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Laboratorio de Fisiología en Recursos Acuáticos

(LAFIRA)

"CARACTERIZACIÓN DEL CANIBALISMO EN LARVAS DE PEJELAGARTO (*Atractosteus tropicus*) Y POSIBLES SUPRESORES".

TESIS

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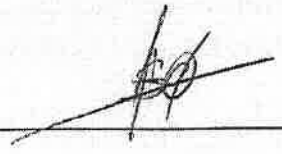

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Para Carmen y Cuco, y Andrés,

A los que ya no están: Mago, Teresa y Chicolino.

Y al guionista.

Se debe tener confianza en uno mismo
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Veni, vidi, vici

ÍNDICE

RESUMEN

Una de las principales problemáticas del sector acuícola es el comportamiento caníbal-agresivo presente en diversas especies de peces con potencial acuícola. Dichos comportamientos se relacionan con altos índices de mortalidad, primordialmente en la etapa larval y/o juvenil. El canibalismo y la agresividad afectan la producción de la especie que lo presenta, elevando los costos y cuidados sobre los organismos, por estos motivos la rentabilidad y viabilidad del cultivo disminuyen. En este sentido, el pejelagarto (*Atractosteus tropicus*) una especie nativa dulceacuícola del suroeste de México, con alta importancia económica debido a su cultivo, pesca, símbolo cultural, objeto artesanal, e importancia ecológica, presenta canibalismo en la etapa larval, con lo que se limita su potencial de cultivo en sistemas acuícolas. El objetivo de este trabajo fue caracterizar el comportamiento caníbal-agresivo de larvas de pejelagarto (*A. tropicus*), partiendo de tres puntos (capítulos) de estudio: 1. Etología, 2. Efecto de la densidad y alimentación, y 3. Efecto de posibles mitigantes. En el capítulo uno, se observó el comportamiento caníbal-agresivo de larvas de *A. tropicus*, de manera grupal (10 larvas) y en parejas (2 larvas). Se describió el tipo de ataque, preferencia de ataque y el efecto de refugios (vegetación artificial y rocas) en las peceras sobre el número de eventos caníbales, de igual manera se evaluaron nueve colores de fondo (blanco, amarillo, naranja, rojo, rosa, morado, azul, azul marino y verde) y su relación con los eventos caníbales y la preferencia del color por parte de las larvas, además de diferencias morfométricas relacionadas al canibalismo y agresividad. En el capítulo dos, se realizó un bioensayo utilizando larvas de 3 días post eclosión (dpe) de *A. tropicus*, en las cuales se evaluaron dos densidades (0.7 y 1.4 larvas por litro) y dos regímenes de alimentación (alimentación y hambruna). Finalmente, en el capítulo tres, se evaluó el efecto de dos posibles mitigantes del canibalismo (Ácido docosahexaenoico (DHA) y Triptófano (Trp)) incluidos en la formulación de la dieta para *A. tropicus*, usándose en las siguientes concentraciones 20, 30 y 40 g Kg^{-1} Algamac 3050® (producto comercial rico en DHA) y 10, 20 y 30 g Kg^{-1} Trp evaluando su efecto con respecto a un control sin mitigante. Para el capítulo dos y tres se evaluó el efecto sobre el comportamiento, crecimiento, supervivencia, bioquímica digestiva, sistema inmune, modificaciones en las estructuras del sistema digestivo y en la expresión de genes asociados a conductas de agresividad. Los resultados para el primer objetivo indican que se produjo un mayor número de ataques en peceras con parejas de *A. tropicus* con

color de fondo blanco (8.50 ± 0.70) y menor para amarillo y púrpura (0.66 ± 0.57, $p < 0.05$). El mayor número de ataques en grupos se observó con los colores de fondo rosa, azul y amarillo, mientras que en menor número en fondo morado. Por otro lado, la presencia de refugios (vegetación artificial) disminuyó los ataques en parejas y en grupo, a diferencia del uso de rocas como refugio. Además, las larvas de *A. tropicus* muestraron preferencia para vegetación artificial. Se encontró que morfológicamente, tanto los juveniles como las larvas (10 dpe) pueden consumir presas mayores que la profundidad de su propio cuerpo (1.59 ± 0.22, 1.00 ± 0.12) y el ancho del cuerpo (1.74 ± 0.29, 0.94 ± 0.12). El ángulo de apertura de la boca fue significativamente mayor en las larvas (10 dpe) (85.63 ± 6.41°), lo que disminuye a medida que aumenta la edad. De un total de 452 eventos en *A. tropicus*, se describieron cuatro comportamientos: interacción (214 eventos, 47 ataques, efectividad del ataque (EA) 21.96%), persecución (127 eventos, 44 ataques, EA 34.64%), escape (62 eventos) y natación rápida (47 eventos). Se registraron tres tipos de ataques: frontal (41.24%), lateral (29.94%) y posterior (28.81%), con tres regiones de ataque: cabeza (31.64%), cuerpo (10.72%) y cola (57.63%). El ataque más frecuente fue cola posterior con 70 eventos (39.55%). La larva atacante presentó una curvatura en forma de S previo al ataque (30.50%). Se determinaron diferencias en el porcentaje de peso (g) y longitud total (cm) entre las larvas atacantes y las larvas atacadas (16.39 ± 10.86 % y 15.23 ± 5.68 %, respectivamente). En el objetivo dos el mejor crecimiento en larvas de *A. tropicus* fue en una densidad de 1.4 larvas/ L con un régimen de alimentación. La baja densidad (0.7 larvas/ L) aumenta el coeficiente de variación (CV) y la talla de heterogeneidad (SH). La mortalidad por canibalismo se presenta al 8 dpe en larvas alimentadas y al 10 dpe en hambruna. El régimen de alimentación de hambruna modifica la actividad enzimática digestiva y metabólica, y la morfología del hígado e intestino. Finalmente, la densidad 0.7 larvas/ L alimentadas presentaron mayor expresión en genes relacionados a agresividad. Los resultados del objetivo 3 señalan que el uso de 10 g/Kg Trp en la dieta mejora la supervivencia evitando los ataques entre larvas caníbales, en comparación al uso del DHA. El uso en la dieta mejora la supervivencia y reduce el canibalismo en larvas de *A. tropicus*. Las larvas caníbales presentaron un mayor crecimiento debido al efecto "jumper" a diferencia de larvas no caníbales. Larvas identificadas como caníbales y no caníbales muestran una diferencia en su actividad enzimática y expresión de genes.

Con estos resultados se confirma que aspectos como el color de fondo y la presencia de refugios influyen en el comportamiento agresivo-caníbal de esta especie, y con esto se pueden generar estrategias de manejo para evitar dichos eventos. Por otro lado, se identifica el periodo de tiempo en el cual los ataques agresivos derivados a canibalismo se relacionan con aspectos morfométricos que les permiten capturar e ingerir a sus propios congéneres. Finalmente, el uso de triptófano puede mitigar de manera efectiva el comportamiento caníbal entre larvas agresivas.

La suma de todos los resultados de este trabajo pauta las estrategias futuras para entender y controlar la agresividad y canibalismo en *A. tropicus* mejorando su cultivo de manera considerable y puede servir como antecedente para analizar estos comportamientos en otras especies de peces con potencial acuícola.

INTRODUCCIÓN

Los avances en la acuicultura en los últimos años se deben principalmente al desarrollo y la investigación de áreas como el cultivo larval, nutrición, reproducción y la incorporación de nuevas tecnologías (Joffre et al., 2017; Kumar et al., 2018; Yue y Shen, 2022). Sin embargo, a pesar de los avances en la producción anual de productos obtenidos por la acuicultura, problemas como la presencia del canibalismo en las larvas de peces es un problema que limita el desarrollo de su cultivo, disminuyendo la rentabilidad económica y aumentando los problemas de sanidad (Sepúlveda-Quiroz et al., 2022). Por este motivo, diversos estudios han abordado el tratar de explicar y entender el canibalismo como una vía para mejorar las condiciones de cultivo de especies con alto potencial. El comportamiento caníbal se define como el acto de capturar e ingerir ya sea completa o parcialmente a un individuo de su misma especie (Pfennig, 1997; Smith y Reay, 1991). En el caso particular de los peces se han registrado aproximadamente 390 especies que presentan este comportamiento, y de igual manera un aproximado de 150 de estas especies presentan canibalismo en condiciones de cautiverio, donde el comportamiento surge principalmente en etapa larval y en algunos casos llega al estadio juvenil y adulto (Pereira et al., 2017).

A nivel global, en sistemas de producción acuícola, el canibalismo es el responsable de una alta mortalidad de larvas y juveniles de peces, por lo que diversos estudios han tratado de entender y explicar dicho comportamiento generando a su vez diversas estrategias para disminuirlo o mitigarlo.

Una variable zootécnica básica que ha sido estudiada es el efecto de los colores de tanque en la conducta de los peces a cultivar, por ejemplo, en mero (*Epinephelus coioides*) el color verde del tanque influye directamente en la conducta de las larvas aumentando el canibalismo (Takeshita y Soyano, 2008), de igual manera en dorada (*Brycon sp.*) las tonalidades azules incrementan el canibalismo (17.08%) afectando la supervivencia (66.25%) de la especie (Costa et al., 2013). Por otro lado, una de las estrategias probadas y con efectos en la disminución del canibalismo es la implementación de refugios en los tanques de cultivo, en ese sentido el usar refugios (piezas de cloruro de polivinilo) o ambientes enriquecidos (tubo de plástico y partículas de plástico) mejora el bienestar en salmón del Atlántico (*Salmo salar*) y juveniles de barramundi

(*Lates calcarifer*) lo que se traduce en la reducción del estrés y comportamientos caníbales, beneficiando así la práctica del cultivo de estas especies (Näslund et al., 2013; Qin et al., 2004).

Además, los efectos de una alimentación inadecuada ya sea nutricionalmente hablando o carente de las raciones necesarias, al igual que una alta o baja densidad de cultivo puede ser relevante con la aparición del canibalismo en larvas de peces. En el caso particular de juveniles de barramundi (*Lates calcarifer*), el canibalismo es mayor a una mayor densidad, disminuyendo este comportamiento al incrementar la frecuencia alimenticia y bajando la densidad (Ribeiro et al., 2015). En larvas de cabrilla (*Paralabrax maculatofasciatus*) el canibalismo fue mayor en las densidades más altas (150 y 200 larvas/ L) (Álvarez-González et al., 2001).

Aunque el uso de técnicas zootécnicas ha funcionado en el control del canibalismo en ciertas especies de peces, y a un grado significativo, el uso de sustancias que funcionen como agentes mitigadores del comportamiento también se ha probado con resultados importantes, especialmente el ácido docosahexaenoico (DHA) y el triptófano. En este sentido, la administración de alto contenido de DHA en la dieta de mero (*E. coioides*), permitió la disminución del comportamiento caníbal como un efecto entre el DHA y la regulación de la serotonina (5-HT) en el cerebro del organismo (Chang et al., 2019). Por otro lado, suministrar triptófano en los peces *Aequidens pulcher y Apteronotus leptorhynchus*, redujo su agresividad (Maler y Ellis, 1987; Munro, 1986). De igual manera, al usar 2, 4, y 6 ppm y 2 y 3% en la dieta, el canibalismo se redujo significativamente (50%) en larvas de pabda (*Ompok bimaculatus*) (Biswas et al., 2018, 2019).

En el sureste de México, el pejelagarto (*Atractosteus tropicus*) es una especie con una alta aceptación y comercio, tanto por ser un pez de consumo tradicional y por su valor cultural. En el desarrollo de su acuicultura, el pejelagarto presenta canibalismo en la etapa larval (10 días post eclosión) y juvenil (Márquez, 2000; Aguilera et al., 2012). En condiciones de laboratorio la supervivencia ronda 24% debido al canibalismo 33% (Frías-Quintana et al., 2017) lo que puede limitar escalar su producción. Aunque se han evaluado diversos regímenes de alimentación (alimento vivo y comercial) y densidades (Palma-Cancino et al., 2019) el canibalismo persiste y aún no se tiene la información suficiente para desarrollar e implementar estrategias de control y mitigación del canibalismo en esta especie.

El objetivo de esta investigación fue describir el comportamiento caníbal de larvas (*A. tropicus*), así como, la evaluación del uso de refugios y color de fondo y su efecto en los ataques caníbales, además, se identificó los efectos de la densidad de cultivo y el régimen de alimentación con la tasa de canibalismo y finalmente se empleó diferentes concentraciones de DHA usando de fuente un producto comercial "Algamac 3050®" (20, 30 y 40 g Kg^{-1}), y tres más con concentraciones de triptófano (10, 20 y 30 g Kg^{-1}), como posibles mitigantes de dichos comportamientos. De forma simultánea se evaluaron los parámetros de crecimiento, actividad enzimática digestiva, estructura del sistema digestivo y genes relacionados a la agresividad (canibalismo).

ANTECEDENTES

Canibalismo en peces

El canibalismo es un comportamiento que se ha registrado en diversos grupos de animales entre ellos: salamandras, arácnidos, pulpos, mamíferos marinos, crustáceos, gasterópodos (Ironside et al., 2019; Johnson et al., 2016; Nishimura, 2018; Polis, 1981; Rosas-Luis et al., 2019; van Neer et al., 2019; Yu et al., 2018).

Como ya se mencionó, el canibalismo también está presente en peces (aproximadamente 390 especies), y por su diversidad de variables involucradas se han realizado diversas formas de clasificar dicho comportamiento, por ejemplo, se han clasificado siete tipos de canibalismo debido a la relación que existe entre la "presa" y el "caníbal" mismas que están agrupadas en tres criterios: estado de desarrollo de la presa (*canibalismo de huevos*), relación genética entre presa-caníbal (*canibalismo filial o entre hermanos*), y finalmente, la relación por edad de la presa y el caníbal (*canibalismo intra-cohorte e inter-cohorte*) (Smith y Reay, 1991).

Por otro lado, existe otra clasificación del canibalismo cuyo fundamento es el tipo de ingesta de los peces caníbales: tipo 1, el pez caníbal es de menor tamaño en relación con el pez presa (pocas presas consumidas por día), tipo 2, el pez caníbal es de mayor tamaño y puede ingerir completamente al pez presa (alto consumo de presas por día), y tipo 3, varios peces están involucrados en un acto caníbal, donde desmiembran a la presa y la consumen (Baras et al. 2000; Baras, 2013; Baras y Jobling, 2002; Cuff, 1980).

Diversos estudios han abordado el comportamiento caníbal presente en peces con la finalidad de encontrar explicaciones y desarrollar estrategias que logren disminuir o mitigar dicho comportamiento, algunas de estas teorías sobre el canibalismo en peces son: el canibalismo como una herramienta ecológica cuya función principal está relacionada con la regulación de las poblaciones un ambiente en particular (Kohlmeier y Ebenhoh, 1995). Por otro lado, Dong y Polis (1992) indican una relación directa entre la presencia del canibalismo con la biodisponibilidad del alimento y el gasto de energía al cazar o alimentarse (teoría del forrajeo óptimo). Además, se ha demostrado que peces identificados como caníbales presentan una mejor eficiencia de conversión alimenticia y tiene una mejor tasa de crecimiento que peces alimentados con otro tipo de alimentos (Ribeiro y Qin, 2015). Finalmente, estudios donde se han realizado observaciones y estudios etológicos de especies de peces caníbales ha permitido

relacionar variables como interacciones entre presa y caníbal durante un evento de canibalismo, preferencia de tallas aptas para consumir, diferentes tipos de ataques, porcentaje de efectividad de ataque, ciclo de depredación, tiempo de seguimiento o comportamientos de persecución de la presa (Baras et al., 2014; Colchen et al., 2019), todo esto en relación a ataques dirigidos y ataques aleatorios, lo que ha permitido la toma de decisiones para elaborar estrategias que mitiguen dicho comportamiento.

Factores detonantes del comportamiento agresivo-canibal en peces

En peces, estudios enfocados en entender y explicar el comportamiento caníbal-agresivo de los organismos ha resultado en una serie de factores ambientales y poblacionales que están relacionados con la presencia de este comportamiento en los organismos: sexo, densidad, domesticación, tamaño de la larva, componente genético, alimentación, condición de luminosidad, temperatura del agua, color y forma del tanque de cultivo, así como la presencia o ausencia de refugios por mencionar algunos (Naumowicz et al., 2017).

Estrategias de mitigación del canibalismo

Colores del tanque y comportamiento caníbal

El uso de colores en el fondo del tanque de cultivo ha tenido ciertos resultados importantes en la mitigación del canibalismo, de esta manera es una de las principales estrategias que se experimentan con finalidad de controlar dicho comportamiento. Otro de sus efectos ha sido relacionado con la supervivencia, índice de conversión alimenticia, salud, crecimiento y color corporal (McLean, 2021). Por ejemplo, en juveniles de trucha ártica (*Salvelinus alpinus*), se registró la mayor agresividad en los tanques experimentales de color blanco en comparación con los tanques de color negro (Höglund et al., 2002). Por otro lado, en los juveniles de bagre africano (*Clarias gariepinus*), una mayor agresividad fue contabilizada al usar tanques de color blanco, azul, verde y rojo en comparación con los tanques de color amarillo (Sallehudin et al., 2017). De igual manera en salmón del pacifico (*Oncorhynchus kisutch*), el uso de tanques de color blanco, azul y gris registraron una mayor tasa de agresividad a diferencia de cuando se empleaban tanques de color negro (Gaffney et al., 2016). Se puede relacionar el color de las larvas con el color del tanque, por lo que un alto contraste entre ambos puede ser directamente proporcional a una mayor tasa de ataques caníbales por la facilidad de observar a su presa por

parte de la larva caníbal, caso contrario cuando la tonalidad del tanque y las larvas es similar, lo que las hace menos visibles a sus posibles atacantes.

Refugios y comportamiento caníbal

En el medio natural los peces prefieren ciertas condiciones bióticas y abióticas para lograr un estado de bienestar. En este sentido, la acuicultura está modificando sus formas de cultivo con la finalidad de proveer o simular las condiciones naturales en un medio de cultivo artificial, y con esto conseguir varias ventajas, entre ellas, reducir el canibalismo. El uso y la implementación de diferentes tipos de refugios artificiales han demostrado la disminución del comportamiento caníbal en ciertas especies de peces, por ejemplo, el uso de refugios disminuyó el canibalismo en juveniles de barramundi (*Lates calcarifer*), donde también se redujo la frecuencia de persecución y mordeduras (Qin et al., 2004). Combinar el tipo de refugio y la densidad usada por tanque también es otra línea de investigación que ha permitido reducir la tasa de canibalismo, por ejemplo, en larvas del pez roca (*Sebastes schlegelii*) el uso de refugios a una alta densidad (ocho por tanque) permitió disminuir el canibalismo (Xi et al., 2017). En bagre (*Cirrius garitpinus*) la implementación de refugios artificiales disminuyó el canibalismo, sin embargo, aumentó la agresividad, debido probablemente a la territorialidad que ejerce esta especie (Hecht y Appelbaum, 1988). El principio del uso de refugios es que fomenta el grado de protección aumentando la cobertura defensiva de las larvas, permitiendo no ser observadas por sus captores, ocultar sus movimientos y evitan ser perseguidos con facilidad.

Densidad y alimentación

Otra variable relacionada con el desarrollo del comportamiento caníbal en larvas de peces es la densidad a la cual son mantenidos en los estanques de cultivo. Por ejemplo, en larvas de cabrilla (*Paralabrax maculatofasciatus*) el canibalismo fue observado en las densidades de cultivo empleadas más altas (150 y 200 larvas/ L) (Álvarez-González et al., 2001). En juveniles de róbalo (*Centropomus undecimalis*) empleando una densidad de 400 peces por tanque, el canibalismo fue mayor lo que generó una alta tasa de mortalidad a diferencia de usar densidades menores (100 y 200 peces por tanque) (Hans et al., 2019). De igual manera, en European sea bass (*Dicentrarchus labrax*) la supervivencia se vio afectada por el canibalismo cuando se

usaron densidades altas de cultivo (15 y 20 post larva/ L), contrario al usar densidades menores (5 y10 post larva/ L) donde la supervivencia fue mayor (Hatziathanasiou et al., 2002).

Sumado a la densidad, la alimentación es otro factor que está relacionado en su conjunto sobre la presencia del canibalismo de las larvas y juveniles de peces. Un caso documentado es en la curvina (*Cynoscion nebulosus*) en donde hay una correlación entre la densidad del cultivo empleada y la alimentación suministrada con la tasa de agresión, en otras palabras, canibalismo (Manley et al., 2014).

Mitigantes

Las estrategias empleadas hasta el momento para intentar disminuir el canibalismo en larvas y juveniles de peces se basan en tener controladas las variables (luminosidad, temperatura, alimento, refugio, turbidez, densidad) en las cuales los organismos se encuentran en bienestar y presentan menor estrés en el estanque de cultivo. De igual manera, se ha buscado encontrar la combinación entre ambas para tener un mejor resultado de supervivencia reduciendo principalmente el canibalismo. Estas prácticas pueden estar catalogadas como estrategias zootécnicas (manejo y cuidado de los peces), sin embargo, mantener las condiciones ya mencionadas o emplear las adecuaciones necesarias puede ser un gasto económico considerable, afectando otras áreas de producción. Una solución para esto es el empleo de compuestos o aditivos que sean sustancialmente más eficientes que las estrategias mencionadas, con esto, los efectos en el aumento de la supervivencia debido a la disminución del canibalismo pueden ser inmediatos sin el gasto (tiempo-económico) de las estrategias zootécnicas.

Estos aditivos o compuestos son principalmente añadidos al alimento y son ingeridos por las larvas y juveniles de peces. Algunos de estos compuestos con resultados importantes en la reducción del canibalismo y agresividad en peces son el ácido docosahexaenoico (DHA) y el triptófano.

El ácido docosahexaenoico (DHA)

El ácido docosahexaenoico (DHA) 22:6 n-3, es un ácido graso polinsaturado de cadena larga (n-3 serie), considerado como un ácido graso primordial para el desarrollo en peces (Watanabe, 1993). Los ácidos grasos en específico los poliinsaturados de cadena larga (LC-PUFA) son de gran importancia nutricional principalmente en las primeras etapas de desarrollo

de las larvas de peces, ya que está relacionado con la reserva de energía, moléculas estructurales y reguladores metabólicos, vinculados a procesos de homeostasis, respuesta al estrés y sistema inmune (Turchini et al., 2022). En el cultivo de mero manchado (*Epinephelus coioides*) el uso de un alimento rico en DHA disminuyó considerablemente el canibalismo, esto debido a la relación que tiene DHA con la regulación de serotonina (5-HT) en el cerebro del organismo (Chang et al., 2019).

Triptófano

El triptófano es un aminoácido esencial, el cual es el precursor de 5-hidroxitriptamina (5-HT) (serotonina) (Leathwood, 1987), con diversas funciones como ser un neurotransmisor encargado del control del apetito (Smith y Seddon, 1998), relacionado a procesos de reproducción (Akiyama et al. 1996), y procesos fisiológicos como la inmunidad y la homeostasis intestinal (Comai et al. 2020). En relación con el canibalismo en peces, la administración del triptófano en los peces *Aequidens pulcher* y *Apteronotus leptorhynchus* redujo conductas agresivas (Maler and Ellis, 1987; Munro, 1986). Caso contrario sucede cuando se administró un inhibidor de la serotonina (p-clorofenilalanina) provocando un aumento de conductas agresivas en *Cichlasoma meeki* (Adams et al., 1996). Un claro efecto de la reducción del canibalismo por la incorporación del triptófano se registró en larvas de pabda (*Ompok bimaculatus*) donde al usar concentraciones de 2, 4, 6 ppm y 2-3% en la dieta redujo 50% en el canibalismo en las larvas (Biswas et al., 2018, 2019).

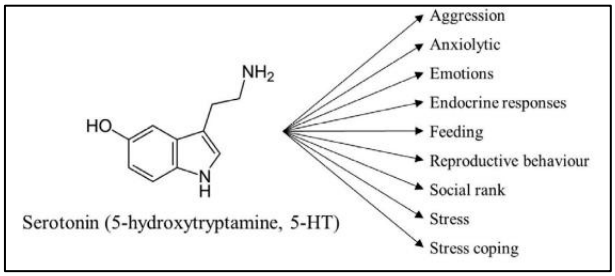

Figura 1.- Funciones en las que participa la serotonina (5-HT) (Backström y Winberg, 2017).

Ruta de acción del DHA y triptófano

La síntesis de la serotonina se lleva a cabo en las neuronas serotoninérgicas, donde el triptófano sirve como precursor. Se inicia la síntesis cuando la enzima triptófano hidrolasa hidroxila el triptófano pasando a L-5-hidroxitriptófano, posteriormente se descarboxila por medio de la enzima L-aminoácido descarboxilasa generando 5-hidroxitriptamina (5-HT). La 5-HT es degradada por la enzima monoamino oxidasa transformándolo en 5-hidroxiindol acetaldehído, y se finaliza cuando la enzima aldehído deshidrogenasa produce ácido 5-hidroxiindolacético (5-HIAA) (Winberg y Nilsson, 1993; Höglund et al., 2019; Sahu et al., 2020).

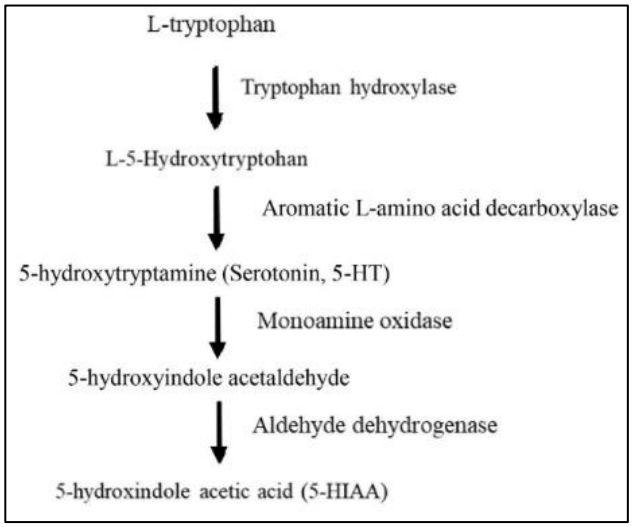

Figura 2.- Ruta de biosíntesis de serotonina (Höglund et al., 2019).

