateo García-Olazábal, Mackenzie Keegan, Patrick Reilly, Kang Du, Alejandra P. Díaz-Loyo, Shreya Banerjee, Danielle Blakkan, David Reich, Peter Andolfatto, Gil G. Rosenthal, Manfred Schartl and Molly Schumer

Science **368** (6492), 731-736.
DOI: 10.1126/science.aba5216

Mapping vertebrate incompatibility alleles

Deleterious gene interactions may underlie the observed hybrid incompatibilities. However, few genes underlying hybrid incompatibilities have been identified, and most of these involve species that do not hybridize in natural conditions. Powell *et al.* used genome sequencing to map genes likely responsible for incompatibilities that reduce fitness in naturally occurring hybrid swordtail fish. These gene combinations result in malignant melanoma, which is found in naturally hybridizing populations but is not present in the parental populations (see the Perspective by Dagilis and Matute). Using genome and population resequencing, the authors performed a genome-wide association study to identify potentially causative mutations. Using an admixture mapping approach that assessed introgression between multiple swordtail fish species, the authors suggest that lineages carry different genes that interact with the same candidate gene, resulting in the observed melanomas and providing insight into convergent hybrid incompatibles that arise between species.

Science, this issue p. 731; see also p. 710

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science* is a registered trademark of AAAS.