El triptófano compite con otros aminoácidos (AA) (valina, isoleucina, leucina, tirosina, fenilalanina y metionina) al intentar ingresar al cerebro, siendo un factor clave el balance entre estos AA y el triptófano en el plasma. El triptófano proveniente de la ingesta de proteína eleva su concentración específica en relación con otros AA, sin embargo, la presencia del resto de AA termina por saturar los transportadores específicos causando un descenso del flujo de triptófano al cerebro. Sin embargo, cuando se ingieren carbohidratos ya sea en combinación con proteínas, la insulina promueve una captación de AA con excepción del triptófano, lo que aumenta su

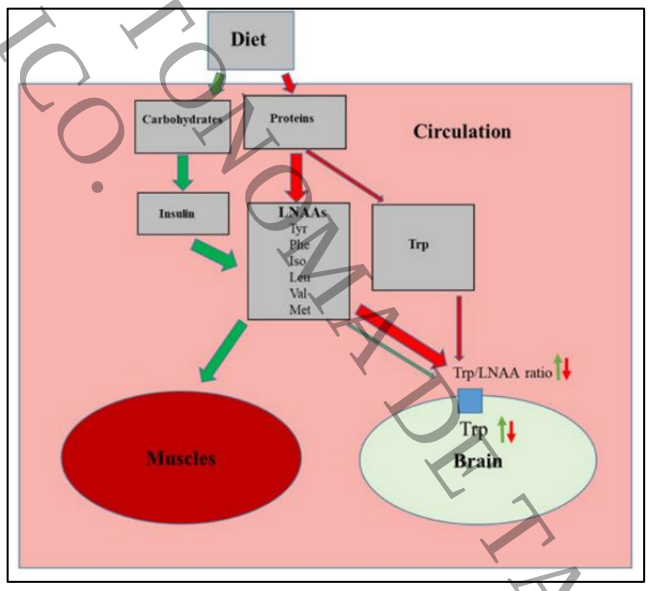

Figura 3.- Efectos de las proteínas y los carbohidratos sobre el flujo de triptófano al cerebro (Höglund et al., 2019).

relación (concentración específica) en comparación a otros AA favoreciendo el flujo de triptófano al cerebro (Höglund et al., 2019).

Por otro lado, el consumo de alimentos ricos en ácidos grasos, particularmente ácidos grasos altamente insaturados (HUFAs), incrementa en la concentración de ácidos grasos n-3/n-6 (grupo del DHA) disponibles en el cerebro. Las bajas concentraciones de estos tipos de ácidos grasos se relacionan con una disminución de dopamina y 5-HT (serotonina) (Fernandes et al., 2017) y un aumento en la agresión debido al aumento de reguladores del estrés como las catecolaminas y citoquinas proinflamatorias (TNF-α e IL-6) (Bradbury et al., 2004). Estas citoquinas aumentan la actividad de la enzima indolamina 2,3-dioxigenasa y hace que el triptófano usado originalmente para sintetizar 5-HT cambie su ruta metabólica y se convierta en quinurenina, lo que resulta en una disminución de los niveles de 5-HT en el cerebro (Husted y Bouzinova, 2016). En ese sentido la disponibilidad de ácidos grasos (n-3/n-6) es importante en el cerebro ya que inhabilita la generación de TNF-α e IL-6, generando un aumento de la disponibilidad de triptófano precursor para la síntesis de 5-HT. Ya en el cerebro, el triptófano favorece el funcionamiento de las enzimas TPH (triptófano descarboxilasa) y AAAD (L-Aminoácido aromático descarboxilasa), las cuales son fundamentales para la biosíntesis de la serotonina principalmente.

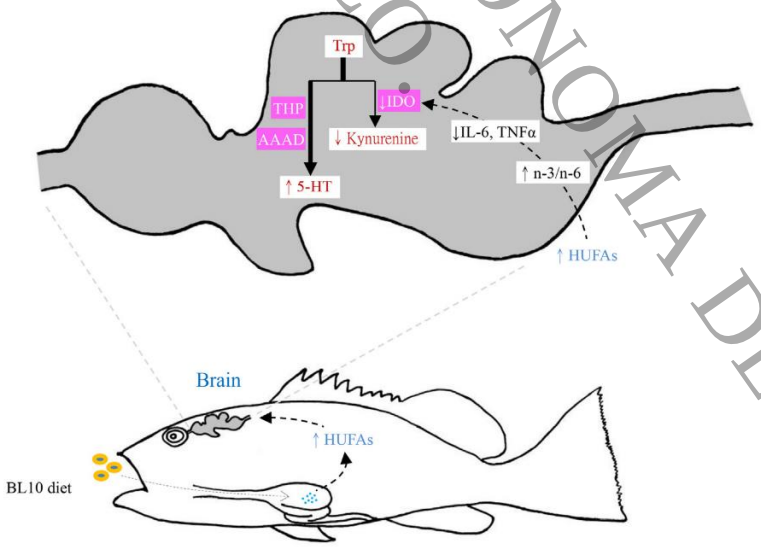

Figura 4.- Mecanismos de acción de los ácidos grasos (DHA) y triptófano en la regulación de la agresividad (Chang et al., 2019).

Neurotransmisores, rutas metabólicas y genes relacionados al canibalismo

Neurotransmisores

Una de las dificultades de estudiar el canibalismo en peces es la poca información y estudios que aborden temas como rutas metabólicas, neurotransmisores específicos o genes relacionados a dicho comportamiento, esto se debe principalmente a que el comportamiento caníbal está inmerso y relacionado con aspectos de agresividad, concepto altamente estudiado.

Los neurotransmisores son moléculas encargadas de la señalización en el sistema nervioso y su función está delimitada por requerir de receptores específicos los cuales producen una reacción química en cadena. Un neurotransmisor puede promover o inhibir la reacción química que desempeña al mismo tiempo. En concreto, los neurotransmisores son de gran importancia debido a su función de mensajeros bioquímico y están relacionados como reguladores del comportamiento, ya que son los encargados en responder a los estímulos externos (Beaver y Walsh, 2011). Se ha reportado que algunos de estos neurotransmisores están relacionados o regulan la agresividad, siendo los más estudiados: dopamina, serotonina (5-HT), histamina, adrenalina, ácido gamma-aminobutírico (GABA) y glutamato (GLU) (Winberg y Nilsson, 1993; von Bohlen y Dermietzel, 2002; Cooper et al., 2003). De igual manera, existen neuromoduladores como las endorfinas, hormona liberadora de gonadotropina (GnRH), óxido nítrico (NO) y vasopresina, que cumplen en ciertos casos la función de los neurotransmisores (von Bohlen y Dermietzel, 2002). Estos moduladores también han sido estudiados debido a estar relacionados a la agresividad (Filby et al., 2010).

Los organismos, al percibir o reaccionar a un estímulo, la biosíntesis del neurotransmisor se realiza en las células nerviosas (neuronas) de forma intracelular. Una vez formado y liberado de la célula, los neurotransmisores se unen con los receptores específicos los cuales tienen la función de acoplarse con proteínas dina comenzando con esto la reacción química especifica. Este proceso puede ser pausado a causa de dos efectos, cuando el neurotransmisor es detenido en la estructura de producción y/o por una degradación enzimática (Beaver y Walsh, 2011), por ejemplo, la monoamino oxidasa (MAO) degrada la serotonina, dopamina y otros neurotransmisores (Zigmond et al., 2003).

A continuación, se describen los neurotransmisores más estudiados con relación a la agresividad reportados hasta el momento con algunos ejemplos:

Glutamato y ácido gamma aminobutírico (GABA)

El ácido gamma-aminobutírico (GABA) es un importante neurotransmisor con una función inhibitoria en el sistema nervioso. Se sintetiza a partir de un proceso de descarboxilación de L-glutamato, por parte de la enzima glutamato descarboxilasa (Wassef et al., 2003). El GABA está relacionado con la modulación de la agresividad y se ha encontrado que sus niveles bajos aumentan el estado de agresividad (de Almeida et al., 2005). Por otro lado, altas concentraciones de los reguladores (benzodiazepinas y los barbitúricos) de los receptores de GABA, reducen el comportamiento agresivo en ratones, caso contrario sucede al disminuir las concentraciones de los reguladores (Miczek et al., 2002, Gowin et al., 2010). El glutamato es el principal neurotransmisor excitador en los mamíferos, se sintetiza a través del ciclo de Krebs y las células gliales desde donde se transporta a las células nerviosas. Este neurotransmisor está relacionado como disparador de procesos agresivos, un aumento en este neurotransmisor puede contribuir a un aumento de la agresión (Beaver y Walsh, 2011; Umukoro et al., 2013).

Serotonina

La serotonina (5-hidroxitriptamina o 5-HT) se produce a partir de L-triptófano dietético, y por acción de la enzima triptófano hidroxilasa se convierte a 5-hidroxitriptófano (5-HTP) (Aldegunde et al., 2000). El caso opuesto ocurre cuando la enzima monoamino oxidasa actúa sobre 5-HT, convirtiéndolo en ácido 5-hidroxiindolacético (5-HIAA) (Winberg et al., 1992). En juveniles de bacalao del Atlántico (*G. morhua*), al administrar 28 g kg^{-1} L-triptófano (precursor de la serotonina) se reduce significativamente los comportamientos agresivos, también influye en la concentración final de ácido hidroxiindolacético (5-HIAA) y 5-HT en los organismos (Hoglund, et al. 2005). En el caso particular del bagre (*Ompok bimaculatus*) usando tres concentraciones (2,4,6 ppm) de triptófano presentaron una disminución significativa del canibalismo del 50% en relación con el control (sin triptófano) (Biswas et al. 2019), atribuido a la producción y aumento de serotonina (5-HT).

Dopamina, epinefrina y norepinefrina

La dopamina, norepinefrina y epinefrina comparten la misma ruta de biosíntesis, estos tres neurotransmisores son conocidas como catecolaminas (Beaver y Walsh, 2011). La dopamina (3-hidroxitiamina), presenta cinco diferentes receptores: D1, D2, D3, D4 y D5 (Hansen y Manahan-Vaughan, 2012), está relacionada en procesos biológicos como el control del movimiento, aprendizaje, el sistema de recompensa y la memoria a largo plazo (Rossato et al., 2009; Arias-Carrión et al., 2010). Con respecto a la agresividad, la biosíntesis de la dopamina se activa cuando el organismo sufre de una amenaza por parte de otro organismo (Ferrari et al., 2003). Como ejemplo, en ratones, sujetos a un bioensayo donde se colocan ratones intrusos en una población ya consolidada, tanto la dopamina y los niveles de serotonina aumentaron ante un estado natural de amenaza y estrés que les produce (Miczek et al., 2004). Los adrenérgicos (norepinefrina y epinefrina) son los principales responsables del estado de alerta y se activan cunado los organismos sufren un evento estresante (Beaver y Walsh, 2011).

Histamina

La histamina se produce en células de origen neuroepitelial, se sintetiza a partir del aminoácido histidina por medio de la enzima histidina descarboxilasa. La histamina participa en la secreción ácida gástrica, inmunomodulación, contracción muscular, vasodilatación, control de la barrera epitelial y endotelial, además de reguladores del comportamiento (regulación de temperatura, ansiedad, aprendizaje, percepción al estrés y dolor) (Brown et al., 2001; Nuutinen y Panula 2010). La importancia de la histamina radica en que es un neurotransmisor que regula neuronas con serotonina, norepinefrina, dopamina y acetilcolina (Haas et al., 2008). En machos dominantes de pez cebra, genes relacionados a la histamina presentaron una sobreexpresión, en este caso los receptores H1 de histamina puede inhibir la síntesis de serotonina (5-HT) (Filby et al., 2010), de igual manera, en este organismo, receptores de histamina (H3) fueron identificados en comportamientos de agresión y la ansiedad (Reichmann et al., 2020).

Rutas metabólicas

En peces, se ha reportado que al menos ocho rutas metabólicas diferentes (sistema-hipotálamo-neurohipofisario, serotonina, somatostatina, dopamina, hipotálamo-pituitaria-intrarrenal, hipotálamo-pituitaria-gonadal, histamina y óxido nítrico) están involucrados con el

canibalismo y la agresividad (Filby et al., 2010), por lo que diferentes neurotransmisores, neuropéptidos y hormonas están relacionados a la vez.

Las hormonas son sustancias químicas secretadas en el torrente sanguíneo que actúan sobre tejidos distantes, generalmente de manera reguladora (Bahadoran et al., 2019), su producción y regulación se da por parte del sistema neuroendocrino de los peces. Este sistema está compuesto por el hipotálamo que controla a la glándula pituitaria integrada por la adenohipófisis y la neurohipófisis. La adenohipófisis es el sitio de síntesis, almacenamiento y liberación de hormonas, por su parte la neurohipófisis funciona como centro de almacenaje y distribución de neuropéptidos (Figura 5) (Oliveira & Gonçalves, 2008).

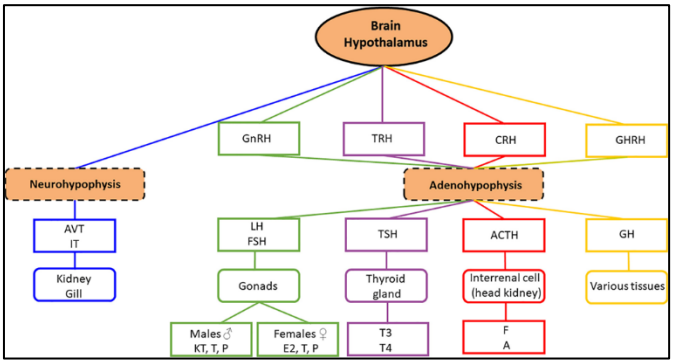

Figura 5.- Representación esquemática de la organización jerárquica del sistema neuroendocrino en peces teleósteos; GnRH: hormona liberadora de gonadotropina; TRH: hormona liberadora de tirotropina; CRH: hormona liberadora de corticotropina; GHRH: hormona liberadora de hormona del crecimiento; LH: hormona luteinizante; FSH: hormona estimulante del folículo; TSH: hormona estimulante de la tiroides; ACTH: hormona adrenocorticotrópica; F: cortisol; A: adrenalina; GH: hormona del crecimiento; AVT: arginina-vasotocina; IT: isotocina; KT: 11-cetotestosterona; T: testosterona; P: 17,20 \ beta - dihidroxipregn-4-en-3-ona; E2: 17ß-estradiol; T3: triyodotironina; T4: tiroxina (Oliveira y Goncalves, 2008).

El comportamiento de la agresividad ha sido muy estudiado en el grupo de los vertebrados, y al hablar de canibalismo en peces tenemos que referenciarlo como parte de la agresividad. Los andrógenos, arginina, vasotocina y oxitocina, son las hormonas más estudiadas con relación en la agresividad en peces (da Silva et al., 2021).

La función principal del andrógeno es la estimulación del crecimiento y la maduración de las gónadas, el desarrollo de características sexuales y la expresión del comportamiento reproductivo en machos principalmente (Patrão et al., 2009). El factor de liberación de corticotropina estimula la glándula pituitaria anterior generando la hormona adrenocorticotrópica que a su vez estimula el tejido intrarrenal para producir cortisol, (Carpenter et al., 2014). En mero de manchas naranjas (*Epinephelus coioides*) la administración de una dieta rica en ácidos grasos (DHA) permitió la disminución del canibalismo intracohorte, además aumentó los niveles de serotonina (5-HT) en el cerebro y se dedujeron los niveles de cortisol en suero, se asume que la biodisponibilidad de DHA modifica la permeabilidad de las células

cerebrales permitiendo la entrada del cortisol, el cual es depurado por sistema hipotálamo-pituitario-adrenal (Chang et al., 2019).

En hembras con diferente estado reproductivo (hembra en pre desove, hembra con huevos, hembra con larvas eclosionadas y hembra con larvas con nado libre) de cíclido sudamericano (*Cichlasoma dimerus*), se les cuantificó el comportamiento agresivo, la concentración de glucocorticoides (cortisol), estrógenos (17b-estradiol) y andrógenos (testosterona y 11-cetotestosterona), como resultado las hembras en el estado pre-desove registraron una mayor agresividad, estrógenos y andrógenos, el cortisol fue similar en todos los estados reproductivos excepto en hembras con huevos donde su concentración fue mayor (Tubert et al., 2012).

Con respecto al canibalismo, en específico al canibalismo filial presente *Rhabdoblennius nitidus*) donde el cuidado parental es del macho y el mismo consume a su propia progenie, se encontró que este comportamiento se relaciona directamente a una componente endocrinóloga. Los machos exhiben ciclos de cría dependientes del andrógeno, la presencia de huevos en el nido es un estímulo clave que regula los niveles de andrógeno, la eliminación de los huevos por un aparente canibalismo es necesario para reiniciar otro periodo de cortejo, y se comprobó a manera experimental al remover los huevos de un tratamiento, lo que provocó al día siguiente un aumento en testosterona y 11-cetotestosterona (Matsumoto et al., 2018).

En el caso de las hormonas no peptídicas como arginina, vasotocina, oxitocina, se han identificado en comportamientos agresivos y sexuales que se presentan en condiciones sociales como jerárquicos o dominancia (Godwin y Thompson, 2012). La importancia de estas hormas radica por su función en el sistema hipotalámico-pituitario-intrarrenal como neuromoduladores de la serotonina por medio de vasotocina arginina y factor liberador de corticotropina (Backström y Winberg, 2017).

Finalmente, otro grupo de hormonas metabólicas como tiroxina, triyodotironina, hormona de crecimiento y somatostatina también han tenido reportes de estar relacionadas con regulaciones de comportamientos agresivos, territorialidad y jerarquía en peces, sin embargo, aún falta más investigación al respecto.

Genes

Se ha utilizado al pez cebra (*Danio rerio*) como organismo modelo, en el cual se analizaron genes relacionados a la agresividad, regiones del cerebro con mayor expresión de genes (hipotálamo y telencéfalo), y la diferencia que existe en la expresión de genes entre machos-hembras dominantes-subordinados, en este caso, estos genes se relacionan en ocho rutas neurológicas (sistema hipotálamo-neurohipofisario (HNS), serotonina (5-HT), somatostatina, dopamina, histamina, óxido nítrico, hipotálamo-pituitaria-intrarrenal (HPI) e hipotálamo-pituitaria-gonadales (HPG)) (Tabla 1) (Filby et al., 2010), la agresividad se relaciona por un carácter sexual y jerárquico de la especie.

Tabla 1.- Genes relacionados a la agresividad en machos dominantes y subordinados de pez cebra (*Danio rerio*) (Filby et al., 2010).

Genes sobre expresados en peces dominantes			
Gen	Nombre	Ruta neurológica	Sexo
avpl	arginine vasopressin-like	HNS	Ambos
avplr1b	arginine vasopressin-like receptor 1b	HNS	Ambos
oxtl	oxytocin-like	HNS	Ambos
tph2	tryptophan hydroxylase 2	5-HT	Hembras
tph1b	tryptophan hydroxylase 1b	5-HT	Machos
htr1a	5-hydroxytryptamine (serotonin) receptor 1A	5-HT	Ambos
slc6a4a	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4a	5-HT	Hembras
maoa	monoamine oxidase a	5-HT	Hembras
sst1	somatostatin 1	Somatostatin	Ambos
sst3	somatostatin 3	Somatostatin	Hembras
sstr1	somatostatin receptor 1	Somatostatin	Machos
th	tyrosine hydroxylase	Dopamine	Ambos
drd2c	dopamine receptor d2c	Dopamine	Machos
slc6a3	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	Dopamine	Ambos
hdc	histidine decarboxylase	Histamine	Machos
hrh2	histamine receptor h2	Histamine	Ambos
nos1	nitric oxide synthase 1 (neuronal)	Nitric oxide	hembra
crh	corticotropin releasing hormone	HPI	Ambos
nr3c1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	HPI	Machos
npy	neuropeptide y	HPI	Ambos
nr3c1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	HPI	Hembras
gnrh3	gonadotropin-releasing hormone 3	HPG	Ambos
esr2a	estrogen receptor 2a	HPG	Ambos

ar	androgen receptor	HPG	Ambos
esr1	estrogen receptor 1	HPG	Hembras
esr2b	estrogen receptor 2b	HPG	Machos
cyp19a1b	cytochrome P450, family 19, subfamily A, polypeptide 1b	HPG	Hembras
Genes sobre expresados en peces subordinados			
drd3	dopamine receptor d3	Dopamine	Machos
hrh2	histamine receptor h2	Histamine	Machos
crh	corticotropin releasing hormone	HPI	Machos
nr3c1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	HPI	Machos
npy	neuropeptide y	HPI	Machos

*HNS: sistema hipotálamo-neurohipofisario. 5-HT: serotonina, HPI: hipotálamo-pituitaria-intrarrenal. HPG: hipotálamo-pituitaria-gonadales.

En el mismo organismo, se demostró que el gen *fgfr1*está relacionado a la agresividad, audacia y la exploración en adultos, originado por la regulación de la histamina desempeñada por este gen (Norton et al., 2011). De igual manera, al realizar un metaanálisis para identificar genes de agresividad en ratones y peces cebra con presencia de comportamientos agresivos y no agresivos, se lograron identificar 70 y 77 genes respectivamente en organismos agresivos. De los cuales siete genes se presentaron en ambos organismos (*Fos, Dusp1, Hdac4, Ier2, Bdnf, Btg2, y Nr4a1*) relacionados a las proteínas FOS, encargadas de modulación de la proliferación, diferenciación y muerte celular (Malki et al., 2016).

En otro trabajo con peces cebras dominantes y subordinados se presentaron diferencia significativa en los genes relacionados al estrés (receptor de glucocorticoides *gr*, receptor de mineralocorticoides *mr*), actividad neuronal (factor neurotrófico derivado del cerebro *bdnf*, *c-fos*), y el sistema serotoninérgico (receptor de hidroxitriptamina (serotonina) 2b *htr2b,* familia de transportadores de solutos 6 (neurotransmisor transportador), miembro 4b *slc6a4b*) (Theodoridi et al., 2017). En machos de un cíclido africano (*Astatotilapia burtoni*) expuestos a un escenario de dominancia y jerarquía por ser colocados en una misma pecera, se identificó que la disminución del gen receptor 1 de la hormona liberadora de corticotropina en el cerebro produce una disminución proporcional al comportamiento agresivo (Chen y Fernald, 2011).

Tabla 2.- Genes relacionados a comportamientos agresivos en pez cebra (*Danio rerio*) (de Abreu et al., 2019).

Gen	Nombre	Descripción
htr1a	Serotonin 1 A receptor	Este gen codifica un receptor acoplado a proteína G para 5-hidroxitriptamina (serotonina) y pertenece a la subfamilia de receptores de 5-hidroxitriptamina.
htr1b	Serotonin 1B receptor	La proteína codificada por este gen es un receptor acoplado a proteína G para la serotonina (5-hidroxitriptamina). La unión de ligandos activa segundos mensajeros que inhiben la actividad del adenilato ciclasa y controlan la liberación de serotonina, dopamina y acetilcolina en el cerebro.
htr2b	Serotonin 2B receptor	Este gen codifica uno de los diferentes receptores de 5-hidroxitriptamina (serotonina) que pertenece a la familia del receptor 1 acoplado a proteína G.
ar	Androgen receptor	Funciona como un factor de transcripción activado por hormonas esteroides y estimula la transcripción de genes que responden a los andrógenos.
avpr1b	Arginine vasopressin receptor 1B	La proteína codificada por este gen actúa como receptor de arginina vasopresina.
bdnf	Brain-derived neurotrophic factor	Este gen codifica un miembro de la familia de proteínas del factor de crecimiento nervioso, promueve la supervivencia neuronal.
slc6a3	Dopamine transporter	Este gen codifica un transportador de dopamina.
c-fos	Fos proto-oncogene	Regula de la proliferación, diferenciación y transformación celular. En algunos casos, la expresión del gen FOS también se ha asociado con la muerte celular apoptótica.
gr	Glucocorticoid receptor	Este gen codifica el receptor de glucocorticoides, y participa en las respuestas inflamatorias, la proliferación celular y la diferenciación en los tejidos diana.
mr	Mineralocorticoid receptor	Este gen codifica el receptor de mineralocorticoides, que media las acciones de la aldosterona sobre el equilibrio de agua y sal dentro de las células diana restringidas.
oxtr	Oxytocin receptor	La proteína codificada por este gen pertenece a la familia de receptores acoplados a proteína G y actúa como receptor de oxitocina.
slc6a4a	Serotonin transporter	Este gen codifica una proteína de membrana integral que transporta el neurotransmisor serotonina desde los espacios sinápticos a las neuronas presinápticas.
sstr1	Somatostatin receptor 1	Receptor de somatostatinas, hormonas peptídicas que regulan diversas funciones celulares como la neurotransmisión, la proliferación celular y la señalización endocrina, además de inhibir la liberación de muchas hormonas y otras proteínas secretoras.
tph1b	Tryptophan hydroxylase 1B	La proteína codificada es una de las dos enzimas triptófano hidroxilasa que catalizan el primer paso limitante en la biosíntesis de la hormona y neurotransmisor serotonina.

th	Tyrosine hydroxylase	La proteína codificada por este gen está involucrada en la conversión de tirosina en dopamina.

A pesar del esfuerzo por conocer los genes relacionados al canibalismo, hasta el momento no se tiene identificados algunos en específico, todas las investigaciones se basan a las interacciones de varios genes en relación con la agresividad, territorialidad, audacia y estrés.

Aspectos morfométricos relacionados al canibalismo

Además de condiciones zootécnicas como lo son la administración de alimento, calidad de la dieta y su frecuencia, o bien la densidad de cultivo y la forma o presencia de refugios que son variables estudiadas para mitigar el canibalismo, otros estudios apuntan a variables morfométricas de las larvas y/o juveniles para entender el canibalismo mediante modelos matemáticos.

Por ejemplo, en mero (*Epinephelus coioides*) y mero gigante (*Epinephelus lanceolatus*) la medición de variables morfométricas como lo son el ancho de la boca, ancho de cuerpo y longitud total, así como la construcción de modelos (regresión lineal) permitió considerar grupos de tallas como una medida de manejo de los organismos con el fin de disminuir el canibalismo (Hseu et al., 2003; Hseu et al., 2004). En ese sentido, en larvas de dourado (*Salminus brasiliensis*) el canibalismo presentado durante la ontogenia está relacionado directamente con la morfometría de las larvas, particularmente el tamaño de la boca y la relación de cabeza-cuerpo, y su vez estas variables determinan la zona de ataque al momento de conductas caníbales, dichas zonas también cambian con el cambio de edad derivado de los cambios morfométricos (Ribeiro y Portela, 2020). Por otro lado, en larvas de barramundi (*Lates calcarifer*), se ha reportado que el canibalismo está relacionado principalmente a la diferencia de tallas entre las larvas del cultivo, sin embargo, los cambios en el crecimiento (alométrico) de la boca en relación con el ancho del cuerpo influye en el canibalismo presente en la etapa larval (Ribeiro y Qin, 2013).

En pejelagarto (*A. tropicus*), se ha descrito los días de aparición del canibalismo en larvas, sin embargo, no se ha cuantificado si alguna variable relacionada a la morfometría está relacionada con dicho comportamiento, por lo que este estudio puede arrojar resultados importantes.

Pejelagarto y el canibalismo

El pejelagarto (*Atractosteus tropicus*) pertenece a la familia *Lepisosteidae* en la cual se encuentran dos géneros: *Lepisosteus* (*Lepisosteus osseus, L. platyrhincus, L. platostomus* y *L. oculatus*) y el género *Atractosteus* (*A. tropicus, A. spatula* y *A. tristoechus*). En México el pejelagarto (o también llamado pez armado o catán) se distribuye en la zona sur, principalmente en los estados de Tabasco, Veracruz, Campeche y Chiapas, así como en Nicaragua, Guatemala y Costa Rica (Márquez-Couturier et al., 2015). Por sus características biológicas, como lo son su alta tolerancia a condiciones de poco oxígeno, el cultivo a alta densidad, facilidad para ser alimentado con dieta comercial, y sobre todo una amplia aceptación en el mercado por parte de los consumidores, el pejelagarto es una especie la cual ha sido usada como una especie de desarrollo en la acuicultura (Couturier et al., 2015). El pejelagarto además de contar con una demanda comercial y ser una fuente de empleo, ecológicamente, es una especie control ya que regula las poblaciones de otros organismos, por lo cual es de importancia biológica y ecológica., Aunado a lo anterior, el desarrollo de su acuicultura también tiene la función de que se disminuya su pesca la cual se calcula en 325 toneladas por año en el estado de Tabasco, México y se opte por el consumo proveniente de la acuicultura y que además se repueble las poblaciones silvestres con esta actividad (Couturier et al., 2015).

En relación con el canibalismo, en el pejelagarto (*A. tropicus*) se ha reportado en el medio natural como en cautiverio, principalmente en etapa larval al día 10 post eclosión presentando un canibalismo entre larvas del mismo desove (Márquez, 2000; Frías-Quintana y Álvarez-González, 2010; Frías-Quintana et al., 2016; Frías-Quintana et al., 2017). Desatender este comportamiento puede derivar a una alta mortalidad, ya sea por el propio consumo de las larvas entre ellas o por enfermedades que se adhieren por las heridas provocadas, en ambas situaciones se baja la rentabilidad de la actividad, y si no se atiende el problema este comportamiento puede seguir hasta etapa juvenil (Aguilera et al., 2012).

Al igual que otros peces, algunos de los factores que están relacionados con la presencia del canibalismo en el pejelagarto (*A. tropicus*) son: diferencia de talla o color de las larvas, mala alimentación o uso de dietas con poco valor nutricional, y larvas con anormalidad en el cultivo (Márquez et al., 2015).

Por otro lado, este comportamiento caníbal también ha sido reportado en catán o alligator gar (*Atractosteus spatula*), especie que pertenece a la misma familia del pejelagarto (*A. tropicus*). En este caso las larvas de *A. spatula* presentan canibalismo alrededor del día 15 post eclosión (Mendoza et al., 2008), y como una estrategia para disminuir dicho comportamiento ha sido emplear compuestos antitiroideos reteniendo el crecimiento y el desarrollo del hocico de las larvas lo que limita el ataque y captura entre los organismos y que se pueda dirigir su alimentación a un consumo más específico como el de una dieta comercial o *Artemia*, disminuyendo así el porcentaje de canibalismo (Clay et al., 2011; Mendoza et al., 2002).

Aunque en pejelagarto (*A. tropicus*) se ha reportado que el canibalismo está presente y disminuye considerablemente la rentabilidad y el bienestar del cultivo, no se han generado hasta el momento líneas de investigación que proporcionen más información acerca de este comportamiento y como disminuir sus efectos. Por lo que aquí presentamos será de gran utilidad y fomentará el desarrollo de estrategias para reducir este comportamiento.

JUSTIFICACIÓN

Actualmente se conoce que en etapas tempranas del desarrollo del pejelagarto (*A. tropicus*) se presenta canibalismo, el cual conlleva a una disminución significativa en el porcentaje de supervivencia que merma su producción. Además, la carencia de información sobre el comportamiento de la especie plantea la necesidad de realizar estudios que permitan describir los factores que se vinculan con el canibalismo, así como sus posibles mecanismos de mitigación.

Este estudio permitirá describir por primera vez, el comportamiento caníbal de *A. tropicus* y se propondrán mecanismos de mitigación con la finalidad de disminuir su efecto negativo en el cultivo; lo que permitirá a los productores obtener una mayor tasa de supervivencia y rentabilidad, además se generarán las bases para estudiar los posibles mecanismos de mitigación del canibalismo en otras especies de interés acuícola.

OBJETIVOS

Objetivo general

Caracterizar el canibalismo en larvas de pejelagarto (*Atractosteus tropicus*) así como emplear triptófano y DHA como posibles mitigantes y su efecto en parámetros de crecimiento y morfofisiológicos.

Objetivos específicos

- Capítulo 1: Describir el comportamiento e interacciones del canibalismo en larvas de pejelagarto (*A. tropicus*) intracohorte bajo el efecto de refugios y color de fondo.
- Capitulo 2: Determinar el efecto de la densidad de cultivo (0.7-1.4 larvas/L) y regímenes de alimentación (alimentación y hambruna) en larvas de pejelagarto (*A. tropicus*).
- Capítulo 3: Evaluar el efecto de la inclusión de Triptófano (10, 20 y 30 g Kg^{-1}) y DHA (20, 30 y 40 g Kg^{-1}, Algamac 3050®) como posible supresor de conductas caníbales en larvas de pejelagarto (*A. tropicus*).

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CAPÍTULOS

Capítulo 1: Attack behavior leading cannibalism in tropical gar (*Atractosteus tropicus*) larvae under different tank colors and shelter type

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Attack behavior leading cannibalism in tropical gar (*Atractosteus tropicus*) larvae under different tank colors and shelter type

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ABSTRACT

Aggressivity expressed as cannibalism in fish larvae is a problem that limits the development of many species in aquaculture, therefore, understanding it and generating strategies to reduce its impact is important. This study described cannibalistic attacks behavior in Tropical gar (*Atractosteus tropicus*) larvae. The larvae were exposed to different tank colors and shelters (rocks and plastic vegetation), in pairs (2 larvae) and in groups (10 larvae). In addition, attacks behavior, types of attacks, and morphometric aspects related to cannibalism were described. In pairs, attacks occurred in greater numbers with white background color (8.50 ± 0.70) and fewer for yellow and purple (0.66 ± 0.57, $p < 0.05$). The largest number of group attacks was observed with the background colors pink, blue and yellow, and purple to a lesser extent. The presence of shelters (artificial vegetation) decreased the attacks in pairs and in groups compared to the use of rocks as refuge. *A. tropicus* larvae show a clear preference for artificial vegetation. It was found that morphologically, both juveniles and larvae (10 Days after hatching (DAH)) can consume prey greater than their own body depth (1.59 ± 0.22, 1.00 ± 0.12) and body width (1.74 ± 0.29, 0.94 ± 0.12). The mouth depth angle was significantly higher in larvae (10 DAH) (85.63 ± 6.41°), which decreases as age increases. A total of 452 events were recorded, four behaviors were described: interaction (214 events, 47 attacks, effectiveness of the attack (EA) 21.96%), chasing (127 events, 44 attacks, EA 34.64%), escape (62 events), and fast swimming (47 events). Three types of attacks were recorded: frontal (41.24%), lateral (29.94%), and posterior (28.81%), with three attack regions: head (31.64%), body (10.72%), and tail (57.63%). The most frequent attack was posterior tail with 70 events (39.55%). Attacker presented a S-like curvature prior to the attack (30.50%). Differences were determined in the percentage of weight (g) and total length (cm) between the attacker larvae and the attacked larvae 16.39 ± 10.86% and 15.23 ± 5.68%, respectively. In conclusion, *A. tropicus* larvae show cannibalism Type I, II, and two variants of Type III. It is suggested that this species is a more efficient cannibal than an interspecific predator. The relation of the greater number of attacks in the white color tanks could be related to the contrast with the bottom, thus, there is a less preference for this color. This information is essential to carry out a more efficient sorting of the larvae during their culture and reduce cannibalism in the larval stage in *A. tropicus*. The results of this study could be useful to understand this behavior in other species.

1. Introduction

Cannibalism is defined as the act of killing and ingesting, either completely or partially, an individual of its own species (Pfennig, 1997; Smith and Reay, 1991). In fish, around 390 species have been documented that show some type of cannibalism and of these, only 150 species have shown this behavior in captivity; most of the time it occurs throughout its life and in others only in the early stages of development

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(larval and/or juvenile) (Pereira et al., 2017). Seven classifications of cannibalism in fish have been reported based on the relationship between the prey and the cannibal, grouped into three criteria: development of the prey (e.g., eggs cannibalism), genetic relationship between prey-cannibal (e.g., filial and sibling cannibalism), and finally, the relationship by age of the prey and the cannibal (e.g., intracohort and intercohort cannibalism) (Smith and Reay, 1991). There are three types of cannibalism depending on the intake of organisms: a) Type I, the cannibal is smaller than the prey, b) Type II, the cannibal is larger and can fully ingest the prey, and c) Type III, several cannibals dismember the prey and consume it (Baras et al., 2000; Baras and Jobling, 2002; Baras, 2013; Cuff, 1980). The works focused on the description of the cannibal behavior in fish, allows to know the different interactions between prey and the cannibal during a cannibalism event, the size preference to consume, the type of attack, the percentage of attack effectiveness, the predation cycle, the prey tracking time. In addition, the relationship between energy expenditure and applications of the optimal foraging theory have also been studied to understand this behavior and generate mitigation strategies (Baras et al., 2014; Colchen et al., 2019; Rao, 2003).

Cannibalism is a behavior that usually causes high mortality rates in fish, particularly during the larval period (Krol and Zieliski, 2015). Consequently, this behavior affects the economic profitability of the aquaculture industry and generates a reduction in animal welfare, the lacerations that this behavior triggers in farming systems and different biotic and abiotic factors (genetic background, sex, hatching, size of larvae, density, animal management, feed, light conditions, temperature, color, shape of tanks, and refuges) have been identified as precursors (Naumowicz et al., 2017). For example, the used of green tank color is related to the increase of mortality by cannibalism in orange spotted grouper (*Epinephelus coioides*) (Takeshita and Soyano, 2008). In Dorada larvae (*Brycon* sp.) blue tanks shows a greater cannibalism and less survival (17.08% and 66.25%) (Costa et al., 2013). The implementation of shelters (polyvinyl chloride pieces) or enriched environments (plastic tube and plastic shredding) can decrease stress and aggressive behaviors in Atlantic salmon (*Salmo salar*) and juvenile Barramundi (*Lates calcarifer*) showed benefits in fish culture (Näslund et al., 2013; Qin et al., 2004). Moreover, in Jundiá (*Rhamdia quelen*) the correct use between tank color and the amount of shelters could result in decreasing stress beahvior (Barcellos et al., 2009) Also, in orange-spotted grouper (*Epinephelus coioides*) and giant grouper (*Epinephelus lanceolatus*) the use of morphometric measurements (mouth width, body depth, and total length) and the models development (linear regression) suggests establishing a management of the larvae as a measure preventive to cannibalism (Hseu et al., 2003; Hseu et al., 2004).

The tropical gar (*Atractosteus tropicus*) inhabits in Central American countries and in southeastern Mexico and is considered a species of great economic and cultural importance in the region (Márquez-Couturier and Vázquez-Navarrete, 2015; Nelson, 2006). However, one of the main limitations for the culture of this species is the cannibalism present in the larval and juvenile stages, both in the wild and in captivity (Aguilera et al., 2012; Márquez, 2000). Survival in these stages can be from 1 to 33% due to cannibalism (Palma-Cancino et al., 2019). In *A. tropicus*, ethological information is limited, there is only one study describing the observation of encounters, attacks, captures, ingestions, and rejections (Escalera-Vázquez et al., 2018). Although there are several studies that point out the problems that cannibalism generates on the productive and survival values of the species (Frías-Quintana et al., 2017; Jiménez-Martínez et al., 2020), there is no work that addresses the ethology and description of cannibal behavior in tropical gar.

The objective of this work was to determine, identify and characterize the type of attack behavior before cannibalism in Tropical gar (*A. tropicus*) larvae, describing attacked-attacker relationships also, to evaluate the effect of tank colors, the use of artificial shelters, and the morphometric changes.

2. Material and methods

2.1. Biological material

This work was carried out at the facilities of the Aquatic Resources Physiology Laboratory (LAFIRA) at the División de Ciencias Biologicas de la Universidad Juárez Autónoma de Tabasco (DACBiol-UJAT), using an *A. tropicus* broodstock from the laboratory. One female and three males were placed in a 2000 L capacity circular tank. The female was induced to spawn by an injection of LHRHa (30 μg/Kg) and spawning 17 h after injection.

2.2. Care and maintenance of the stock of larvae

For this study, larvae were placed in three tanks of 70 L connected to a RAS (recirculated aquaculture system), a 0.5 HP water pump (Jacuzzi, JWPA5D-230A, Delavan, WI), and a 1500 L reservoir for solids deposition and a biological filter. Feeding started at 3 DAH with a co-feeding (diet and *Artemia* nauplii) for five days; the diet used was the proposed by Frías-Quintana et al. (2016) (44% protein, 14% lipids), manufactured following Álvarez-González et al. (2001). Diet was grounded until reaching a desire particle size (co-feeding 0.5 mm, weaning 0.7 mm) for larvae. Larvae were fed four times per day (8:00, 12:00, 16:00, 20:00 h) ad libitum. Water quality variables like temperature (27.72 ± 0.7 °C) and dissolved oxygen (4.9 ± 0.2 mg/L) were checked using a multiparameter (YSI 85; OH), and pH (7.1 ± 0.1) was checked using a pH-meter (HANNA HI 991001, Romania) every week.

2.3. Experimental design

In this study, a total of 362 larvae were used (0.022 ± 0.003 g mean weight and 1.52 ± 0.10 cm total length). Four bioassays were evaluated simultaneously to identify cannibalistic behavior in larvae of *A. tropicus*. The bioassays allowed to evaluate the following factors: a) the effect the tank colors bottom by using nine colors (red, dark blue, purple, green, white, orange, blue, pink, and yellow) distributed randomly (modified from Sallehudin et al., 2017); b) the absence or presence of shelters, where the presence of rocks and artificial vegetation was evaluated. Both factors (bottom tank color, and shelters) were evaluated in pairs and in groups: 1) pairs, two larvae were placed by tank (15 × 10 × 8 cm) (11 replicates, 22 different larvae per color), in which the nine colors were evaluated (Fig. 1a), and the presence of shelter using artificial rocks and vegetation by separate (11 replicates, 22 different larvae per shelter type) (Fig. 1b); 2) groups, ten larvae were placed by tank (30 × 30 × 10 cm) (by triplicate), which had the nine colors in the tank bottom (30 larvae) (Fig. 1c) and 30 different larvae were used for shelter preference as described above but with artificial rocks and vegetation (Fig. 1d). Larval color preference was determined by counting the total time (seconds) of presence by larvae in each color in the recordings (15 min, by triplicate); the preference of the larvae for the shelter was determined by counting the total time (seconds) of presence by larvae in each shelter in the recordings (15 min, by triplicate), placing them with a medium distribution towards one end of the tanks (Fig. 1d). To carry out the bioassays, the larvae were randomly collected from the stock tanks and placed in the corresponding tanks and remained 30 min for acclimatation before starting each experiment.

The morphometric relationships between the size of various structures of the body of *A. tropicus* at different ages was evaluated to determine its relationship to cannibalistic behavior. For this, 30 organisms of four different stages were used: larvae (4 DAH), larvae (10 DAH), juveniles (150 DAH) and adults (1 year age and 187 DAH) from the LAFIRA stock. The organisms were anesthetized with clove oil (0.1 mL/L) to avoid injuries. To determine anatomical measurements in larvae, a stereoscopic microscope was used (Carl Zeiss mod. Stemi DV4, Germany) and a micrometer to determine the scale, while an ichthyometer was used for juveniles and adults. At all ages, the organisms were

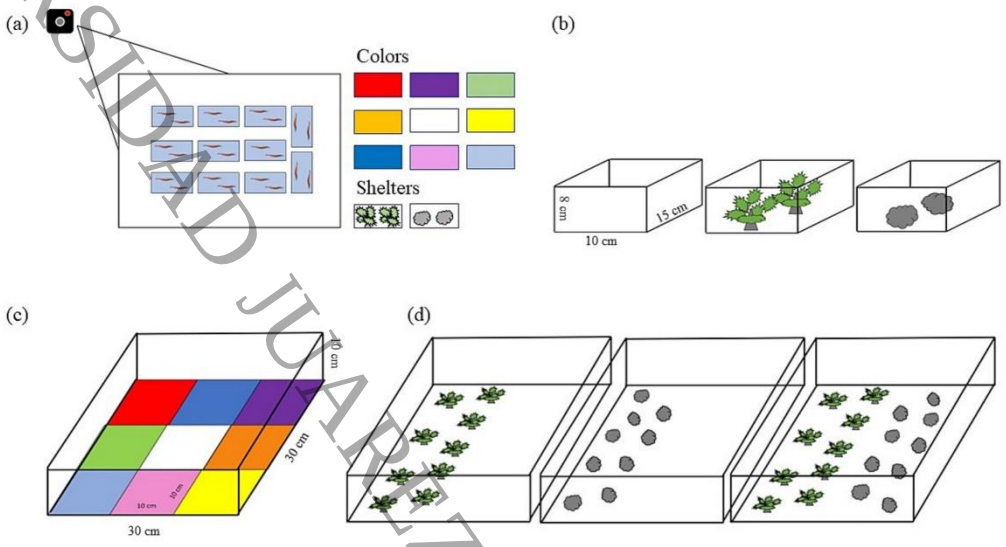

Fig. 1. Description of the fish tanks and experiments: (a) colors, shelters, and distribution of fish tanks for individual challenges, (b) individual tank dimensions and tanks with rocks and artificial vegetation, (c) group fish tanks with nine colors in the bottom, (d) challenges with artificial vegetation, rocks, and both as shelters in group tanks.

photographed, and measurements were made using the software ImageJ 1.51j8 (U.S. National Institutes of Health, Bethesda, MD). Thirteen measurements were made for each organism (cm): 1) total length, 2) standard length, 3) body depth, 4) eye diameter, 5) body width, 6) width between eyes, 7) head length, 8) length mouth to eyes, 9) mouth width, 10) mouth depth, 11) length upper jaw, 12) length lower jaw, 13) mouth

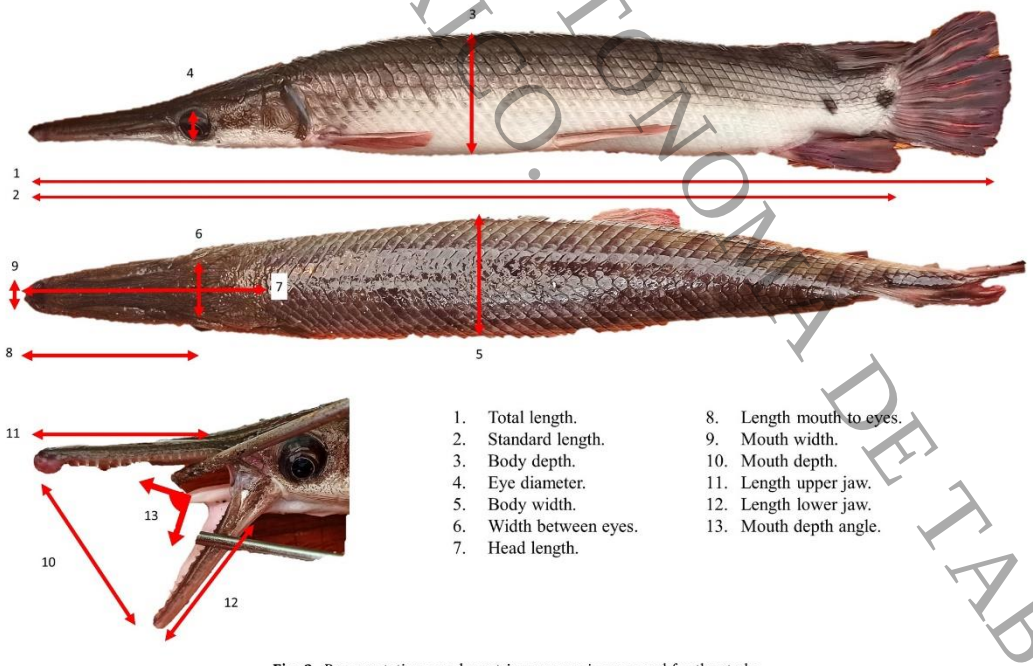

Fig. 2. Representative morphometric measures images used for the study.

depth angle (Fig. 2). Moreover, we analyzed the morphological ability of the organisms from different development stage to swallow a sibling with the following formula mouth depth (cm) / body depth (cm) (MBD), mouth depth (cm) / body width (cm) (MBW).

2.4. Behavioral quantification

The larvae in each bioassay were recorded for 15 min with two video cameras (GoPro Hero4 and GoPro Hero7 Silver, China). The software Openshot 2.5.1 (OpenShot Studios, LLC) and Lightworks 2021.1 were used (LWKS Software Ltd) for video edition. For the observation and behavior analysis of the larvae, Boris (Friard and Gamba, 2016) and TRACKER 5.1.5 (Copyright: Douglas Brown, Wolfgang Christian, Robert M Hanson) software were used. With the same recorded material, an analysis of aggressive behavior was carried out, by observing and counting the attacks and recording and counting the following actions related to aggressivity and cannibalism: origin and position of the attack, region of preference for the attack, behavior. Attacks that do not come from an identified behavior were classified as "random" attacks. Houde's description was used (Houde, 1972; Houde, 2001) to identify the changes in position type "S" in larvae.

The attacker larva (cannibal) and the attacked larva (prey) were counted, separated, weighted, and sized at the time of the attack. The individual weight of each organism was recorded using an analytical balance (A&D Company, Limited mod.HR-250 AZ, Korea). These organisms were photographed in a container with a transparent bottom and a scale (graph paper, 0.01 mm) using a stereoscopic microscope (Carl Zeiss mod. Stemi DV4, Germany). The photographs were analyzed with the software ImageJ 1.51j8 (U.S. National Institutes of Health, Bethesda, MD) to determine total length of larvae. We used the classification of Baras et al. (2000); Baras and Jobling (2002); Baras (2013); and Cuff (1980) to identify the type of cannibalism from *A. tropicus* larvae. Capture was defined when the cannibal attacks, bites, holds, and partially engulfs the prey.

2.5. Statistical analysis

The total time (seconds) of presence by larvae in each color, the total time (seconds) of presence by larvae in each shelter, and the number of attacks were tested for normality (Kolmogorov–Smirnov) and homoscedasticity (Barttlet) tests, respectively. Kruskal-Wallis test was carried out for bottom color and shelter preference analysis in the grouped experiment. In pair bioassays, one-way ANOVA was carried out for number of attacks in each color and shelter, a posteriori test of unequal N HSD (Tukey) was used. A student's *t*-test was used to compare the differences in weight and total length between attacker and attacked larvae. Furthermore, a principal component analysis (PCA) was used for the analysis of the 13 morphometric measurements as these changes with age.

Additionally, according to Van Snik et al. (1997) a log10 linearization was performed using the morphometrics relations: 1) total length, 2) standard length, 10) mouth depth, 11) length upper jaw, 12) length lower jaw for the comparison of 4 and 10 DAH larvae; and the log10 linearization of the morphometrics relations: 1) total length, 2) standard length, 4) eye diameter, 9) mouth width for the comparison of juveniles and adults through a covariance analysis (Zar, 1999). All tests were performed using the software Prism V. 9.0 with a significance value of 0.05.

3. Results

3.1. Description of attacks

Three types of attacks were identified according to the position and origin of the attack between the larvae: 1) Frontal, 2) Lateral and 3) Posterior. In addition, there were three regions of preference for the attack: a) Head, b) Body, and c) Tail. The attacks identified as Posterior recorded a total of 73 events (41.24%), followed by Lateral with 53 (29.94%), finally Frontal with 51 (28.81%). The zone with higher preference was Tail with 102 events (57.63%), Head with 56 (31.64%) and finally Body with 19 (10.72%). The most frequent attack was Posterior Tail with 70 events (39.55%). Of the 177 registered attacks, in six of them, the attacker fish captured the other fish. In the Posterior Tail attack three captures were recorded, followed by two captures in the Frontal Head attack, and finally only one capture in the Lateral Tail attack. Three captures were registered after an interaction and three more after a Chasing (Table 1).

3.2. Color and aggressive behavior

In the individual tests, two larvae were placed per tank with a specific background color (red, dark blue, purple, green, white, orange, blue, pink, and yellow). The highest average number of attacks was recorded in fish tanks with a white bottom (8.50 ± 0.70 attacks), with a significant difference from all other colors ($p < 0.05$). It continued in number of attacks on the red and dark blue bottoms with 5.50 ± 0.70 and 4.00 ± 1.41 respectively. The number of attacks on green and pink bottoms (1.33 ± 1.15), orange and blue (1.00 ± 1.00), did not show any difference between them. The lowest number of attacks were recorded in yellow and purple fish tanks bottom with an average of 0.66 ± 0.57 (Table 2). Only one capture was recorded on the red tank bottom with a

Table 1
Attack types and captures observed in Tropical gar (*A. tropicus*) larvae.

Attacks		Description	Events (#)	Percent (%)	Captures (#)
Frontal	Head	Both fish are facing each other, one bites the head of the other.	50	28.25	2
	Body	Both fish are facing each other, the attacker swims slightly over the other fish, turns and bites in the middle of the body.	1	0.56	–
Lateral	Head	The attacker fish is perpendicular to the other fish, attacking the lateral side of the attacked fish biting the head.	6	3.39	–
	Body	The attacker fish is perpendicular to the other fish, attacking from the side of the attacked fish biting the head.	15	8.47	–
	Tail	The attacker fish is perpendicular to other fish, and attacks by the side of the attacked fish, biting the caudal fin or peduncle of the fish	32	18.08	1
Posterior	Body	The attacker fish is behind the other fish, increases swimming speed, and attacks by biting the middle of the other fish	3	1.69	–
	Tail	The attacker fish is behind the other fish, approaches, and attacks by biting the caudal fin or the peduncle of the fish	70	39.55	3
Captures		The attacker fish bites and holds the prey fish for a certain time	6	3.38	–

Table 2
Effect of bottom color and shelters in relationship with attacks in Tropical gar (*A. tropicus*) larvae.

Colors	Red	Dark blue	Purple	Green	White	Orange	Blue	Pink	Yellow
Pairs									
Attacks (mean ± *SD*)	5.50 ± 0.70^{b}	4.00 ± 1.41^{bc}	0.66 ± 0.57^{d}	1.33 ± 1.15^{cd}	8.50 ± 0.70^{a}	1.00 ± 1.00^{d}	1.00 ± 1.00^{d}	1.33 ± 1.15^{cd}	0.66 ± 0.57^{d}
Group									
Total Attacks (%)	5(9.25%)	5(9.25%)	2(3.70%)	4(7.40%)	7(12.96%)	7(12.96%)	8(14.81%)	8(14.81%)	8(14.81%)
Shelters									
Pairs	Rocks				Artificial vegetation				
Attacks (mean ± *SD*)	7.00 ± 1.41				5.00 ± 1.41				
Group									
Total Attacks (%)	8 (61.53%)				5 (38.46%)				

Significant differences are indicated by different letters ($p < 0.05$).

posterior tail-type attack. Six types of attacks were identified, the most frequent was Posterior Tail (46.29%), followed by Frontal Head (22.22%), Lateral Tail (16.66%), Lateral Head and Lateral Body (5.55%) and Posterior Body (3.70%). In the pairs of larvae tests, the type of attack Body Head was not observed.

The total time of presence by each larvae in the different colors is shown in Fig. 3a. The highest preference was recorded in tanks with a yellow bottom (30.88%), with significant difference over the remaining colors ($p < 0.05$) except for blue (18.89%), followed by red (11.13%), purple (9.49%), pink (8.20%), green (8.14%), dark blue (6.44%) colors tanks bottom. The lowest preference was recorded in the white bottom tank color (1.60%) with no significance difference ($p > 0.05$) with orange bottom tank color (5.23%) (Fig. 3b). The highest number of attacks were recorded for blue, pink, and yellow bottom tank color with eight events each (14.81%), followed by white and orange bottom with seven attacks each (12.96%), red and dark blue bottom with five attacks each (9.25%), green bottom with four attacks (7.40%) and finally, purple bottom with two attacks (3.70%) with no significance difference ($p > 0.05$) (Table 2). Of these 54 attacks, six attack types were identified, distributed as follows: the type of attack with the highest record was Posterior Tail with 26 events (48.14%), followed by Frontal Head attack with 15 (27.77%), and Lateral Tail with nine (16.66%), Lateral Body only registered two events (3.70%) and finally only one event for frontal Body and Posterior Body attack (1.85%). Of all the attacks, only two captures were registered, one in the red color bottom (Lateral Tail) and one for the white color bottom tank (Posterior Tail).

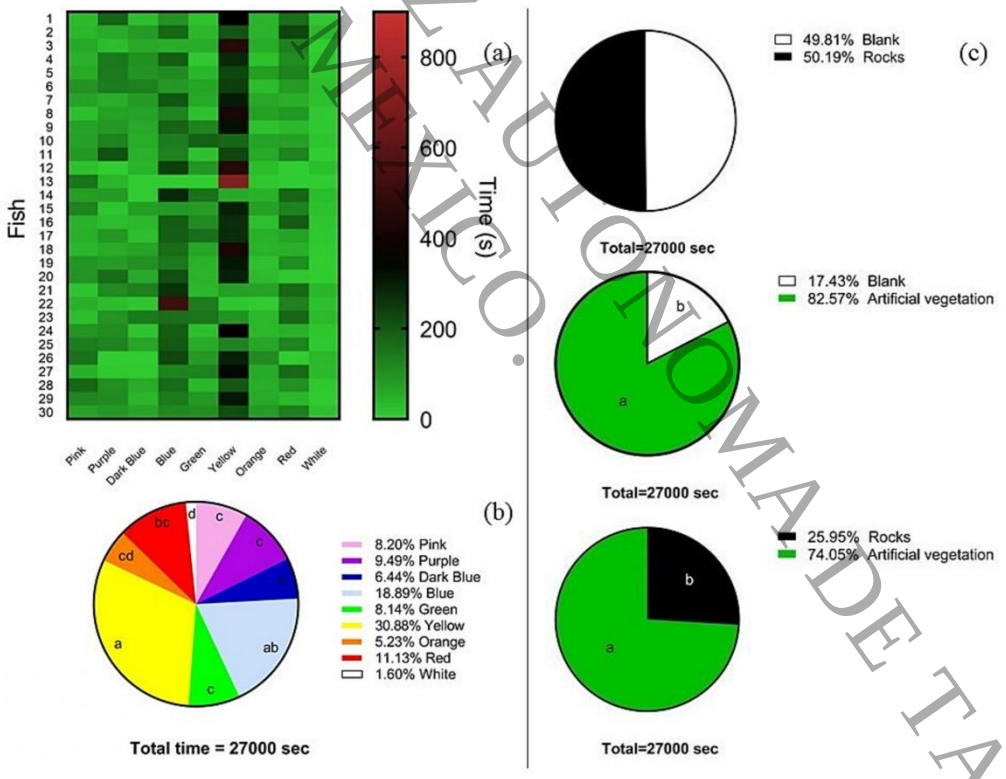

Fig. 3. Bottom color tank and shelters preference of Tropical gar (*A. tropicus*) larvae.

3.3. Refuges and aggressive behavior

In pairs fish tanks with rocks, there were 7.00 ± 1.41 attacks in average. In fish tanks with plastic vegetation, 5.00 ± 1.41 average attacks were recorded, however, there was no significant difference regarding the use of rocks ($p > 0.05$) (Table 2). The presence of shelters also influenced the number of types of attacks. In fish tanks with rocks, five types of attacks were identified: Frontal Head, Posterior Tail, and Lateral Head, Body, and Tail, and in fish tanks with plastic vegetation, there were only three types of attacks: Frontal Head, Lateral Body, and Posterior Tail.

For group tanks test there was no significance difference between preference for rocks shelter (50.19%) and empty side of tank (blank) (49.81%) ($p > 0.05$). Although, the preference for artificial vegetation shelter was (82.57%) was higher to the preference of the blank (17.43%) ($p < 0.05$). Finally, when comparing the preference between artificial vegetation shelter (74.05%) and rocks shelter (25.95%), the highest preference was found in artificial vegetation with significance difference ($p < 0.05$) (Fig. 3c). In the case of attacks, the highest number was found in the tanks with rocks shelter (61.53%) compared with artificial vegetation shelter (38.46%) with no significance difference ($p > 0.05$) (Table 2). Only four types of attacks were recorded, the most frequent was Frontal Head with six (46.15%), followed by Posterior Tail and Lateral Tail both with three (23.07%) and Lateral Head with one (7.69%).

3.4. Morphometric relationship

The morphometric relationships present at different ages in *A. tropicus* allowed to correlate the appearance of cannibalism with the anatomical measurements at different development stages. The MBD relationship with respect to the four stages and cannibalism was greater between the juveniles with 1.59 ± 0.22. Which indicates that anatomically, these fish can eat 1.59 ± 0.22 times the size of its body depth. The larvae (10 DAH) have the anatomical capacity to consume 1.00 ± 0.12 their body depth. Larvae (4 DAH) and adults are anatomically incapable of consuming its body depth (0.47 ± 0.09 and 0.88 ± 0.13 respectively). The tendency is similar in the MBW, where juveniles are capable of consuming 1.74 ± 0.29 times its body width, in larvae (10 DAH) and adults, this ability decreased, 0.94 ± 0.12 and 0.90 ± 0.13, larvae are only capable of consuming 0.49 ± 0.10 times its body width. Regarding the mouth opening angle, this was significantly higher in larvae (10 DAH) with 85.63 ± 6.41°, the larvae presented an angle of 73.25 ± 6.50°, juveniles of 63.44 ± 5.56° and adults 54.88 ± 5.79°. In the analysis of main components (PCA) (Fig. 4), two groups were observed delimitated by the age of the organisms. The morphometric relationships 1 vs 10, 1 vs 11, 1 vs 12, 2 vs 10, 2 vs 11 and 2 vs 12 for larvae (4 and 10 DAH), while the relationships 1 vs 4, 1 vs 9, 2 vs 4, and 2 vs 9 are grouped into juveniles and adults.

As a complement of the analysis, the linearization of the morphometric relation rate from the variables: 10) mouth depth, 11) length upper jaw, 12) length lower jaw allows to confirm that larvae of 4 and 10 DAH showed differential rates ($p < 0.05$) in relation with the variables 1) total length and 2) standard length (Fig. 5). Regarding juveniles (150 DAH) and adults (552 DAH) the linearization of the morphometric relation rate from the variables: 4) eye diameter and 9) mouth width allows to confirm that juveniles and adults showed differential rates ($p < 0.05$) in relation with the variables 1) total length and 2) standard length (Fig. 6).

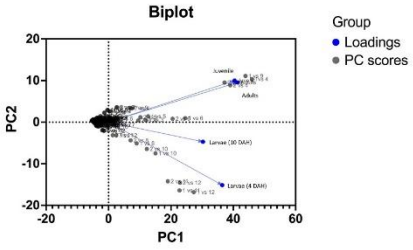

Fig. 4. Principal Component Analysis (PCA) of morphometric relationships in four different stages of Tropical gar (*A. tropicus*).

3.5. Identification and quantification of behavior types

A total of 62 recordings were obtained with a total time of 15.96 h. From these recordings, four types of behaviors were identified among the *A. tropicus* larvae, which were described as: 1) Interaction, 2) Chasing, 3) Escape, and 4) Fast Swimming (Table 3). A total of 452 events were recorded, of which Interaction had the largest number of records with 214 events, followed by Chasing with 127, Escapes with 62 and finally Fast Swimming with 49 events. A total of 177 attacks were recorded of which 47 (26.55%) of them occurred during Interaction and 44 (24.85%) attacks were recorded at the end of Chasing. The rest of the attacks (86, 48.58%) did not show a pattern to adjust to our classification, were named as random attacks. Of the total events registered for Interaction, only 21.96% ended in attack, 64% Chasing events ended in direct attacks (Table 3). The average duration in the Interaction was 4.6 ± 1.77 s, which maximum and minimum duration was 8 and 3 s. The average duration of Chasing was 35 ± 28 s, where there was a maximum duration of 201 s and a minimum of 4 s.

Another behavior identified in cannibal larvae was the change in body position before each attack and capture made. Moments before the attack, the cannibal larva picks up its caudal fin resembling a letter "S". Of the 177 recorded aggressions, the change in the position of the cannibal's body was identified on 54 occasions (30.50%) and registered in five of the six catches (83.33%). The average duration from cannibal capture to release was 204.5 ± 207.9 s, being the maximum 571 s, and minimum 2 s. There was no significant difference between the duration of the capture and the type of attack by the number of events that were registered ($p > 0.05$).

3.6. Characterization of cannibal behavior in A. tropicus

The attackers (15 larvae) registered an average weight of 0.032 ± 0.001 g and the attacked (15 larvae) 0.027 ± 0.0008 g (mean ± SEM) ($p < 0.0042$). For average length, attackers showed 2.092 ± 0.025 cm and the attacked 1.772 ± 0.036 cm (mean ± SEM) ($p < 0.0001$). The average difference in weight and total length between attacker and the attacked larvae was 0.005 ± 0.004 g and 0.32 ± 0.123 cm (mean ± SD), which corresponds to a difference of 16.39 ± 10.86% in weight and 15.23 ± 5.68% in length (Fig. 7). It was identified that the larvae of *A. tropicus* predominantly presented a type II cannibalism, followed by type I, and two records of possible variants of type III cannibalism: Variant I: a prey is eaten by two cannibals, one of them gobbling it by the head and the other by the tail, in this variant, two or more cannibals can participate, however, unlike type III, there is no dismemberment of the prey; in the end, only one cannibal consumes the prey and the other cannibals involved desist from the attack. Variant II (carousel shape): three organisms are involved, the prey is eaten by the tail by a cannibal, and in turn, this cannibal is simultaneously eaten by the tail by another cannibal (Fig. 8).

4. Discussion

4.1. Tank colors vs aggressive behavior

One of the strategies used to mitigate cannibalism and aggressiveness in fish larvae has been the use of different tank colors, with effects over

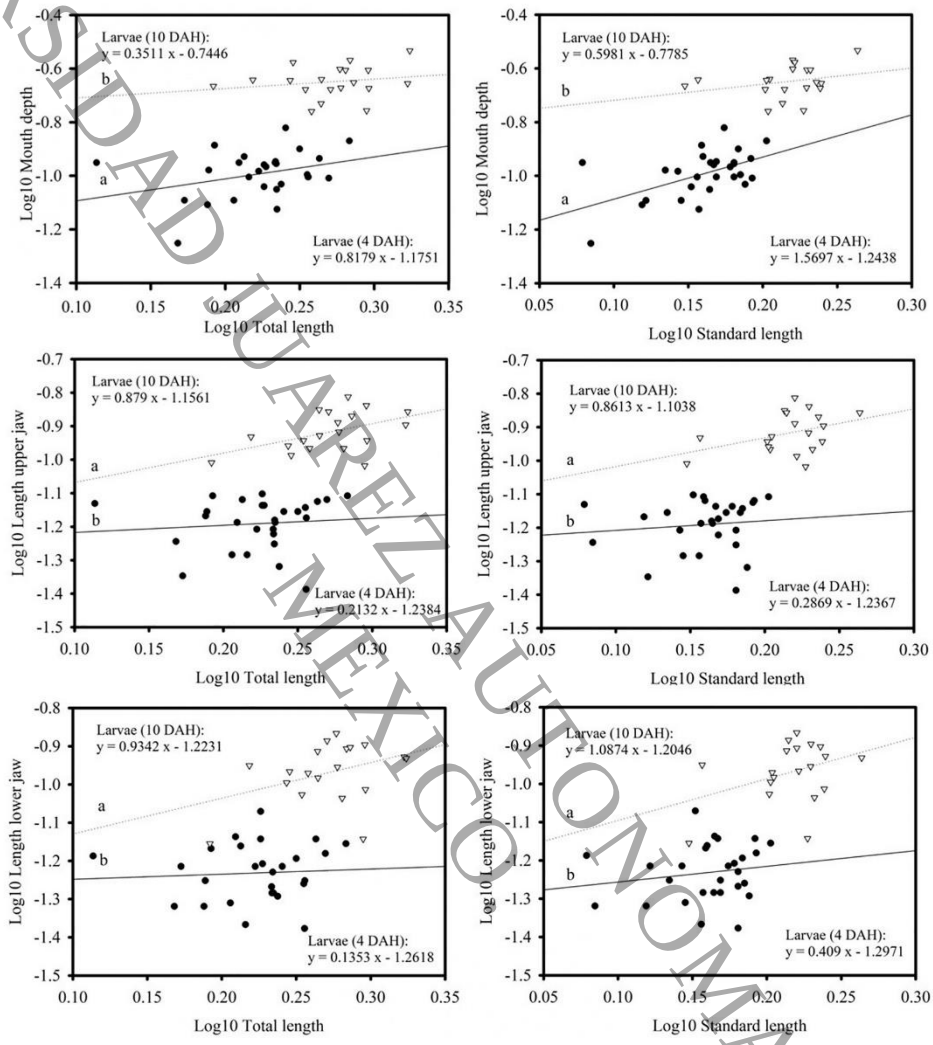

Fig. 5. Linearization of the morphometric relationship rate (mouth depth, length upper jaw, length lower jaw, total length, and standard length) in larvae (4 and 10 DAH). Black circles represent 4 DAH larvae. Triangles represent 10 DAH larvae. Significant differences within the larvae are indicated by different letters ($p < 0.05$).

survival, feed conversion ratio, health, growth, and body color (McLean, 2021). In the present study, different bottom colors tanks were used to determine the preference of *A. tropicus* larvae and to evaluate their effect on the number of attacks. Our results show that *A. tropicus* larvae grouped in pairs, present a greater number of aggressions on the white bottom tank, where the dark tone of the larvae contrasts with the white color, becoming more visible. This result coincides with the lowest percentage of preference for the white bottom color tank in the group tests. In contrast, the lowest number of aggressions was recorded on the purple bottom tank (the darkest color evaluated), as well as in the group tests. Our results showed a similar tendency related to the clear tone and aggressivity behavior as well as in juveniles of Arctic charr (*Salvelinus alpinus*) where the highest aggressivity was found in white tanks compared with black tanks (Höglund et al., 2002). Similar occurred in African catfish (*Clarias gariepinus*) juveniles, there was more aggressivity in white, blue, green, and red color tanks compared to the yellow color tanks (Sallehudin et al., 2017). Also, in Coho Salmon (*Oncorhynchus kisutch*), the highest aggressivity was found in white, blue, and grey color in contrast with a less aggressive behavior in black color (Gaffney et al., 2016). This tendency could be related to the increase of larvae density and to behavioral factors, social context by density effect or social hierarchies of the species (Castanheira et al., 2016; Polverino et al., 2016), this contrast with pairs evaluations where in the clear tone tanks, the attacks decrease (excluding white color), this could be related to the random selection of the larvae and its different sizes, where probably this differences did not motivate attacks. Also, more research is

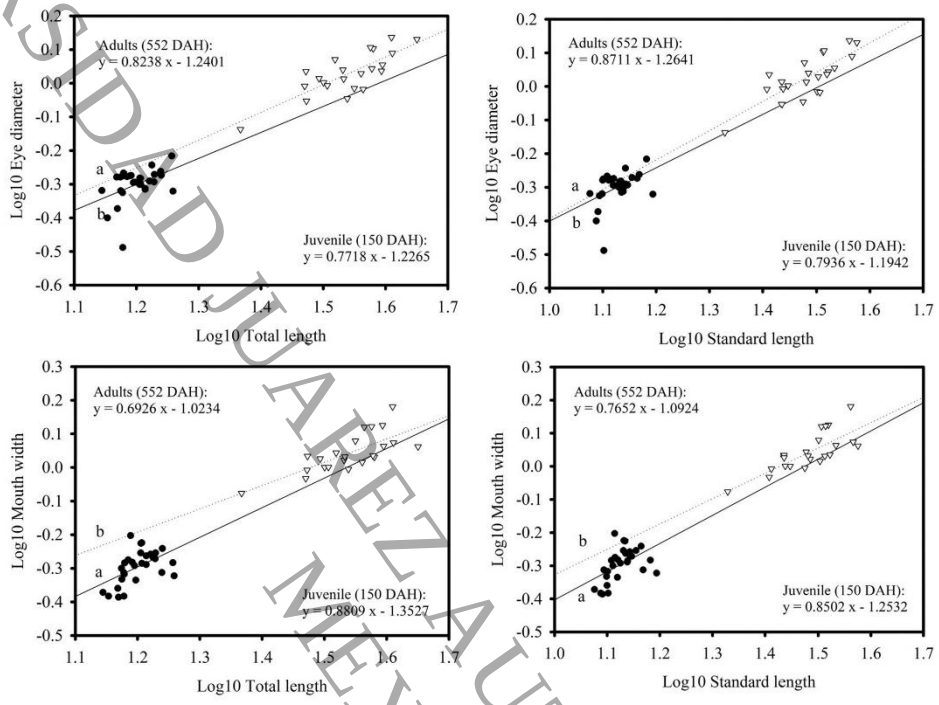

Fig. 6. Linearization of the morphometric relationship rate (eye diameter, mouth width, total length, and standard length) in juvenile (150 DAH) and adults (552 DAH). Black circles represent juvenile. Triangles represent adults. Significant differences within the larvae are indicated by different letters ($p < 0.05$).

Table 3
Behaviors, number of events and attacks observed in Tropical gar (*A. tropicus*) larvae.

Behaviors	Description	Events (%)	Attacks (%)
Interaction	Two fish with a frontal, lateral or posterior encounter, follow by swimming around and finally encounter breaks up.	214 (47.34)	Yes:47 (21.96) No: 167 (78.03)
Chasing	A fish follows another for several seconds, in some cases is captured and they have an interaction or attack.	127 (28.09)	Yes: 44 (34.64) No: 83 (65.35)
Escape	A fish participates in an interaction or comes from a chasing, escapes with a sudden fast swim.	62 (13.71)	No attacks
Fast swimming	A resting fish with no risk of being attack, generates an instant fast swim for a short period of time.	49 (10.84)	No attacks

need it on eye anatomy, light adaptation, colors, and movements perception could influence the number of attacks (Colchen et al., 2020).

4.2. Refuges vs aggressive behavior

In this study, we observed that the larvae of *A. tropicus* prefer the areas with artificial vegetation, and there are fewer attacks compared to the areas with rocks. To simulate natural environments (artificial vegetation) could foster a state of well-being and avoid aggressive behavior (Márquez-Couturier et al., 2015), or the degree of protection (refuge), which allows a greater coverage of defense, hiding the predator movements in comparison to rock refuges. Similar studies show the benefits of using artificial shelters, for example, the use of refuges decreased cannibalism in Barramundi juveniles (*Lates calcarifer*), where the chasing and biting frequency were reduced (Qin et al., 2004). Also, the use of plastic shelters in sharptooth catfish (*Cirrius garitpinus*) decreased cannibalism, but there was an increase in aggressiveness, probably due to territoriality of these species (Hecht and Appelbaum, 1988). And using a high-density shelter (8 per tank) had a direct effect on decreasing cannibalism in black rockfish (*Sebastes schlegelii*) frys (Xi et al., 2017). Although, our results showed a tendency to decrease attacks when using artificial vegetation, further studies are needed in relation to straight cannibalism, shelter density and distribution.

4.3. Relationship between morphometrics and aggressive behavior in A. tropicus

The information obtained regarding the morphometric relationships present at different ages in *A. tropicus*, allows to correlate the appearance of a possible cannibalism with the differences in anatomical measurements that allow a larvae or juvenile to consume a smaller sibling of its own cohort. A greater mouth opening angle in early stages means a greater number of possible preys to consume in the natural environment, which translates into a greater amount of feed obtained. However, it should be noted that the presence of cannibalism cannot be considered a bad thing from an ecological point of view, since it increases the chances

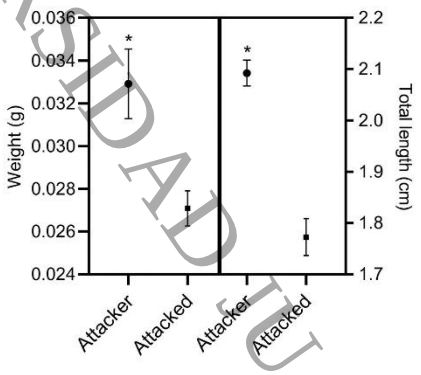

Fig. 7. Differences in weight (g) and total length (cm) between attacker and attacked larvae of *A. tropicus*. Values are mean ± SEM ($n = 30$).

of survival of the species by obtaining energy from the consumption of its siblings and increasing its growth rate (Gallagher and Dick, 2015). The high rate of cannibalism during the first days of ontogeny in dourado (*Salminus brasiliensis*) resulted in a 85.7 ± 3.8% and 97.7 ± 0.5% of mortality at 5 and 10 (DAH), this is related to the morphometry of the larvae, specifically the mouth size and relative shallow head/body depth, and in turn conditions the attack zone, preferring the caudal fin and changing to an attack on the head with increasing age (Ribeiro and Portella, 2020). The larvae of *A. tropicus* show a preference for attacks to the posterior zone. The Principal component analysis (PCA) of the morphometric relationships rate and the morphometric relation rate in *A. tropicus* indicate a change between the different measurements at the four ages. The relationship between total length, standard length, eye diameter, mouth depth, mouth width, upper and lower jaw length and their interaction show changes in the morphology of larvae with respect to juveniles and adults. These changes may be determined by two primary needs in the larvae: the ability to feed (development of structures in the head) and the development of mobility (growth of the tail) as methods of defense against possible predators (Osse et al., 1997). In the case of barramundi larvae (*Lates calcarifer*), although cannibalism is attributed to a difference between sizes, also the allometric growth of the mouth regarding body depth defines cannibal behavior in early stages (Ribeiro and Qin, 2013). Growth patterns are related to morphological characteristics in allometric or isometric growth (Van Snik et al., 1997) In *A. tropicus* larvae the changes in the morphometric relationships due to allometric or isometric growth can generate specific morphological conditions (MBD and MBW) for cannibalism to take place. Therefore, delimiting until what age the larvae or juveniles have the morphological capacity that facilitates cannibalism, can be useful for the management of organisms.

4.4. Aggressivity of A. tropicus larvae

One aspect to highlight in this study is the application of tools to describe and analyze cannibalistic and aggressive behavior in *A. tropicus*. In pikeperch (*Sander lucioperca*) seven aggressive and cannibalistic behaviors were recorded: Orientation, Approach S-shape, Attack without an S-shape, Attack after an S-shape, Capture, and Pursuit. In addition, there was a change in the preference of prey ingestions with respect to time, being tail-first >60%, after the 48 dph, head-first increased (Colchen et al., 2019). In this work with *A. tropicus*, we observed the interaction and chasing were the conducts that show attacks, and the most common was Posterior Tail. Regarding aggressive behavior, this has been reported in Amazonian fish (*Astronotus ocellatus*) (Gonçalves-de-Freitas and Mariguela, 2006), African cichlid (*Neolamprologus pulcher*) (Sopinka et al., 2009), and zebrafish (*Danio rerio*) (Zabegalov et al., 2019). In relation to cannibalism, this has also been reported on pikeperch (*Sander lucioperca*) (Colchen et al., 2019), and

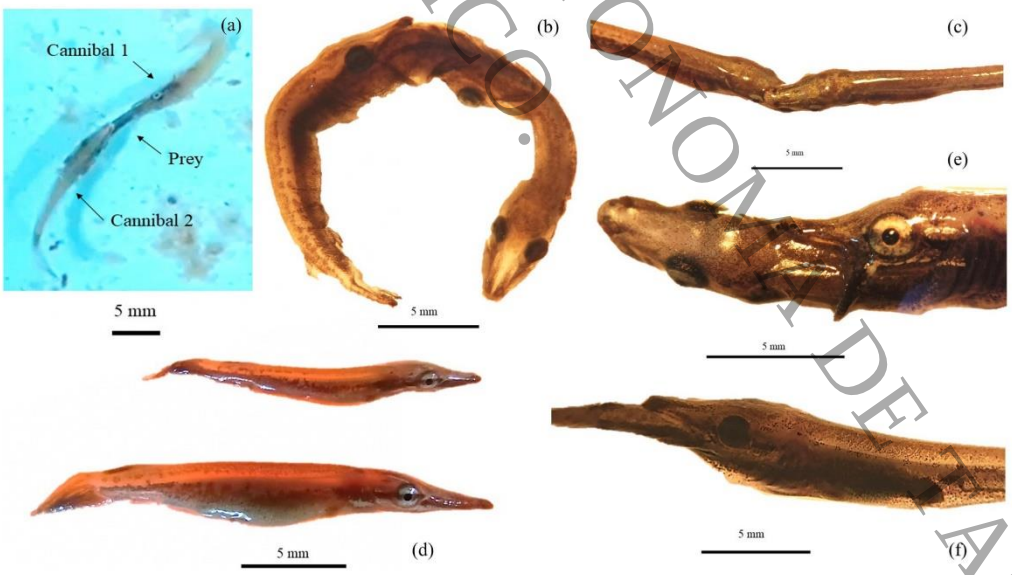

Fig. 8. Representative images of cannibalism in Tropical gar (*A. tropicus*) larvae: (a) cannibalism type III, variant I: a attacked is eaten by two attackers at the same time, the first one from the head and the second one from tail, (b) cannibalism type III, variant II: three or more larvae practice cannibalism, (c) frontal capture, (d) weight ant total length differences between a attacked and an attacker larvae, (e) posterior capture, partially eaten, (f) frontal capture, partially eaten.

matrinxã (*Brycon amazonicus*) (de Souza et al., 2014). However, even though approximately at least 390 species that present some types of cannibalism are registered (Pereira et al., 2017), there are still few ethological studies, which makes it difficult to understand the behavior, to subsequently try to mitigate it, specifically in *A. tropicus*, such behavior had not been previously described.

It has been described that, in fish with aggressive, certain movement patterns, body position or some other behavior can be observed in direct relation to this behavior. Some fish use hunting strategies that allow them to get their feed more effectively, while other fish show changes in body posture prior to an aggressive event. Fukuhara (1986) reported that the larvae of Japanese flounder (*Paralichthys olivaceus*) take two positions: Omega (Ω) and S-flex. The name of Omega (Ω) posture is due to the similarity that fish adopt with this form. This posture has also been reported in spotted halibut (*Verasper variegatus*) (Sabate et al., 2008), matrinxã (*Brycon amazonicus*) (de Souza et al., 2014), and is directly related to aggressiveness (Sakakura, 2006). On the other hand, the "S" type movement is another example of the change in posture prior to an attack. This change in posture of the attacking fish is characterized by its similarity to the shape "S", by which, it attacks the prey with a sudden and violent swim propelled by its tail (Houde, 1972, 2001). This type of movement has been identified in scaled sardine (*Harengula pensacolae*) (Houde and Palko, 1970), Pacific mackerel, (*Scomber japonicus*) (Hunter and Kimbrell, 1980) and pikeperch (*Sander lucioperca*) (Colchen et al., 2019). In this study, it was observed that the larvae of *A. tropicus* adopted the posture in the form of "S" in the recorded attacks.

Regarding the characterization of the type of cannibalistic behavior, our results showed that *A. tropicus* presents cannibal behavior type II, I and two variants of type III according to Baras et al. (2000), Baras and Jobling (2002), Baras (2013), and Cuff (1980) (Fig. 7). In a previous work, Escalera-Vázquez et al. (2018) propose that *A. tropicus* exposed to different live prey (*Artemia fransiscana*, *Daphnia pulex*, and *Moina macrocopa*), becomes an active predator as a carnivorous fish, and can adapt to choose other types of prey of a wide variety of sizes, showing an affinity for *Artemia*. Despite the good acceptance and availability of *Artemia* nauplii (Frías-Quintana et al., 2010; Palma-Cancino et al., 2019) as feed for *A. tropicus* (Escalera-Vázquez et al., 2018), is very common to find cannibalism in the specie, we assume that *A. tropicus* larvae could be more efficient cannibal that interspecific predator (Byström et al., 2013) due to the high number of attacks that could result in cannibal events.

Several reports describe that the difference between length/weight can trigger the presence of cannibalistic behavior. For example, in African catfish (*Heterobranchus longifili*), cannibals select undersized prey (close to optimal prey size) (Baras et al., 2014). Atlantic cod (*Gadus morhua*) showed cannibalism when the body length ratio was 1:1.5 (Folkvord, 1992). In the larviculture of giant grouper (*Epinephelus lanceolatus*) was recommended to avoid the length differences between prey and cannibal, a difference in length over 30% increase the probability to show cannibalism (Hseu et al., 2004). Black rockfish (*Sebastes schlegelii*) fry showed a relationship between cannibalism rate and the difference in length, when the difference is <25 mm, the cannibalism rate was 0.4 and when the size difference was increased (35 mm), the cannibalism rate was over 0.8 (Xi et al., 2017). Regarding *A. tropicus* larvae, our results showed the presence of cannibalism when there is a difference in weight and length present between cannibals and prey; this allows the sorting of organisms of different sizes, as a preventive measure to avoid or control cannibalism in production systems (Márquez-Couturier et al., 2015).

The difference in the percentages of weight and size presented by cannibals and prey on the initial day (10 DAH) of the presence of cannibal behavior, added to what was reported by Burggren et al. (2016), where juvenile fish of *A. tropicus* that have a size >3.0–4.0 cm can present a double difference in wet body mass caused by problems both for feeding and assimilation. This increase in wet body mass may be related to cannibalism, specifically due to the "jumper" effect, jumper cannibals, which increase their weight and size more quickly due to the action of eating their sibling (Baras et al., 2011), further increasing the variability between sizes and wet body mass within a cohort, which can translate into a considerable affectation of survival (Abdulraheem et al., 2012; Okeke, 2014). Therefore, one way to prevent cannibalism is the sorting of "jumper" organisms from culture tanks (Ibrahim and Naggar, 2010; Prinsloo et al., 1989).

Regarding our results in *A. tropicus* for MBD, MBW, and mouth depth angle, these morphometric factors can delimit the size preference of possible preys or conspecifics (Sabatés and Saiz, 2000), a range of preference that can vary according to age and morphometric changes. Aspects that may be important in larviculture for the administration of artificial diets or live feed for the stage and species.

Finally, Dong and Polis (1992) mentioned that the presence of cannibalism may be related to feed bioavailability and energy expenditure when hunting or feeding (optimal foraging theory). It has been observed in some species that in cannibal individuals, the feed conversion efficiency and growth performance is higher compared to organisms fed with formulated diet (Ribeiro and Qin, 2015). However, it is necessary to carry out studies on bioenergetics to understand other aspects that could help explain cannibalism in *A. tropicus*, and from which strategies can be developed to mitigate this behavior.

5. Conclusion

This study addressed for the first time the characterization of cannibalism in *A. tropicus*. In the evaluation of the effect of the colors in the bottom of the fish tanks; it was recorded that, in the pair challenges, the greatest number of attacks occurred in white, possibly due to the contrast of color with respect to the dark larvae. In group challenges, *A. tropicus* larvae preferred yellow color. On the other hand, it was distinguished that the presence of artificial vegetation used as shelter reduced the number of attacks in both challenges. Morphologically, larvae (10 DAH) and juveniles can consume their own body depth and body width, related to their high mouth opening angle at these ages. The description of cannibal behavior in *A. tropicus* larvae was evaluated, whereby four types of interaction behaviors were identified: interaction, chasing, escape, and fast swimming. The highest number of cannibal attacks occurred in Interaction. Three types of attacks were also recorded: frontal, lateral and posterior; and three attack regions: head, body, and tail. The type of attack that presented the highest frequency was the Posterior Tail. In addition, it was identified that the cannibal larvae change the shape of their body, adopting an "S" type position before making an attack. Likewise, it was established that the larvae of *A. tropicus* present cannibalism Type I, II, and two variants of Type III. The average difference in weight and size between the cannibal and its prey was determined, which was 16.39 ± 10.86% and 15.23 ± 5.68%, respectively. Further studies combining Artemia and *A. tropicus* larvae are recommended to further understand the functional responses between the two and determine the affinity for efficient cannibal or efficient interspecific predator. The results obtained are important to consider carrying out a more efficient sorting of *A. tropicus* larvae thus, reducing cannibalism in the culture. It is recommended to use ethology as a tool to obtain more information that contributes to understanding the cannibalism present in this species and that promotes the development of effective mitigation strategies.

Availability of data and material

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Ethics approval

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (NOM-062-ZOO-

1999, 2001).

Authors' contributions statement

Each member of the authors team made a significant contribution to this study. All the authors are aware of and agree with the content of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Capítulo 2: Stocking density and starvation in Tropical gar (*Atractosteus tropicus*) larvae and their effect on aggressivity: intra-cohort cannibalism.

Intra-cohort cannibalism, aggressivity and starvation in tropical gar (*Atractosteus tropicus*) larvae: Effect of stocking density

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Highlights

- The best growth in *Atractosteus tropicus* larvae was at 1.4 larvae/ L fed and decrease the frequency of attacks.
- Low density (0.7 larvae/ L)increases coefficient of variation (CV) and size heterogeneity (SH) in *A. tropicus* larvae.
- Cannibalism mortality occurs at 8 DAH in fed larvae and at 10 DAH in starvation.
- Starvation modifies digestive and metabolic enzyme activity, and liver and intestine morphology.
- The density 0.7 larvae/L fed showed higher expression in genes related to aggressiveness.

Abstract

Cannibalism in early periods of the fish development is a biological phenomenon affecting their culture, so seeding density and feeding schemes play a fundamental role in its optimal control. The objective of this research was to evaluate two initial stocking densities, 0.7 larvae/L (D7) and 1.4 larvae/L (D14), which were fed (F) and not fed (NF) in tropical gar larvae (*Atractosteus tropicus*) on growth, attack frequency, survival, digestive and metabolic enzyme activity, digestive morphology, and gene expression. A total of 420 larvae (0.022 ± 0.003 g; 1.52 ± 0.10 cm) were placed in 70 L tanks connected to a recirculation system. Treatment F larvae were fed a formulated food (44 % protein and 14 % lipids). D14-F larvae had the highest final weight, total length, and specific growth rate (SGR) ($p<0.05$). The highest survival percentage was in D7-F larvae (46.19 ± 2.74), and the lowest was in D7-NF (19.04 ± 2.19) ($p<0.05$). The highest frequency of attacks occurred in D14-F larvae (26.668 ± 0.824), with D14-NF larvae having the lowest frequency of attacks (8.675 ± 0.183) ($p<0.05$). The coefficient of variation (CV) and size heterogeneity (SH) was higher in D7-F larvae (13.56 ± 1.80; 0.81 ± 0.10) ($p<0.05$). The F larvae showed the highest frequency of attacks at 8 days post-hatching (DAH), while the NF larvae showed it until 10 DAH. The highest frequency of attacks occurred when the difference in weight between the attacker and attacked was 16.44 ± 9.75% and in total length 12.28 ± 4.57 % ($p<0.05$). Concerning digestive enzyme activity, acid and alkaline protease, trypsin, and lipase activities presented significant differences ($p<0.05$) between both factors (density and feeding regime), as well as the interaction between them. D14-F larvae presented the highest Glutathione peroxidase (GPX)

activity (95.51 ± 9.008) ($p<0.05$), while D7-NF larvae registered the highest catalase (CAT) activity (6.885 ± 0.038). Superoxide dismutase (SOD) activities only showed significant differences between F (23.67 ± 0.90) and NF (17.72 ± 0.99) larvae ($p<0.05$). The liver presented the highest percentage of area with melanomacrophage centers (MMC) in D7-NF larvae (11.368 ± 0.268). Hepatocytes with the highest area (7.422 ± 0.237 μm^2) and enterocytes height (12.479 ± 0.305 µm) were recorded in D14-F larvae ($p<0.05$). Catalase expression was lower in D7-F larvae ($p<0.05$). Likewise, D7-F larvae showed higher expression in *cck* ($p<0.05$). On the other hand, D14-F larvae showed the lowest expression of *gpx* for the other treatments ($p<0.05$). The expression of *sod1* and *sod2* of the larvae of treatments D7-F and D14-F presented the highest expressions ($p<0.05$). D7-F larvae presented higher expression of *avp1*, *crh*, *htr1*, *sstr1*, *th* and *tph1* genes ($p<0.05$). Our results show that *A. tropicus* larvae D14-F present the best growth and morphometric parameters, reduce CV and SH, which shows a more homogeneous growth and decreases the frequency of attacks; however, it is necessary to analyze other physiological and social factors that could be related to the aggressiveness and cannibalism of the species.

KEYWORDS

Aggressivity, behavior, intracohort cannibalism, fish larvae issues, fish larvae management.

1. INTRODUCTION

Aggressiveness in early developmental stages has been reported in several studies, which sometimes results in high intra-cohort cannibalism and death of fish (Baras and Jobling, 2002; Chang et al., 2019; Colchen et al., 2019), defined as an event in which a fish attacks and frequently consumes siblings, both belonging to the same age or cohort (Smith and Reay, 1991). This behavior represents a severe problem in the profitability of aquaculture crops by decreasing survival in the production of offspring (Sepúlveda-Quiroz et al., 2022). There are different variables related to cannibalism during culture; some are the absence of shelter, temperature, tank color, light periods, feeding, and seeding density in the tanks (Naumowicz et al., 2017). In spotted seatrout (*Cynoscion nebulosus*), a direct relationship between density and

feeding is shown, which causes a high percentage of aggressiveness during culture (Manley et al., 2014). In spotted sand bass (*Paralubrax maculatofasciutus*) larvae, cannibalism was observed at the highest densities used (150 and 200 larvae/L) (Álvarez-González et al., 2001a). Similarly, when using four experimental densities (5, 10, 15, and 20 post larvae/ L) in European sea bass (*Dicentrarchus labrax*), survival decreased significantly at 15 and 20 post larvae L^{-1} mainly because the cannibalism (Hatziathanasiou et al., 2002). In juvenile common snook (*Centropomus undecimalis*) culture at a density of 400 fish per tank, mortality due to cannibalism is higher and with a significant difference concerning lower densities (100 and 200 fish per tank) (Hans et al., 2019). Similarly, it has been observed that in barramundi (*Lates calcarifer*) juveniles, cannibalism increases at the highest culture density, which was improved by increasing the feeding frequency and decreasing the seeding density (Ribeiro et al., 2015).

On the other hand, tropical gar (*Atractosteus tropicus*) is a fish distributed in southeastern Mexico and Central America and represents a species of great ecological and economic importance (Márquez-Couturier and Vázquez-Navarrete, 2015; Nelson, 2006). It presents cannibalism in early life stages (larvae and juveniles), both in controlled environments and in the wild (Aguilera et al., 2012; Márquez, 2000). Despite scientific contributions related to digestive physiology, culture, and nutrition in this species (Frías-Quintana et al., 2010, 2015, 2016; Guerrero-Zárate et al., 2014, 2019; Huerta-Ortiz et al., 2018; Jiménez-Martínez et al., 2018; Martínez-Cárdenas et al., 2020; Nájera-Arzola et al., 2018; Nieves-Rodríguez et al., 2018; Sepúlveda-Quiroz et al., 2020), cannibalism continues to be a bottleneck on the percentage of survival obtained in culture. For example, under laboratory conditions, Frías-Quintana et al. (2017) reported cannibalism and survival values of 33% and 24%, respectively. In contrast, Jiménez-Martínez et al. (2020), when administering a low concentration of polyunsaturated fatty acid, observed an increase in cannibalism (40%) and fluctuations in survival between 15 and 30%. Likewise, Palma-Cancino et al. (2019) implemented a co-feeding (commercial diet and *Artemia*) during the larval stage and recorded cannibalistic behaviors at 10 days post-hatching with survival no higher than 32%. Therefore, this work aimed to identify the relationship between different culture densities, feeding and starvation, growth, attack frequency, survival, digestive and metabolic enzyme activity, digestive morphology, and *avp1*, *crh*, *htr1*, *sstr1*, *th* and *tph1* gene expression in *A. tropicus* larvae.

2. MATERIAL AND METHODS

2.1 Biological material

Larvae were obtained by induced spawning by injecting the female with the hormone LHRHa (30 µg/kg) using a ratio of one female to three males. The fish induction was done in a 2000-L circular tank in the Laboratory of Physiology in Aquatic Resources (LAFIRA) of the DACBiol-UJAT. After hatching, the larvae were transferred to 70 L tanks. For this study, 420 larvae (0.022 ± 0.003 g mean weight and 1.52 ± 0.10 cm total length) of *A. tropicus* were used.

2.2 Experimental design

A bifactorial experiment was designed where the first factor evaluated was the initial seeding density with two treatments, 0.7 (D7) and 1.4 (D14) larvae L^{-1}, and the second factor was the feeding regime with two treatments, fed (F) and non-fed (NF) larvae. For this bioassay, 35 larvae per experimental unit were used, and each treatment was evaluated in triplicate. All experimental units were connected to a recirculation system by a 0.5-HP water pump (Jacuzzi, JWPA5D-230A, Delavan, WI) and a 1,500 L reservoir for solids deposition and biological filter. The feeding of the larvae of the treatments (F) began at 3 DAH (first day of the experiment), to which were supplied a co-feeding of formulated diet (44% protein and 14% lipids) as proposed by Frías-Quintana et al. (2016) and *Artemia* nauplii, for four days to later administer only the formulated diet. Food was fed four times per day (8:00, 12:00, 16:00, and 20:00) ad libitum. The diet was manufactured following the protocol of Álvarez-González et al. (2001b). Partial water replacement of 10% per day was performed by siphoning to remove feces and uneaten feed. Water quality was quantified daily (mean ± standard deviation, SD), mean temperature (28.12 ± 0.9°C), dissolved oxygen (4.9 ± 0.7 mg/L) determined by an oximeter (YSI 85; OH), pH (7.0 ± 0.4) with a potentiometer (HANNA HI 991001, Romania).

2.3 Growth indexes and feed quality

Two biometries were performed, the first at the beginning of the experiment (2 DAH) and the final twelve days later (14 DAH). An analytical balance (A&D Company, Limited mod.HR-250AZ, Korea) was used to quantify the individual weight of each larvae. The total

length was calculated by taking photographs of the analyzed organisms using ImageJ 1.51j8 software (U.S. National Institutes of Health, Bethesda, MD). Productive values were calculated as Survival (S): (final fish number/initial fish number) × 100; specific growth rate (SGR): [(ln final weight - ln initial weight)/days] × 100. Besides, the coefficient of variation (CV) (%): (standard deviation of individual weight/ mean individual weight) × 100, and size heterogeneity (SH): (final coefficient of variation/ initial coefficient of variation) were calculated. The percentage of spawning deformity was calculated using the following formula: Deformity (D) (fish with deformity/initial fish number) x 100. Deformed organisms were identified by high dark pigmentation, erratic swimming, and apparent malnutrition, which was checked using a stereo microscope (Carl Zeiss mod. Stemi DV4, Germany).

2.4 Attack frequency and cannibalism

Attack frequency and cannibalism were quantified through direct observations every 30 min before and one hour after each feeding, replicating the observation time in NF treatments. Larvae exhibiting the following conditions were considered as attack-cannibalistic behavior: Bite attack (one fish attacks another fish by biting without the attacker ingesting the prey), partial cannibalism (one fish partially ingests another fish), and complete cannibalism (complete ingestion of one fish to another fish). The formula (fish with cannibalistic behavior / initial fish number) x 100) was used to quantify aggressiveness in each treatment. The attacking larvae (cannibals) and their attacked larvae (prey) were counted, separated, and their weight and length were obtained. Subsequently, they were sacrificed by thermal shock and preserved according to the corresponding analysis.

2.5 Collection of biological samples

At the end of the experiment, a total of 12 *A. tropicus* larvae per treatment were sacrificed by thermal shock. One larva per replicate was fixed in the Davison solution for histological analysis, and the other two larvae per replicate were frozen at -80°C for digestive enzymatic activity. Finally, one larvae per replicate was preserved in RNAlater at -80°C for gene expression analysis. In all studies, whole larvae were stored at -80°C.

2.6 Digestive enzyme activity

Before the digestive enzyme analysis, larvae were manually dissected in a cold plate to eliminate the tail and head. Each sample (approximately 100 mg) was manually macerated in 1.5 mL Eppendorf tubes with 500 mL of distilled water to determine the digestive enzyme activities. Subsequently, the sample was centrifuged at 16, 000 g per minute at 4°C for 15 min, recovering the supernatant. Aliquots of 30 µL were taken from this supernatant and preserved at -80°C. Soluble protein was determined by Bradford technique (1976). Quantification of acid proteases used 0.5% hemoglobin as substrate solubilized in 100 mM glycine-HCl pH 2. Alkaline proteases were quantified using 0.5% casein solubilized in 50 mM Tris-HCl and 10 mM CaCl at pH 9 (Sarath et al., 1989). In both techniques, samples were incubated at 37°C, and the reaction was stopped with 0.5 mL of 20% trichloroacetic acid and centrifuged at 16,000 g per minute; absorbance was read at 280 nm. The extinction coefficient (ε) for calculating acid and alkaline protease activity was 0.005 µM mL^{-1} cm^{-1}. To quantify Trypsin activity, 1 mM BAPNA (Nα-Benzoyl-DL-DL-Arginine-P-nitroanilide) was used as substrate dissolved in 50 mM Tris-HCl, pH 8 at 37°C, and absorbance was quantified at 410 nm using an ε of 8,800 ml/µM cm (Erlanger et al., 1961). The method of Maroux et al. (1973), was used to determine Leucine aminopeptidase activity, with 0.1 M leucine p-nitroanilide as substrate dissolved in DMSO with 50 mM sodium phosphate, pH 7.2, incubated at 37°C, the absorbance was quantified at 410 nm with an ε of 8,800 µM mL^{-1} cm^{-1}. Lipase activity was determined using β-naphthyl acetate (100 mM) as substrate dissolved in 50 mM Tris-HCl at pH 7.5 with sodium taurocholate (100 mM) at 37°C, stopping the reaction with 0.72 N TCA. Fast Blue (100 mM) and a 1:1 ethanol/ethyl acetate mixture was added, and absorbance was quantified at 540 nm using ε d 0.02 µM mL^{-1} cm^{-1} (Versaw et al., 1989). The enzyme activity was determined using the following equations: 1) units by mL (U mL^{-1}) = [Δabs × final reaction volume (mL)] / [ε × time (min) × extract volume (mL)]$^{-1}$, where (ε) molar extinction coefficient; specific activity (U mg $protein^{-1}$) = U mL x mg of soluble $protein^{-1}$.

2.7 Antioxidant enzyme activity

Glutathione peroxidase (GPX) activity was performed according to the supplier (Cayman Chemical, USA). The larvae were rinsed with PBS solution (pH 7.2), then the tissue was cold homogenized (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1 mM DTT). It was centrifuged at 10,000 g (15 min) at 4°C. Finally, the supernatant was obtained. The activity

was calculated by the following equation: GPX activity = ((ΔA340/min)/(0.00373μM^{-1}) × (0.19 ml) x 0.02 mL^{-1})) × sample dilution. Catalase Assay Kit (Cayman Chemical, USA) was used to determine CAT activity following the supplier's protocol. The larvae were washed with PBS solution (pH 7.4). They were cold massaged with buffer (50 mM potassium phosphate, pH 7.0, 1 mM EDTA). The supernatant obtained was centrifuged at 10,000 g (15 min) at 4°C. The following equation calculated the activity: CAT activity = (μM of sample x 20 min^{-1}) × sample dilution. Finally, SOD activity was obtained by Superoxide dismutase Assay kit (Cayman Chemical, USA), following the supplier's recommendations. The larvae were washed with PBS solution (pH 7.4). The samples were cold homogenized with 20 mM HEPES buffer (pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose). The following equation obtained the activity: SOD (U mL^{-1}) = ((sample LR - y-intercept/slope) × (0.23 mL x 0.01 mL^{-1})) × sample dilution.

2.8 Histological analysis

The whole larvae were fixed in Davidson solution, then dehydrated using a gradient of alcohols (70%, 80%, 90%, 96%, and 100%), ending with the inclusion of the sample in kerosene. Slices of 7-μm were made and stained with eosin and hematoxylin (H&E). Photographs of the samples were taken with a camera (Axiocam ERc 5s, Zeiss) attached to an optical microscope (Primo Star, Zeiss). In the liver, melanomacrophage centers (MMC) percentage and hepatocyte area (μm^2) were quantified. In the intestine, enterocyte height (μm) was quantified. The different parameters were calculated with Image Pro Plus software (Media Cybernetics, Inc., WA).

2.9 RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

The supplier's recommendations were used to extract Total RNA from larvae using Trizol (Invitrogen, Waltham, MA). The concentration and purity of RNA samples were assessed by the ratio between the absorbance at 260 and 280 nm in a spectrophotometer (Jenway GenovaNano, Cole-Parmer, Staffordshire, UK). RNA (1 μg) was reverse-transcribed (RT) using the SuperScript II kit (Invitrogen), with a final volume of 20 μL. RT reactions were performed in a thermocycler (Mastercycler nexus GSX1, Eppendorf AG, Hamburg, Germany). The standard RT program used was as follows: 5 min at 65°C, 10 min at 25°C, 50 min at 42°C (cDNA strand extension), 15 min at 70°C (reverse transcriptase inactivation) and

finally, 20 min at 37°C. Catalase (*cat*), glutathione peroxidase (*gpx1*), superoxide dismutase 1 and 2 (*sod1* and *sod2*), cholecystokinin (*cck*), and somatostatin receptor 1 (*sstr1*), tyrosine hydroxylase (*th*), histidine decarboxylase (*hdc*), corticotropin-releasing hormone (*crh*), 5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled (*htr1a*), arginine vasopressin induced 1 (*avpi1*), and tryptophan hydroxylase 1 (*tph1*) for *A. tropicus* for were designed from the species transcriptome (NCBI Accession: PRJNA395289) (Martinez-Burguete et al., 2021) (Table 1). The RT-qPCR was performed in a CFX96 Real-Time System (BioRad, Hercules, CA) using 5 µL of EvaGreen Supermix (BioRad), 0.5 µL primers mix, and 4.5 µL of cDNA for a final volume of 10 µL. The RT-qPCR program was used at 50°C for 2 min, 95°C 10 s, followed by 40 cycles at 95°C for 15 s and 62°C 1 min. *β-actin* was used as a reference gene. Relative gene expression was calculated as fold-change compared with control and using −ΔΔCt formula (Livak and Schmittgen, 2001).

2.10 Statistics analysis

Normality (Kolmogorov–Smirnov) and homoscedasticity (Barttlet) were tested to know the effect of factors (density and feed regime) a Multiple ANOVA was carried out for all the analysis, and in case of finding differences, a posteriori test of unequal N HSD (Tukey) was used. A student's t test was used to compare the differences in weight and total length between attacker and attacked larvae. Gene expression data were analyzed using a nonparametric Kruskal–Wallis and Nemenyi posteriori tests. All tests were performed using the software Prism V. 9.0 with a significance value of 0.05.

3. RESULTS

3.1 Growth indexes and survival

Larvae showed significant differences in the stocking density and feeding schedule ($p<0.05$) for the final weight, total length, SGR, and survival. The interaction of the two factors (density and feeding (D-F)), D14-F larvae recorded higher final weight (0.028 ± 0.0008) (Figure 1a), final total length (1.92 ± 0.03) (Figure 1b), and SGR (4.03 ± 0.30) ($p <0.05$). At the same time, the highest percentage of survival was recorded at D7-F (46.05 ± 1.95 %) and D14-F (43.09 ± 2.25%), a significant difference ($p <0.05$) with the rest of the

treatments. Regarding aggressivity, no significant differences were found for density (D7: 18.25 ± 0.23 %; D14: 17.67 ± 0.25 %) ($p > 0.05$). On the other hand, aggressivity was higher in F larvae (25.71 ± 0.25 %) with respect to NF larvae (10.21 ± 0.23 %) with significant differences ($p<0.05$). For the D-F interaction, significant differences were found among all treatments, where D14-F larvae had a higher aggressivity percentage (26.93 ± 3.51 %), followed by D7-F larvae (24.19 ± 3.50 %). The lowest percentage was recorded in NF larvae (D7: 11.74 ± 0.44 %; D14: 8.67 ± 0.18 %) (Figure 1c). CV and SH showed no differences ($p > 0.05$) with respect to density (CV: D7: 9.34 ± 0.45, D14: 8.07 ± 0.45; SH: D7: 0.56 ± 0.02, D14: 0.48 ± 0.02). However, CV and SH did show differences ($p <0.05$) with respect to feeding, registering the highest value in F larvae (9.57 ± 0.45; 0.57 ± 0.02) as opposed to NF larvae (7.83 ± 0.45; 0.47 ± 0.02). For the D-F interaction, the highest CV value was for D7-F (13.56 ± 1.80) and D14-NF (10.54 ± 1.24) larvae, and the lowest in D7-NF (5.12 ± 0.37) and D14-F (5.59 ± 0.24) larvae ($p <0.05$). The D-F interaction in SH presented the same trend, where the highest value was presented in larvae D7-F (0.81 ± 0.10) and the lowest in larvae D7-NF (0.30 ± 0.02) and D14-F (0.33 ± 0.01) ($p <0.05$) (Table 2).

3.2 Attacks and cannibalism

The larvae of treatment F initiated aggressive and cannibalistic behavior after 8 DAH, and two days later, it was observed in the NF larvae. During seven days (day 8-14 DAH), there was an increase in the intensity of aggressive behavior in the larvae (Figure 2a), which considerably modified their survival (Figure 2b). The larvae identified as attackers (cannibals) weighed 0.031 ± 0.0009 g, and the attacked larvae (prey) weighed 0.027 ± 0.0005 (mean ± SEM) ($p < 0.0007$). The mean total length was 1.98 ± 0.02 cm and 1.75 ± 0.02 cm for the attacker and attacked larvae, respectively ($p <0.0001$) (mean ± SEM). Aggressiveness was present between the attacker and attacked when the difference between their weight was 0.004 ± 0.003 g and 0.22 ± 0.085 cm in length, which corresponds to a difference of 16.44 ± 9.75 % and 12.28 ± 4.57 %, respectively (Figure 3). The average spawning deformity percentage was 4.00 ± 1.54 %.

3.3 Digestive enzyme activity

The acid and alkaline protease, trypsin, and lipases presented significant differences ($p < 0.05$) for the stocking density, feeding schedule, and D-F interaction. Acid protease activity was highest in larvae at a stocking density of D7 (0.105193 ± 0.0001) as opposed to D14 (0.101524 ± 0.001). In turn, NF larvae showed higher activity (0.116 ± 0.0001) than F larvae (0.090 ± 0.0001). In the D-F interaction, D7-NF larvae had the highest enzyme activity (0.129 ± 0.0001), with D7-F larvae having the lowest activity (0.0812 ± 0.0002). For alkaline protease, D7 larvae recorded an activity of 0.035 ± 0.0004, lower than D14 larvae (0.043 ± 0.0004). On the other hand, F larvae recorded the activity of 0.033 ± 0.0004, lower than NF larvae (0.046 ± 0.0004). In the D-F interaction, D14-NF larvae showed higher activity (0.0514 ± 0.0003), while D7-F larvae showed the lowest activity (0.0301 ± 0.0018). In trypsin, D14 larvae showed the highest activity (1.184 ± 0.044) for the stocking density of D7 (0.867 ± 0.044). On the other hand, NF larvae registered the highest activity (1.125 ± 0.044) concerning F larvae (0.962 ± 0.044). The D-F interaction, D14-F (1.236 ± 0.123), D14-NF (1.131 ± 0.066), and D7-NF (1.118 ± 0.057) larvae recorded the highest activity. In lipase activities, D14 larvae recorded the highest activity (0.186 ± 0.001) than larvae at D7 (0.130 ± 0.001). On the other hand, NF larvae had the highest activity (0.204 ± 0.001) relative to F larvae (0.112 ± 0.001). In the D-F interaction, D14-NF larvae had the highest enzyme activity (0.2223 ± 0.0037); the stocking density of D7-F showed the lowest activity (0.0725 ± 0.0001). Leucine aminopeptidase activities did not show significant differences ($p > 0.05$) in the stocking density, feeding regime, and D-F interaction, although D14-F (0.499 ± 0.023) and D14-NF (0.468 ± 0.033) larvae were higher ($p < 0.05$) than D7-F (0.329 ± 0.029) and D7-NF (0.361 ± 0.032) larvae (Table 3).

3.4 Antioxidant enzyme activities

The results of antioxidant enzyme activities are shown in Table 4. GPX activities did not show a significant difference ($p>0.05$) for the stocking density (D7: 62.07 ± 2.75, D14: 64.24 ± 2.75); however, in the feeding schedule, there was a difference ($p <0.05$) (F: 75.34 ± 2.75, NF: 50.97 ± 2.75). In the D-F interaction, significant differences were observed ($p <0.05$), with the highest activity in the D14-F larvae (95.51 ± 9.008) and the lowest activity in

the D14-NF larvae (32.980 ± 1.385). CAT activity presented significant differences ($p < 0.05$) for the stocking density (D7: 6.67 ± 0.01, D14: 6.27 ± 0.01), feeding schedule (F: 6.36 ± 0.01, NF: 6. 58 ± 0.01) and D-F interaction, where the highest activity was presented in D7-NF larvae (6.885 ± 0.038), and the lowest activities were D14-F (6.270 ± 0.017) and D14-NF (6.277 ± 0.023). SOD activity only showed a difference ($p < 0.05$) with the feeding schedule, unlike density (D7: 19.07 ± 0.90, D14: 22.32 ± 0.99), where F larvae showed the highest activity (23.67 ± 0.90) and NF larvae the lowest activity (17.72 ± 0.99). In the D-F interaction, D14-F larvae (26.61 ± 2.276) are different ($p < 0.05$) with D7-NF (17.410 ± 2.602) and D14-NF (18.042 ± 2.520).

3.5 Histological analysis

In the liver, the MMC area showed significant differences concerning density, feeding schedule, and D-F interaction ($p < 0.05$). Larvae D14 showed a greater MMC area (7.22 ± 0.14) for the stocking density of D7 (4.93 ± 0.14). The F larvae treatment showed a greater MMC area (9.93 ± 0.14) than the NF larvae (2.22 ± 0.14). In the D-F interaction, D7-NF larvae showed the largest MMC area (11.368 ± 0.268) and the smallest D7-F larvae (1.361 ± 0.498). Hepatocyte area was highest for the stocking density of D14 (5.60 ± 0.17) and least for D7 (4.60 ± 0.17). The hepatocyte area in the F treatment was greater (6.58 ± 0.17) than in the NF treatment (3.53 ± 0.17) (Figure 4). In D-F interaction, D14-F larvae showed higher hepatocyte area (7.422 ± 0.237), and with lower D7-NF (3.778 ± 0.510) and D14-NF (3.281 ± 0.626). In the intestine, the highest enterocyte height was found at density D7 (10.24 ± 0.15) in contrast to D14 (10.97 ± 0.15). On the other hand, F larvae (11.85 ± 0.15) presented the biggest enterocyte height, in contrast to NF larvae (9.36 ± 0.15) (Figure 5). In the D-F interaction, D14-F larvae presented the greatest enterocyte height (12.479 ± 0.305), being the lowest in D7-NF (9.468 ± 0.683) and D14-NF (9.262 ± 0.058) larvae (Table 5).

3.6 Gene expression

The relative expression of *cat*, *cck*, *gpx*, *sod1*, and *sod2* genes showed significant differences ($p < 0.05$) in the D-F interaction. Larvae D14-F and D14-NF showed the highest expression,

while the lowest gene expression was in the D7-NF treatment ($p < 0.05$). The highest expression of *cck* was recorded by the D7-F, while the lowest gene expression was for larvae from the D7-NF and D14-F treatments ($p < 0.05$). The expression of *gpx* was higher for larvae of treatments D7-F, D7-NF, and D14-NF, with no difference among them ($p > 0.05$), while larvae for the treatment D14-F presented the lowest expression ($p < 0.05$). For *sod1*, larvae from the D7-F treatment showed the highest expression, while larvae from the D14-F and D7-NF treatments showed the lowest expression ($p < 0.05$). Finally, in *sod2*, the highest expression was recorded for larvae of the D14-F treatment, while the lowest expression was recorded for larvae of the D7-F, D7-NF, and D14-NF treatments ($p < 0.05$) (Figure 6, Table 6).

The relative expression of *avpi1*, *crh*, *htr1*, *sstr1*, *th*, and *tph1* genes showed significant differences ($p < 0.05$) in the D-F interaction. The larvae of the D7-F treatment presented the highest expression in *avpi1*, *crh*, *htr1*, *sstr1*, and *th*. On the other hand, larvae from the D7-NF treatment showed the lowest expression for the *avpi1* gene ($p < 0.05$). Additionally, larvae from the D14-F treatment recorded the lowest expression for the *crh* gene ($p < 0.05$). In contrast, the lowest expression of *htr1* occurred in larvae from treatment D14-NF ($p < 0.05$). Larvae of treatment D14-F showed the lowest expression of the *sstr1* gene ($p < 0.05$). Also, larvae from the D14-N, D14-NF, and D7-NF treatments showed the lowest *th* gene expression. For the *tph1* gene, larvae from the D7-F treatment showed the highest expression, although they were statistically equal to larvae from the D14-F and D14-NF treatments ($p > 0.05$), while the lowest expression was for larvae from the D7-NF treatment ($p < 0.05$). Finally, there were no significant differences in *hdc* expression ($p > 0.05$) (Figure 7, Table 6).

4. DISCUSSION

4.1 Growth indexes, aggressivity and cannibalism

In the search to explain cannibalism and aggressiveness in fish and to diminish its effects that harm the profitability and welfare of the culture, different abiotic and biotic factors directly related to cannibalism have been pointed out, among them density (Hecht and Pienaar, 1993; Naumowicz et al., 2017). Aggressive behaviors and cannibalism in fish triggered by density are related to increased competition for food, territoriality, hierarchy, and the likelihood of

cannibal-prey encounters (Baras and Jobling, 2002; Sloman and Armstrong, 2002; Smith and Reay, 1991; Sogard and Olla, 1994; de Souza et al., 2014). Also, in fish larvae, starvation delimits the early development of larvae and is related to their growth, survival, and ecological conditions, such as population dynamics and fisheries recruitment (Shan et al., 2008). Nutritional aspects such as food availability and optimal foraging are related to cannibalistic behavior in fish (Dong and Polis, 1992).

In the case of common snook (*Centropomus undecimalis*), when using culture densities of 1, 2, and 4 individuals L^{-1}, a relationship between higher density and higher mortality caused by cannibalistic behavior is reported (Hans et al., 2019). In *Heteroclarias* hybrid larvae (♀*Clarias gariepinus* x ♂*Heterobranchus spp*), it was identified that the higher the density (0.2, 0.4, 0.8, 0.8, 1.6, and 2.4 larvae L^{-1}) the lower the survival (100, 95, 85, 22.5 and 13%) respectively, with a direct relationship to cannibalistic lesions (Okeke, 2014). Likewise, in matrinxã (*Brycon amazonicus*) larvae exposed to a high density of 60 larvae L^{-1}, the aggressive events were higher compared to larvae at a lower density of 20 larvae L^{-1} (de Barros et al., 2019). However, it has also been observed in Atlantic cod (*Gadus morhua*), Vundu catfish (*Heterobranchus longifilis*), and European chub (*Leuciscus cephalus*), that implementing a high density in culture can decrease aggressive and cannibalistic behaviors (Baskerville-Bridges and Kling 2000; Imorou Toko et al., 2008; Żarski et al., 2008), this because cannibalistic organisms cannot select the most optimal prey (Baras and Jobling, 2002). In our result, *A. tropicus* larvae fed and at a density of D14 growth parameters were better than the rest of the treatments; in addition, survival was higher in larvae fed with no difference between both densities.

The cannibalistic condition in certain fish larvae presents advantages over the rest of the culture. For example, in barramundi (*Lates calcarifer*), the cannibalistic behavior, feed conversion efficiency and growth performance are higher than organisms fed with formulated diet than fresh fish diet (Ribeiro and Qin, 2015). On the other hand, the "jumper" effect is directly related to cannibalistic organisms and their rapid increase in weight and size by the action of ingestion of conspecifics (Baras et al., 2011), which increases the variability of size and weight within the population, this variability increases the probability of further attacks decreasing the survival of the culture considerably (Abdulraheem et al., 2012). Our results

indicate that *A. tropicus* larvae fed at a density of D14 have a lower CV and SH, which can be reflected in uniform growth without the presence of jumper larvae, and a high feeding efficiency.

Variability in weight or length is a trigger for increased aggressiveness and cannibalism Atlantic cod (*Gadus morhua*), African catfish (*Heterobranchus longifili*), giant grouper (*Epinephelus lanceolatus*), and black rockfish (*Sebastes schlegelii*) (Baras et al., 2014; Folkvord, 1992; Hseu et al., 2004; Xi et al., 2017), either by the growth rate of the organisms or by the very presence of cannibalism and the jumper effect (Baras et al., 2011; Kestemont et al., 2003). Therefore, one of the strategies to reduce cannibalism is separation by weight or size. The importance between density and CV of the culture is shown in the work of Corrêa and Cerqueira (2007), where when testing different densities (1.5, 3, and 6 fish L^{-1}) and homogeneous (CV: 8 %) and heterogeneous (CV: 15 %) populations, the highest percentage of cannibalism was reported in high densities and heterogeneous groups.

On the other hand, in *A. tropicus*, a study related to improving nutrition using three different potato starch-based diets (16, 22, 28 %), the lowest cannibalism rate was 33 %, and the highest survival rate was 22.8 %, observed in the larvae fed with 28 % potato starch (Frías-Quintana et al., 2017). Similarly, Jiménez-Martínez et al. (2020) with a density of D14 and different lipid sources (fish oil, soybean lecithin, corn oil, canola oil, and olive oil), observed the best treatments using fish oil and lecithin with 95 and 90 % survival and around 5 % cannibalism in *A. tropicus* larvae. However, a size separation was performed as a cannibalism-mitigating strategy. Regarding a production scheme (4.28 larvae L^{-1}), Palma-Cancino et al. (2019) using different diets (commercial trout diet, experimental diet, co-feeding (commercial trout diet, and *Artemia* nauplii) found survival of 1.0, 5.56 % and 13.55 % respectively; lower cannibalism (5.44 %) was with the use of co-feeding. Aguilera et al. (2012) reported that the cannibalism provoked a survival of 50, 47 and 48 % in larvae of 34 DAH larvae fed with *Artemia* nauplii, frozen *Artemia*, and an artificial diet, respectively, at a density of 12.5 larvae L^{-1}. Finally, in *A. tropicus* juveniles (3.80 ± 0.11 g; 9.15 ± 0. 16 cm), the use of 200 fish m^{-3} is recommended for ornamental purposes or food production; however, no presence of aggressive behavior related to cannibalism is reported (Martínez-Cárdenas et al., 2020).

Our results in F larvae, despite the administration of Artemia nauplii that have a high affinity to be consumed by *A. tropicus* larvae (Escalera-Vázquez et al., 2018; Palma-Cancino et al., 2019; Saenz et al., 2018), and as well as administering a diet with a specific formulation for *A. tropicus*, the appearance of cannibalism may be related to morphometric changes during their development, since at day 10 DAH larvae are anatomically capable of eating their conspecifics, due to the size, breadth, and opening angle of the mandible as well as the ratios: mouth depth/body depth (MBD) and mouth depth/body width (MBW) (Sepúlveda-Quiroz et al., 2023), in turn, the two-day lag concerning NF larvae and the appearance of aggressive cannibalistic events may be related to the low development due to the starvation condition, so that morphometrically they are not capable of consuming their congeners influenced by MBD and MBW.

The effects of starvation on *A. tropicus* larvae are scarce; it has only been reported that all starved larvae die at day 15 DAH at a density of 12.5 larvae L^{-1} (Aguilera et al... 2012), observing in our results that at a lower density 0.7 and 1.4 larvae L^{-1}, survival was 19.04 ± 2.19 % and 27.14 ± 1.16 % respectively at day 14 DAH, very possibly due to a lower probability of aggression or cannibalism among larvae. On the other hand, it was observed in larvae of harp tooth catfish (*Clarias gariepinus*) a direct relationship between the high percentage of cannibalism in starved larvae compared to fed larvae; however, this trend is reversed, starved larvae decreased cannibalism compared to fed larvae after six days due to a weakening process by the effect of starvation (Hecht and Appelbaum, 1988).

4.2 Digestive enzyme activities

The effects of starvation on *A. tropicus* larvae and their digestive enzyme activity (alkaline and acid phosphatases, total alkaline proteolytic, pepsin, lipase, trypsin, chymotrypsin, and aminopeptidase) were reported by Aguilera et al. (2012), the activity at day 14 DAH for total alkaline proteolytic, pepsin, trypsin and aminopeptidase was lower for fed larvae, however, for chymotrypsin, alkaline phosphatase, acid phosphatase, and lipase their activity was higher in larvae fed with different treatments (*Artemia* nauplii, commercial artificial diet). Our results show a similarity in lipase, where NF larvae presented higher activity. However, they differ concerning trypsin, where NF larvae registered the highest

activity. In other species, such as carp (*Labeo rohita*), protease, amylase, and lipase activity decreased by a 7-day starvation effect compared to fed fingerlings (Dar et al., 2018). In Atlantic salmon (*Salmo salar*), the effects of starvation duration (40 days) are observed in the modulation of digestive enzymes (maltase, lactase, and leucine aminopeptidase), presenting a decrease of specific enzyme activity in maltase and leucine aminopeptidase (Krogdahl and Bakke-McKellep, 2005). In the case of density, in juveniles of Japanese flounder (*Paralichthys olivaceus*), trypsin activity varies according to culture density (Bolasina, 2006).

4.3 Antioxidant response

In periods of starvation, fish can use the endogenous energy (glycogen) accumulated in their tissues (liver, white muscle, and skeletal muscle) as a biochemical strategy that can help them survive a limited period, with which their metabolism and locomotion can decrease (Morales et al., 2012; Mustafa and Mittal, 1982; Navarro and Gutiérrez, 1995). The function of antioxidant enzymes in the case of CAT is to break down hydrogen peroxide to molecular oxygen and water, and SOD catalyzes the superoxide anion dismutation to hydrogen peroxide and oxygen. GPX detoxifies hydrogen peroxide into water (Halliwell and Gutteridge, 2007). An imbalance of these enzymes can generate the presence of oxidative stress damaging cells and molecules in individuals, which can lead to a process of apoptosis (Chowdhury and Saikia, 2020); in addition, it has been reported that starvation processes in fish favor the oxidative effect due to aerobic metabolism and the decrease of antioxidant defenses (Morales et al., 2012).

Our results showed that the feeding factor presents differences in the three enzymes evaluated (CAT, SOD, and GPX), with higher activity in F larvae in SOD and GPX, and only in CAT activity for NF larvae. CAT was the only enzyme evaluated, with the difference between densities being higher in D7. Likewise, the expression of the genes *cat*, *gpx1*, *sod1*, *sod2*, and *cck*, showed differences in the interaction of density and feeding factors. In Adriatic sturgeon (*Acipenser naccarii*) and rainbow trout (*Oncorhynchus mykiss*), under 72-day starvation, there was a decrease in the antioxidant enzyme activities of SOD, CAT, and GPX in both species (Furné et al., 2009). On the other hand, in common dentex (*Dentex dentex*) the activity of the antioxidative enzymes SOD, CAT, and GPX in starved fish significantly

increased to respect control fish (Morales et al., 2004). In shi drum (*Umbrina cirrosa*), it was observed that starvation processes are related to higher consumption of fatty acids as an energy source, increasing oxidative stress, also observed a decrease in burst swimming (Hidalgo et al., 2017), derived from the metabolic changes involved in different pathways. On the other hand, in Yangtze sturgeon (*Acipenser dabryanus*), the activities of CAT and SOD decreased compared to fed organisms (Yang et al., 2019). Likewise, in seabreams (*Sparus aurata*), there is an increase in SOD, glutathione reductase, and glutathione peroxidase activities, and a decrease in CAT due to starvation (Pascual et al., 2003).

4.4 Histological analysis

The effects of feeding on the liver and intestine of *A. tropicus* larvae are significant. MMCs are a histological indicator of the immune system in fish (Steinel and Bolnick, 2017), and their size and abundance are related to environmental stressors (Agius and Roberts, 2003). Under starvation conditions, decrease the hepatosomatic index, hepatocyte nucleus size, and nuclear height of the intestinal epithelium in *Rhynchocypris oxycephalus*, compared to fed organisms (Lee et al., 1999), likewise, when comparing the hepatocytes of fed with non-fed olive flounder (*Paralichthys olivaceus*) it is suggested that due to the alterations (reduction in cell and nucleus size, apparent loss of nucleoli, condensation of chromatin, loss of stored glycogen, reduction of endoplasmic reticulum profile, increase in the number of electron-dense bodies, and increased mitochondrial size) found in non-fed organisms, hepatocytes can be an indicator of starvation (Hur et al., 2006). The liver is one of the organs most altered by starvation due to glycogen flux, which can be depleted in extreme cases or depleted to a minimal level, maintained and/or recovered due to gluconeogenesis (McCue 2010). In Atlantic salmon (*Salmo salar*), the effects of starvation (40 days), decreases in mass and length of the stomach and pyloric caeca and only mass in the three sections of the intestine (Krogdahl and Bakke-McKellep, 2005). Similarly, in miiuy croaker (*Miichthys miiuy*), the effects of starvation are notorious in the cells of organs such as the liver, stomach, pancreas, and intestine (Shan et al., 2015).

In *A. tropicus* juveniles, Sepúlveda-Quiroz et al. (2020) recorded a higher percentage of MMC (1.61 ± 0.19) in organisms fed with a commercial diet formulated for another species

(trout), a lower percentage of MMC was observed (0.32 ± 0.05) fed with a diet formulated for the species. These results point to adequate feeding and a diet formulated specifically for the requirements of the species and that the liver in *A. tropicus* can be used as a critical organ when trying to know the nutritional condition of the organisms.

4.5 Aggressive gene expression

The results of the expression of genes related to aggressiveness, except *hdc*, show significant differences in the interaction of the two factors (density and feed regimen) evaluated in this study. Although genes related to cannibalistic behavior have not yet been reported, genes and metabolic pathways related to aggressive behavior have been described in zebrafish (*Danio rerio*), seven neurological pathways were identified (hypothalamic-neurohypophysial-system (HNS), serotonin (5-HT), somatostatin, dopamine, hypothalamic-pituitary-interrenal (HPI), hypothalamic-pituitary-gonadal (HPG) and histamine) concerning expressed genes and aggression, whose expression varies according to specific brain regions (hypothalamus, hindbrain, telencephalon, and, optic tectum), sex and social hierarchy (Filby et al., 2010). Among the most relevant genes involved in these metabolic pathways are *sstr1*, *th*, *hdc*, *crh*, *htr1a*, *avpi1*, and *tph1*, whose relationship with aggressive behavior has already been reported in humans and animals (mice and fish) (de Abreu et al., 2019).

In other work, neurotransmitter response influences the expression of these genes, where the HPI pathway is involved in processes such as stress response (Rosengren et al., 2018; Wendelaar, 1997), with corticotropin-releasing hormone (CRH; or corticotropin-releasing factor (CRF)) being an activator of this pathway (Conrad et al., 2011). In rainbow trout (*Oncorhynchus mykiss*), CRF administration decreased the number of seizures and increased the concentration of serotonin, 5-hydroxyindolacetic acid (5-HIAA), and dopamine (Carpenter et al., 2007, 2009). Tryptophan functions as a substrate in synthesizing serotonin (5-HT), a neurotransmitter that regulates aggressive behavior (Nelson and Trainor, 2007; Sahu et al., 2020; Winberg and Nilsson, 1993). The relative mRNA abundance of 5-HT transporters (htr1a, htr2a) in dominant and subordinate males of *Astatotilapia burtoni* was higher in subordinates than in dominant males, related to 5-HT production (Loveland et al., 2014). The use of a specific agonist (8-OH-DPAT) at serotonin receptors (HTR1A) decreases aggression

in fighting fish (*Betta splendens*) (Clotfelter et al., 2007). The *avpl* (arginine vasopressin-like) gene is related to aggression and social interactions, and in dominant male zebrafish (*Danio rerio*), overexpression has been reported (Caldwell et al., 2008; Filby et al., 2010), in addition to genes involved in sexual genes (*cyp19a1b*, *cyp17*, *hsd11b2*, *hsd17b3*, and *ar*) and aggression (*avplr1b*, *tph1b*, *htr1a*, *sst1*, *sstr1*, *th*, and *slc6a3*) were identified, with higher expression than in subordinate males (Filby et al., 2012).

Somatostatin is related to regulating aggression in the dominant male cichlid *Astatotilapia burtoni* (Trainor and Hofmann, 2006). Dopamine is activated when the organism is threatened by another (Ferrari et al., 2003). Histamine is a regulator of behavior (Brown et al., 2001; Nuutinen and Panula, 2010); its importance lies in the fact that it is a neurotransmitter that regulates serotonin, norepinephrine, dopamine, and acetylcholine (Haas et al., 2008). In zebrafish (*Danio rerio*), histamine receptors (H3) were identified in aggression behaviors and anxiety (Reichmann et al., 2020). In addition, in adults, histamine regulates the *fgfr1* gene related to aggressiveness, boldness, and exploration (Norton et al., 2011).

Although in most of the works, the genes expressed correspond to adult organisms influenced by hierarchical conditions, territoriality, reproduction, or sex, conditions that may differ from those presented in larvae with cannibalistic behaviors. The existence of other genes (*gr*, *mr*, *bdnf*, *c-fos*, *htr2b*, *slc6a4b*, *oxtr*, *hrt1aa*, *slc6a4*, *fos*, *dusp1*, *hdac4*, *ier2*, *btg2*, and *nr4a1*) related to aggressiveness (Chen and Fernald, 2011; Malki et al., 2016; Parker et al., 2014; Theodoridi et al., 2017) can be used to learn more information at the larval stage of *A. tropicus*.

5. CONCLUSION

The results obtained in this work add to the search for factors related to aggressive behavior because of cannibalism to find strategies to reduce its presence in *A. tropicus* larvae. In this sense, the highest density tested in this study presented the best values in growth and a decrease in CV and SH, allowing a consistent growth of the larvae. This adequate stocking density is an essential condition for reducing cannibalism. In *A. tropicus* larvae, starvation does not favor cannibalism as in other species since it occurred two days later than in fed

larvae. In addition, the interaction of the factors evaluated (density and feeding) modified the digestive and antioxidant and digestive enzyme activity and the morphology of the intestine and liver, the latter organ clearly showing the effects of starvation. The expression of genes related to aggressive and cannibalistic behaviors is present in *A. tropicus* larvae. Finally, the results obtained in CV and SH of D14-F larvae and their increased growth and aggressiveness events increased, but ingestion among larvae was decreased (intracohort cannibalism). It is recommended that more factors be evaluated concerning cannibalism in *A. tropicus* and more genes related to aggressiveness. With this, the possibility of designing a multifactorial culture can be carried out with a high probability of mitigating aggressiveness and cannibalism.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (NOM-062-ZOO-1999, 2001).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Table 1. Oligonucleotide design for real-time polymerase chain reaction (qPCR) of aggressive genes in *A. tropicus* larvae.

Protein	**Gen**	**Primers (5'-3')**	**Alignment temperature (°C)**
Catalase	*cat*	FW: GGCATCGTCACATGAATGGC RV: GGCGTTGTACAGGTCCTTGA	59.97
Glutathione peroxidase	*gpx1*	FW: AAGGAGCAGTTGCCTTACCC RV: AAAGGGCCTGTCTACTTGGC	59.96
Superoxide dismutase 1	*sod1*	FW: TGAAGGTGTGGGGAAGCATT RV: CATCGGCCACACCATCTTTG	59.5
Superoxide dismutase 2	*sod2*	FW: TGGCTGGGTTTCGAGAGAGA RV: GTAGGCATGCTCCCAGACAT	60
Cholecystokinin	*cck*	FW: GCGCCAACCACAGGATAAAA RV: ACAGAAGTCGGGGCAAAATCT	60
Somatostatin receptor 1	*sstr1*	FW: CCTCAGCATTGACCGCTACA RV: AATACCGCCATCCACTGACG	60
Tyrosine hydroxylase	*th*	FW: GGACCAGATGTACCAGCCAG	59

		RV: GCAGTTCATCCCTCGCAGAT	
Histidine decarboxylase	*hdc*	FW: GCATTTCGACTGCACTGCTT RV: CTTCGGCTGAGTGGGATCTG	59
Corticotropin releasing hormone	*crh*	FW: AACGTCAACAGGGCTTTCCA RV: TCTTCCCGTCAGGTCTTCCA	60
5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled	*htr1a*	FW: AAGCGCAGTGTGGAACCTAA RV: GCTGTCGGGGTATTAGGCAG	60
Arginine vasopressin induced 1	*avpi1*	FW: AGGGAGGACCACTGAAGATGA RV: CCAGCAGAGGACAAGTCTGC	60
Tryptophan hydroxylase 1	*tph1*	FW: CCCCCGTATCGAGTTCACAG RV: AGGGGCAGGTTCTTGAGGTA	60

Table 2.- Growth performance and feed utilization indexes (mean ± standard deviation, *SD*) of *A. tropicus* larve with different density, feed regimen, and interaction.

Density	**D7**		**D14**		Multifactorial Anova				
Feeding regime	**Fed**	**Non-fed**	**Fed**	**Non-fed**	Source of variation	df	MS	*F*	*P*

Final weight (g)	0.025±0.0002b	0.024±0.0001c	0.028±0.0008a	0.026±0.0002b	Density	1	0.0000259006	111.35	0.0000
					Feeding regime	1	0.0000140098	60.23	0.0000
					Density × feeding regime	1	0.00000185666	7.98	0.0153
Final total length (cm)	1.85±0.032b	1.75±0.013c	1.92±0.030a	1.76±0.018c	Density	1	0.00717629	11.13	0.0059
					Feeding regime	1	0.0682419	105.83	0.0000
					Density × feeding regime	1	0.00335005	5.20	0.0417
SGR	3.31±0.32b	2.30±0.13c	4.03±0.30a	2.43±0.18c	Density	1	0.717629	11.13	0.0059
					Feeding regime	1	6.82419	105.83	0.0000
					Density × feeding regime	1	0.335005	5.20	0.0417
Survival (%)	46.19±2.74a	19.04±2.19c	43.095±2.25a	27.14±1.16b	Density	1	24.9929	5.31	0.0399
					Feeding regime	1	1857.26	394.72	0.0000
					Density × feeding regime	1	125.211	26.61	0.0002

Aggressivity (%)	24.758±0.825b	11.747±0.449c	26.668±0.824a	8.675± 0.183d	Density	1	1.07854	2.86	0.1251
					Feeding regime	1	768.965	2038.18	0.0000
					Density × feeding regime	1	19.861	52.64	0.0000
CV	13.56±1.80a	5.12±0.37b	5.59±0.24b	10.54±1.24a	Density	1	4.87994	3.90	0.0836
					Feeding regime	1	9.10756	7.29	0.0271
					Density × feeding regime	1	134.505	107.60	0.0000
SH	0.81±0.10a	0.30±0.02c	0.33±0.01c	0.63±0.07b	Density	1	0.0177142	3.90	0.0836
					Feeding regime	1	0.0330605	7.29	0.0271
					Density × feeding regime	1	0.488252	107.60	0.0000

SGR: specific growth rate, CV: Coefficient of variation, SH: Size heterogeneity. Significant differences within the treatment are indicated by different letters ($p < 0.05$).

Table 3.- Digestive enzymatic activities (mean ± standard deviation, SD) of *A. tropicus* larvae with different density, feed regimen, and interaction.

Density	**D7**	**D14**	Multifactorial Anova

Feeding regime	**F**	**NF**	**F**	**NF**	Source of variation	df	MS	*F*	*P*
Acid protease	0.0812±0.0002d	0.129±0.0001a	0.0996±0.0005c	0.1034±0.0002b	Density	1	0.0000403872	251.07	0.0000
					Feeding regime	1	0.00201026	12496.74	0.0000
					Density × feeding regime	1	0.00145864	9067.60	0.0000
Alkaline protease	0.0301±0.0018d	0.0409±0.0001b	0.0361±0.0001c	0.0514±0.0003a	Density	1	0.000205035	146.77	0.0000
					Feeding regime	1	0.000514233	368.09	0.0000
					Density × feeding regime	1	0.0000147567	10.56	0.0117
Trypsin	0.616±0.158b	1.118±0.057a	1.236±0.123a	1.131±0.066a	Density	1	0.300606	25.00	0.0011
					Feeding regime	1	0.117964	9.81	0.0140
					Density × feeding regime	1	0.275553	22.92	0.0014
Leucine aminopeptidase	0.329±0.029b	0.361±0.032b	0.499±0.023a	0.468±0.033a	Density	1	0.0577524	63.19	0.0000
					Feeding regime	1	0.0000013622	0.00	0.9702
					Density × feeding regime	1	0.00292993	3.21	0.1112
Lipases	0.0725±0.0001d	0.1874±0.0057b	0.1516±0.0003c	0.2223±0.0037a	Density	1	0.00972427	545.28	0.0000
					Feeding regime	1	0.0258206	1447.87	0.0000
					Density ×	1	0.00146748	82.29	0.0000

					feeding regime				

Enzymatic activities: u/mg protein. Significant differences within the treatment are indicated by different letters ($p< 0.05$).

Table 4.- Antioxidant enzyme activities (mean ± standard deviation, SD) of *A. tropicus* larvae with different density, feed regimen, and interaction.

Density	**D7**		**D14**		Multifactorial Anova				
Feeding regime	**F**	**NF**	**F**	**NF**	Source of variation	df	MS	*F*	*P*
GPX	55.185±6.003bc	68.960±1.470b	95.51±9.008a	32.980±1.385c	Density	1	9.43951	0.31	0.6033
					Feeding regime	1	1188.53	39.20	0.0033
					Density × feeding regime	1	2911.23	96.02	0.0006
CAT	6.457±0.017b	6.885±0.038a	6.270±0.017c	6.277±0.023c	Density	1	0.473721	710.43	0.0000
					Feeding regime	1	0.117964	9.81	0.0140
					Density × feeding regime	1	0.275553	22.92	0.0014
SOD	20.745±1.138ab	17.410±2.602b	26.61±2.276a	18.042±2.520b	Density	1	23.0277	5.87	0.0599
					Feeding regime	1	77.2743	19.71	0.0068
					Density × feeding regime	1	14.934	3.81	0.1085

GPX: Glutathione peroxidase, CAT: Catalase, SOD: Superoxide dismutase. Significant differences within the treatment are indicated by different letters ($p< 0.05$).

Table 5.- Morphological analysis (mean ± standard deviation, SD) of the gut and liver in *A. tropicus* larvae with different density, feed regimen, and interaction.

Density	**D7**		**D14**		Multifactorial Anova				
Feeding regime	**F**	**NF**	**F**	**NF**	Source of variation	df	MS	*F*	*P*
Area MMC (%/area)	1.361±0.498d	11.368±0.268a	3.089±0.099c	8.507±0.439b	Density	1	15.7941	120.70	0.0000
					Feeding regime	1	178.436	1363.59	0.0000
					Density × feeding regime	1	0.96167	7.35	0.0266
Hepatocytes area (μm^2)	5.738±0.207b	3.778±0.510c	7.422±0.237a	3.281±0.626c	Density	1	3.56939	18.97	0.0024
					Feeding regime	1	27.9127	148.37	0.0000
					Density × feeding regime	1	1.05787	5.62	0.0452
Enterocytes height (µm)	11.222±0.132b	9.468±0.683c	12.479±0.305a	9.262±0.058d	Density	1	1.60562	11.04	0.0105
					Feeding regime	1	18.5396	127.49	0.0000
					Density × feeding regime	1	0.829144	5.70	0.0440

MMC: Melanomacrophage centres. Significant differences within the treatment are indicated by different letters ($p< 0.05$).

Table 6.- Multifactorial analysis summary in metabolic and aggressive relative expression of *A. tropicus* larvae with different density, feed regimen, and interaction.

Relative expression metabolic genes	Factor	Multifactorial analysis			
		df	MS	*F*	*P*
cat	Density	1	0.00209279	44.24	0.0002
	Feeding regime	1	0.000209147	4.42	0.0687
	Density × feeding regime	1	0.000343212	7.25	0.0273
cck	Density	1	0.0826263	13.49	0.0063

	Feeding regime	1	0.027411	4.48	0.0671
	Density × feeding regime	1	0.604293	98.69	0.0000
gpx	Density	1	0.308879	9.21	0.0162
	Feeding regime	1	0.117234	3.49	0.0985
	Density × feeding regime	1	0.313065	9.33	0.0157
sod1	Density	1	0.00000002	34.26	0.0004
	Feeding regime	1	0.00000002	32.65	0.0004
	Density × feeding regime	1	0.00000015	244.14	0.0000
sod2	Density	1	0.0000037	28.97	0.0007
	Feeding regime	1	0.0000077	59.47	0.0001
	Density × feeding regime	1	0.0000031	24.25	0.0012
Aggressive genes					
avpi1	Density	1	0.0052414	8.84	0.0178
	Feeding regime	1	0.206349	347.90	0.0000
	Density × feeding regime	1	0.471485	794.91	0.0000
crh	Density	1	0.51638	234.70	0.0000
	Feeding regime	1	0.0170623	7.75	0.0237
	Density × feeding regime	1	0.299845	136.28	0.0000
hdc	Density	1	0.000203005	0.10	0.7600
	Feeding regime	1	0.00173602	0.85	0.3823
	Density × feeding regime	1	0.00590327	2.91	0.1266
htr1	Density	1	4.6808	1050.43	0.0000
	Feeding regime	1	2.1192	475.57	0.0000
	Density × feeding regime	1	0.125914	28.26	0.0007
sstr1	Density	1	0.485125	40.63	0.0002
	Feeding regime	1	0.0000662648	0.01	0.9424
	Density × feeding regime	1	0.285941	23.95	0.0012
th	Density	1	0.106078	9.52	0.0150
	Feeding regime	1	0.117911	10.58	0.0116
	Density × feeding regime	1	0.401061	35.99	0.0003
tph1	Density	1	0.0241889	52.52	0.0001
	Feeding regime	1	0.058161	110.33	0.0000
	Density × feeding regime	1	0.0520085	112.92	0.0000

Figure legends

Figure 1. Growth in weight (g) (a), total length (cm) (b), survival and aggressivity (%) (c) of *A. tropicus* larvae in different density and feed regiment. Values are mean ± SD. Significant differences within the treatments are indicated by different letters ($p < 0.05$).

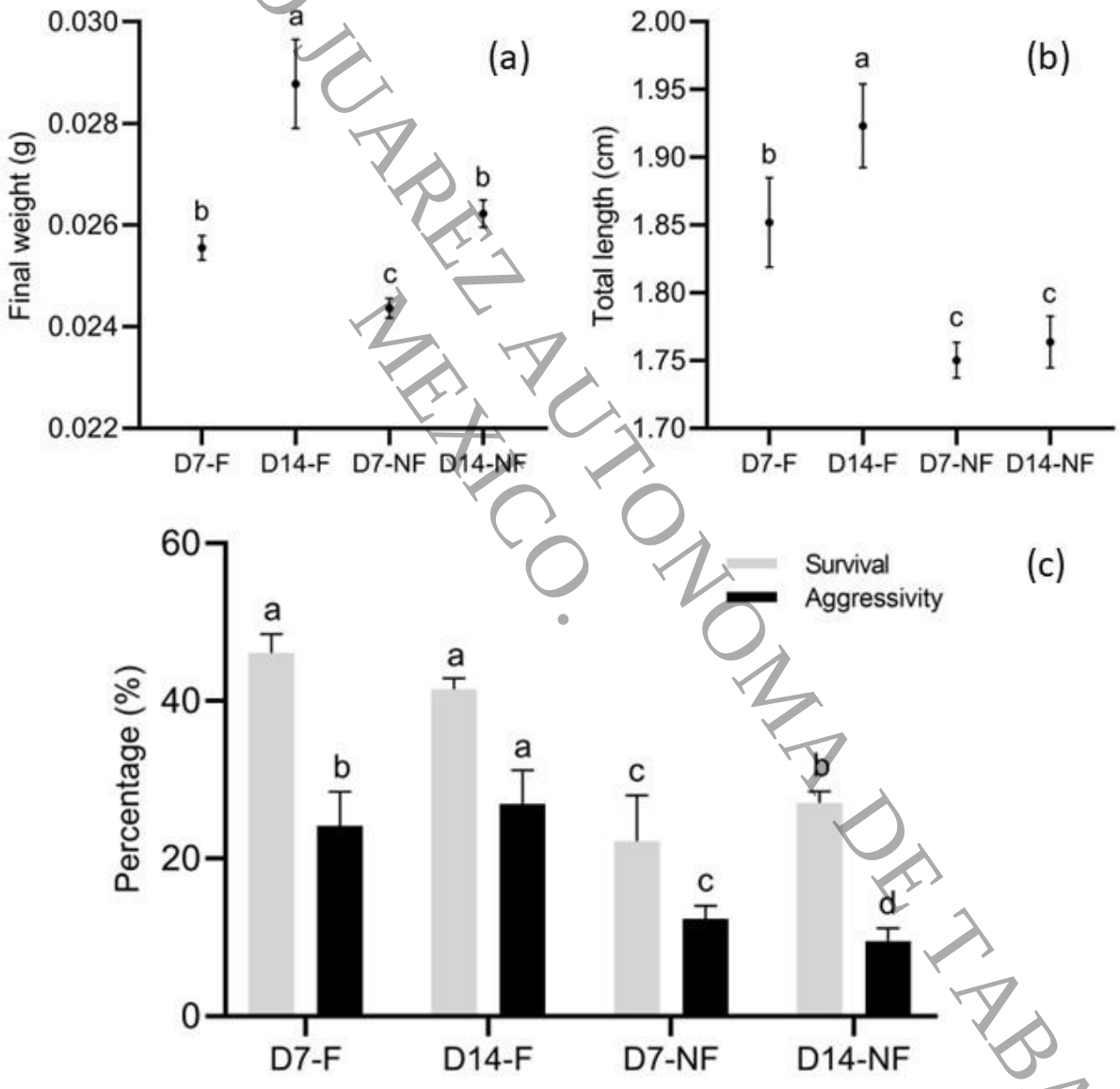


Figure 2. Aggressivity effect in *A. tropicus* larvae culture: (a) Survival curve of *A. tropicus* larvae with different density and feed regiment. (b) Development of Aggressivity in *A. tropicus* larvae during the trial versus the time on each treatment.

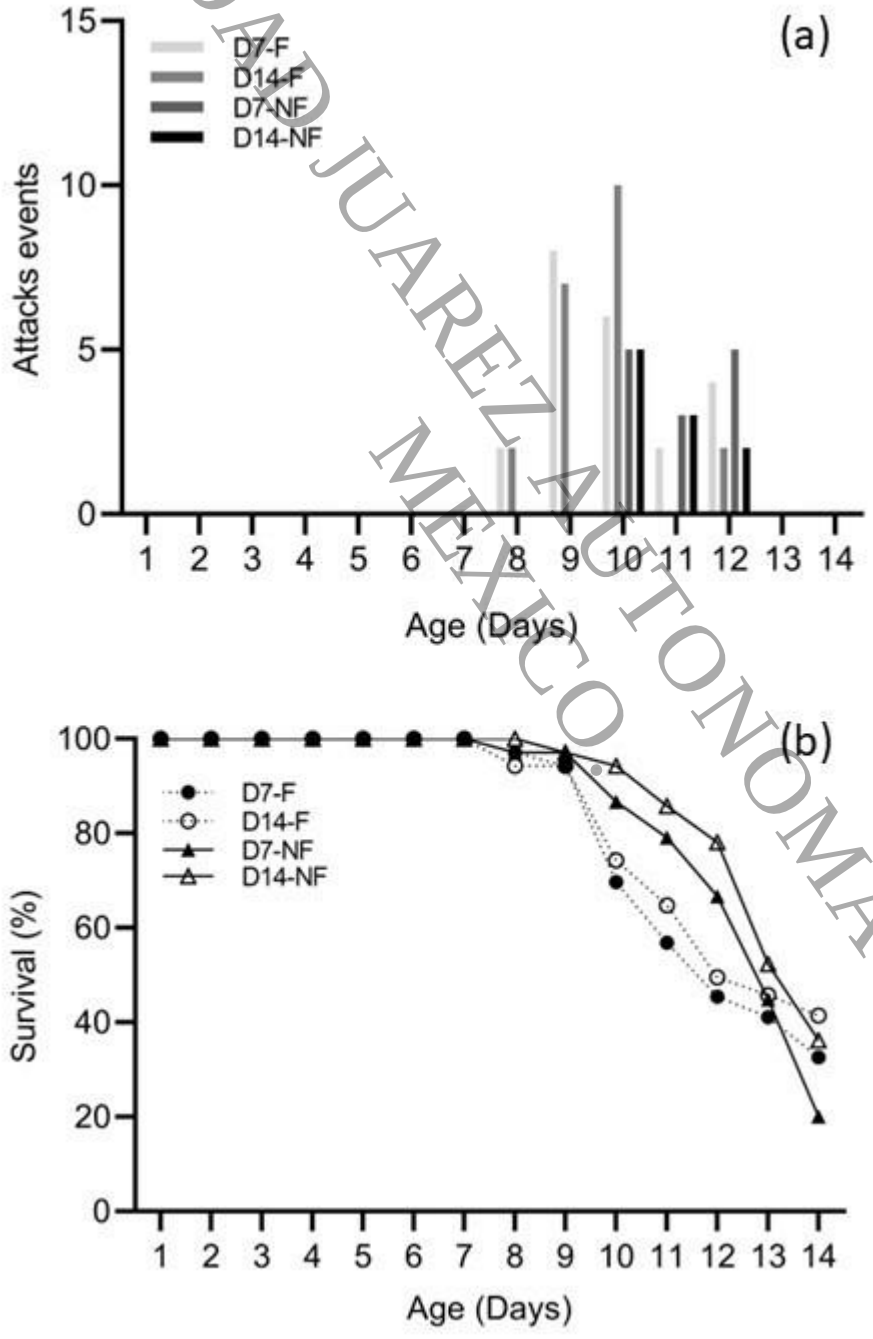


Figure 3. Differences between attacker (cannibal) and attacked (prey) of *A. tropicus* larvae in weight and total length (n = 30, values are mean ± *SEM*). Significant differences within the larvae are indicated by asterisk ($p < 0.05$).

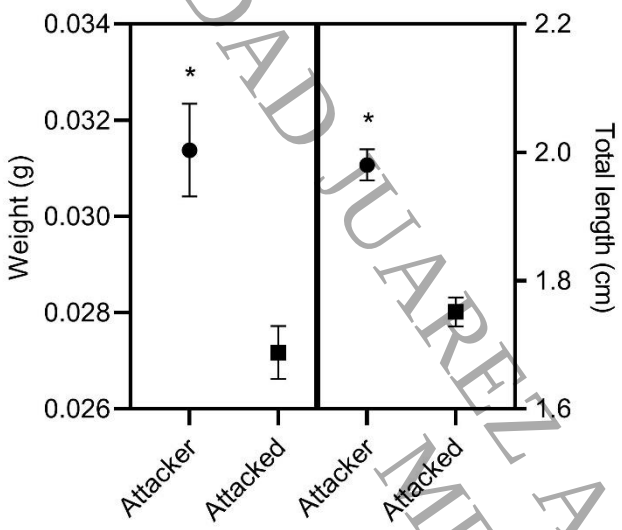


Figure 4. Representative histological images of the liver in *A. tropicus* larvae. Morphological analysis of the liver in *A. tropicus* larvae: (a) D7-NF, (b) D14-NF, (c) D7-F, (d) D14-F. MMC (%/Area) (black arrow), hepatocytes area (μm^2) (yellow arrow) (H&E). All the microphotograph has the same scale bar (10 µm).

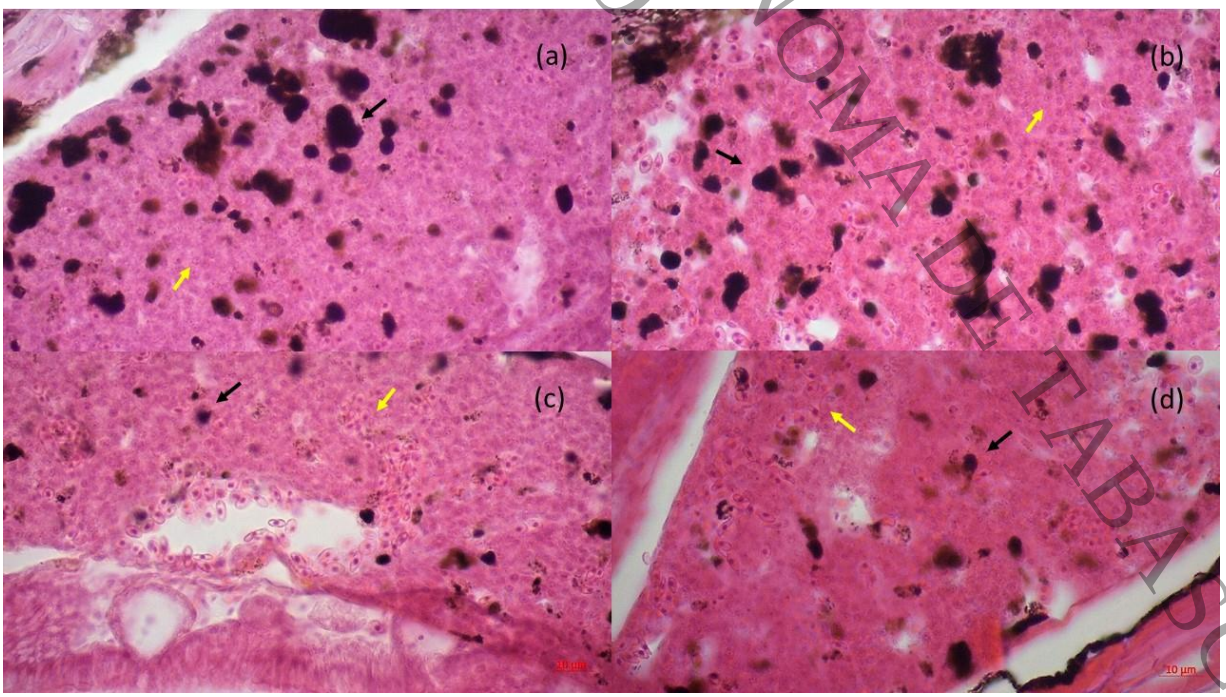


Figure 5. Representative histological images of the digestive system in *A. tropicus* larvae. Morphological analysis of the gut in *A. tropicus* larvae: (a) D7-NF, (b) D14-NF, (c) D7-F, (d) D14-F. Enterocytes height (µm) (black arrow) (H&E). All the microphotograph has the same scale bar (50 µm).

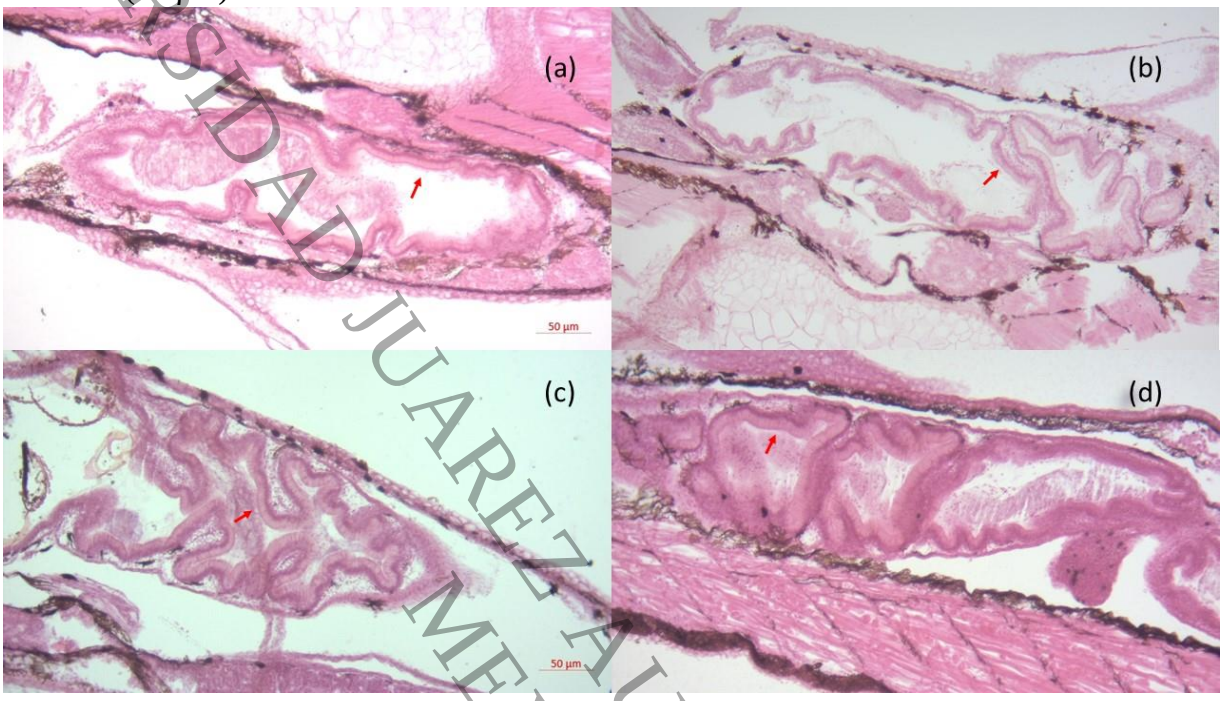


Figure 6. Relative metabolic gene expression in *A. tropicus* larvae with different density and feed regiment. *cck, gpx, cat, sod1 y 2*. Relative mRNA levels of the indicated genes were measured by RT-qPCR using β-actin as the reference gen. Data are presented as fold-changes in the mRNA levels. (n = 3, mean ± *SD*). Significant differences with respect to all treatments are indicated by different letters ($p < 0.05$).

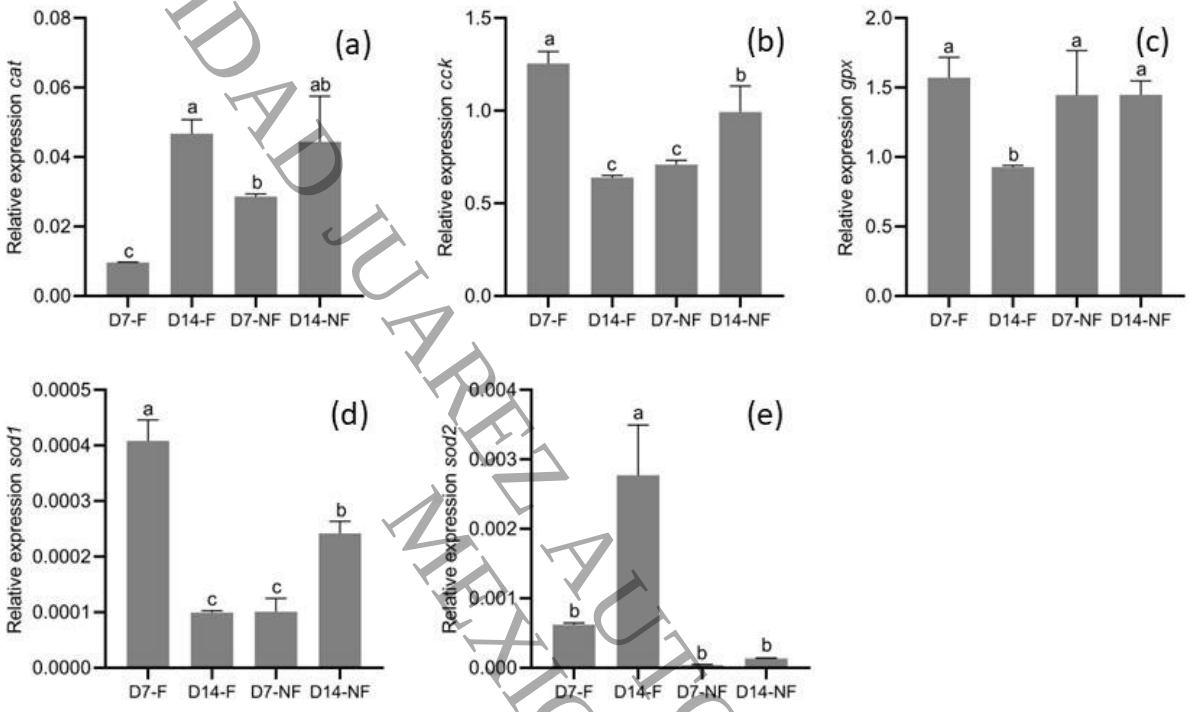
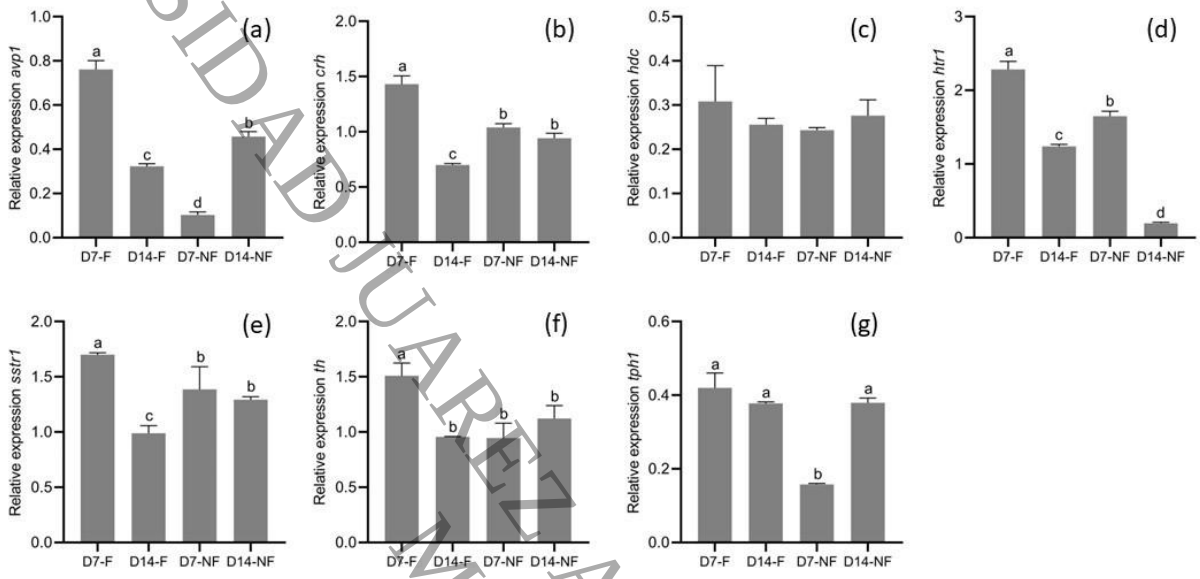
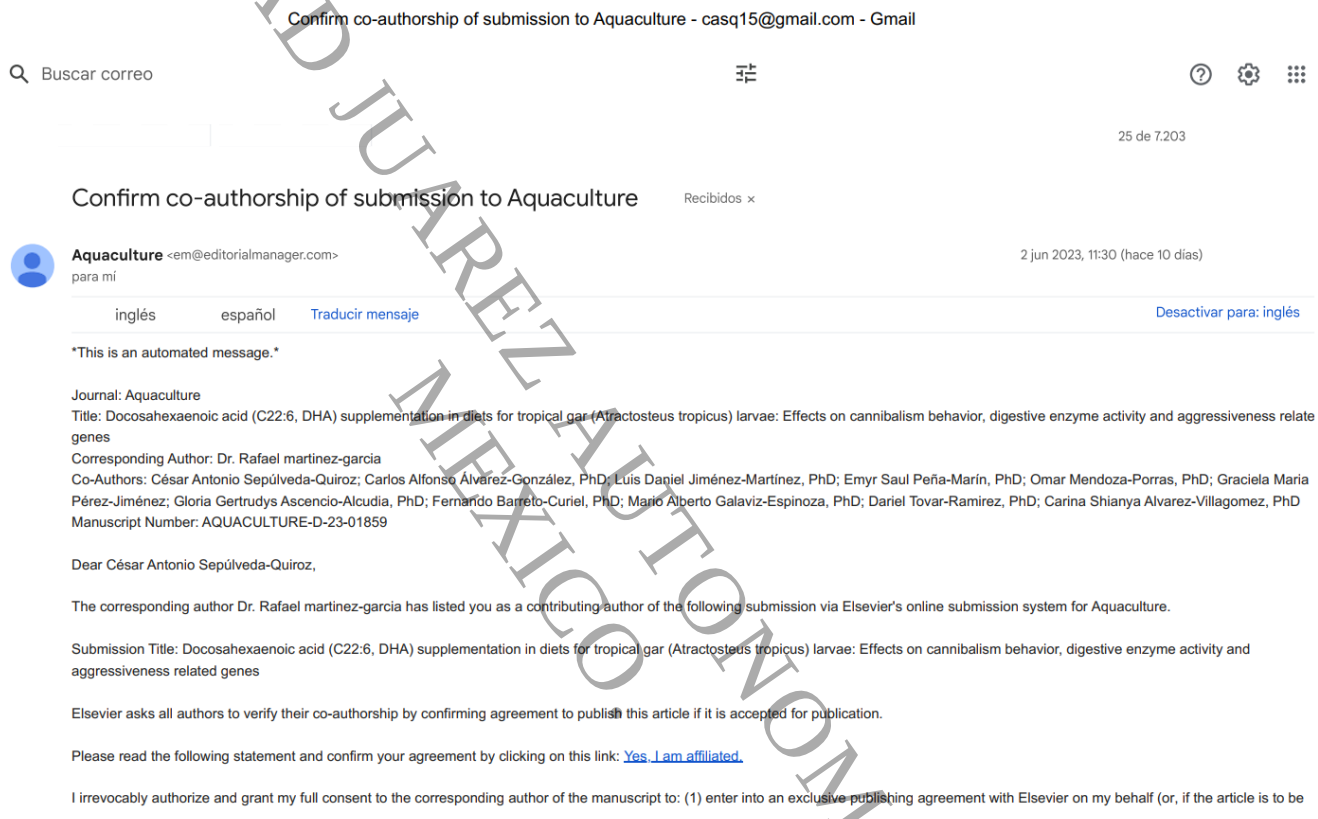


Figure 7. Relative aggressiveness gene expression in *A. tropicus* larvae with different density and feed regiment. *sstr1*, *th*, *hdc*, *crh*, *htr1a*, *avpi1*, and *tph1*. Relative mRNA levels of the indicated genes were measured by RT-qPCR using β-actin as the reference gen. Data are presented as fold-changes in the mRNA levels. (n = 3, mean ± *SD*). Significant differences with respect to all treatments are indicated by different letters ($p < 0.05$).



Capítulo 3: Docosahexanoic acid (C22:6, DHA) supplementation in diets for Tropical gar (*Atractosteus tropicus*) larvae: Effects on cannibalism behavior.



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Corresponding Author: Dr. Rafael martinez-garcia
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Docosahexaenoic acid (C22:6, DHA) supplementation in diets for tropical gar (*Atractosteus tropicus*) larvae: Effects on cannibalism behavior, digestive enzyme activity and aggressiveness related genes

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Abstract

Cannibalism is a behavioral problem that significantly affects growth, survival, and welfare in fish larviculture settings. Aquaculture of tropical gar (*Atractosteus tropicus*), an ancient species, has grown as an industry in the last decades, however, cannibalism negatively affects production. Here, we evaluated growth, survival, cannibalism, behavior, digestive enzyme activity, and genes related to aggression and/or cannibalism in *A. tropicus* larvae fed a diet supplemented with docosahexaenoic acid (DHA) (20,30,40 g/Kg Algamac 3050®) compared to larvae fed a control diet (CD). These diets were tested in larvae at 0-13 days post hatching (DAH) (first stage) and in a cohort of cannibalistic larvae at 14-24 DAH (second stage). In the first stage, the 40 g/Kg DHA treatment showed trends in survival (30.5 ± 4.94 %) and in cannibalism reduction (64.37 ± 0.87 %) with respect to CD (28.25 ± 1.76%; 64.75 ±1.76%). This treatment showed higher lipase activity. CD presented higher alkaline protease and leucine aminopeptidase activity. The difference in weight and size between cannibal and non-cannibal larvae was 9.18 ± 9.49 % (0.002 ± 0.002 g) and 6.74 ± 4.82 % (0.119 ± 0.085 cm) respectively. Cannibals and non-cannibals showed significant differences in enzymatic activity. In cannibalistic larvae, the increase of DHA (30 and 40 g/Kg DHA) in the diet induced overexpression in *crh, hdc, htr1, tph1* and *sstr1*. In the second stage, survival of the 40 g/Kg DHA (35.00 ± 7.07 %) cohort was significantly higher compared to CD (23.33 ± 5.77 %) while cannibalism was lower in 40 g/Kg DHA (68.33 ± 7.63%) compared to CD (76.66 ± 5.77%). Significant differences were found in alkaline protease and trypsin between the CD and 40 g/Kg DHA treatments. Cannibal larvae fed with 40 g/Kg DHA presented overexpression of *avp1, crh, gnrh, sstr1, htr1, th,* and under expression in *hdc* and *tph1* compared to CD ($p < 0.05$). Additionally, the use of artificial vegetation eliminated attacks between cannibals. These results are essential to develop strategies to prevent and/or mitigate cannibalism at larval stage and improve *A. tropicus* aquaculture.

Keywords: Mitigation, Survival, Fish larvae, Cannibalism, Behavior.

1. Introduction

Fish cannibalism has been described in various species and occurs more frequently in larvae and juveniles (Pereira et al., 2017). There are various types of fish cannibalism in the wild (e.g. eggs cannibalism, filial cannibalism), however, in a controlled environment such as aquaculture, occurs in relation to the age of the prey and cannibal, for example: intracohort (fish of the same age) or intercohort (fish of different ages) cannibalism (Smith and Reay, 1991). These types of cannibalism are responsible for high mortalities due to attacks and injuries among the fish that negatively affects the profitability of aquaculture (Król and Zieliski, 2015; Naumowicz et al., 2017). As such, cannibalism is a bottleneck for the exploitation of more and diverse fish species. Population factors (genetic background, sex, hatching, size of larvae, density, and domestication) and environmental factors (feed, light conditions, temperature, color and shape of tanks, supplementation, and refuges) are known stressors for fish that result in cannibalism as an aggressive behavior (Naumowicz et al., 2017). Therefore, there is a need to study the molecular routes or genes underlying aggressive behavior in fish. For example, the role of neurotransmitters as biochemical messengers is related to behavior regulation in the presence of external stimuli (Beaver and Walsh, 2011). Differential regulation of neurotransmitter such as dopamine, serotonin (5-HT), histamine, adrenaline, gamma-aminobutyric acid (GABA), and glutamate (GLU) are known to regulate aggressivity (Winberg and Nilsson, 1993; von Bohlen and Dermietzel, 2002; Cooper et al., 2003; Popova, 2006). Other neuromodulators that include endorphins, gonadotropin-releasing hormone (GnRH), nitric oxide (NO) and vasopressin play, in certain cases, roles of neurotransmitters and are associated to behavior (von Bohlen and Dermietzel, 2002). Around eight metabolic pathways: hypothalamic-neuropituitary system (HNS), serotonin (5-HT), somatostatin, dopamine, histamine, nitric oxide, hypothalamic-pituitary-intrarenal (HPI) and hypothalamic-pituitary-gonadal (HPG) have been linked to aggressive behavior in fish and to expression of specific genes (Filby et al., 2010). About 77 genes associated to aggressivity have been identified in zebra fish (*Danio rerio*) (Malki et al., 2016). Factors such as social hierarchy, sex and stress are related to the modulation of gene expression related to aggression (Chen and Fernald, 2011; Norton et al., 2011; Theodoridi et al., 2017). However, there is a knowledge gap in understanding how expression of specific genes modulates aggressiveness and cannibalism in more fish species.

In this context, tropical gar (*Atractosteus tropicus*) as an ancient fish (*Lepisosteidae*) belongs to the *Atractosteus* genus as well as alligator gar (*Atractosteus spatula*) and Cuban gar (*Atractosteus tristoechus*). *A. tropicus* is distributed in the southeast of Mexico and part of Central America where is considered a species of high importance due to its economic value and its ecological role (Márquez-Couturier and Vázquez-Navarrete, 2015; Nelson, 2006). The most recent research on *A. tropicus* has focused on nutritional aspects, such as the use of optimal ingredients for the formulation and preparation of diets (Guerrero-Zarate et al., 2019; Frías-Quintana et al., 2010). In specific, inclusion of prebiotics like mannan oligosaccharides (MOS), β-Glucans, and fructooligosaccharides (FOS) that have been deemed beneficial feed additives (Nájera-Arzola et al., 2018; Nieves-Rodríguez et al., 2018; Sepúlveda-Quiroz et al., 2020). Also, the development of the digestive tract and activity of digestive enzymes have been described in *A. tropicus* (Frías-Quintana et al. 2015; Guerrero-Zarate et al., 2014). In combination, this research has generated fundamental knowledge for the improvement of *A. tropicus* aquaculture. However, cannibalism at the larval stage (10 DAH) remains a challenge. Cannibalism occurs in other fish species including Atlantic cod (*Gadus morhua*), grouper (*Epinephelus coioides* and *E. lancelatus*), barramundi (*Lates calcarifer*), common snook (*Centropomus undecimalis*), Eurasian perch (*Perca fluviatilis*), and African catfish (*Heterobranchus longifilis*) (Adams and Wolfe, 2006; Baras et al., 2014; Folkvord, 1992; Hseu, 2002; Hseu et al., 2004; Król et al., 2019; Qin et al., 2004). This behavior has been detected in both wild and captive organisms (Márquez, 2000; Aguilera et al., 2012). Frías-Quintana et al. (2017) reported survival and cannibalism values of 24% and 33% under laboratory conditions. This result generates low confidence in the aquaculture of the species, due to the high risk of economic loss. To try to reduce this problem, Palma-Cancino et al. (2019) implemented a co-feeding strategy (commercial diet and *Artemia*) during the larval stage of *A. tropicus*, however, cannibalism was observed at complete intake (at 11 DAH) and incomplete (at 21 DAH) levels that reduced survival to 33%. It was observed that cannibals performing complete ingestion and preys affected by incomplete cannibalism did not survive due to body damage. As recently indicated, *A. tropicus* cannibalism is related to morphometric conditions that allow swallowing preys of its own size from 10 DAH (Sepúlveda-Quiroz et al., 2023). Likewise, ethological studies identified zones of preferential attack wherein the use of artificial shelters (artificial vegetation) decreased attack numbers while a greater number of

attacks were observed when a white background was implemented (Sepúlveda-Quiroz et al., 2023).

Long chain polyunsaturated fatty acids (LC-PUFA) have great relevance in nutrition of fish larvae in the form of energy reserves, structural molecules, metabolic regulators, homeostatic processes, stress response and immune system (Turchini et al., 2022). Docosahexaenoic acid (DHA) 22:6 n-3 is a long-chain polyunsaturated fatty acid (n-3 series), considered an essential fatty acid for development in fish (Watanabe, 1993). Recently, it was reported that supplementation of *Aurantiochytrium sp.* with high DHA content led to cannibalism reduction in orange-spotted groupers (*E. coioides*), mainly due to the relationship of DHA with the regulation of 5-HT (serotonin) in the brain of the grouper (Chang et al., 2019). As such, DHA is a candidate for the mitigation of *A. tropicus* cannibalism.

The objective of this wok was to evaluate the effect of DHA over cannibalistic behavior when supplemented at 20, 30, 40 g/Kg in the diet of tropical gar larvae (*A. tropicus*). Likewise, the rate of cannibalism, survival, growth, digestive enzyme activity, expression of genes related to neurotransmitters linked to behavior were analyzed.

2. Material and Methods

2.1. Biological material

Larvae from *A. tropicus* were obtained by spawning induction of a female using the LHRHa hormone (30 µg/Kg). Subsequently, three males and a female were placed in a circular tank of 2000 L capacity where spawning occurred 17 h later. Broodstock from Aquatic Resources Physiology Laboratory (LAFIRA) from DACBiol-UJAT was utilized for this experiment.

2.2. Experimental design

In the first stage, DHA rich Algamac® was supplemented in the diet at 20, 30, 40 g/Kg to enable comparisons against a control diet (CD) without DHA. Larvae (n = 200, weight: 0.018 ± 0.001 g; total length: 1.28 ± 0.09 cm) were placed in circular-shaped 70 L tank. Each treatment was carried out in triplicate. The culture tanks were connected to a recirculation system powered by a 0.5-HP water pump (Jacuzzi, JWPA5D-230A, Delavan, WI) and a 1,500 L reservoir for solids deposition and biological filter. The feeding regime was as follows: after

the absorption of its yolk sac (3 days after hatching, DAH), the diet corresponding to each treatment and *Artemia* nauplii were administered for additional five days. At the end of the co-feeding, they were manually fed only with the formulated feed, four times per day (8:00, 12:00, 16:00, 20:00 hrs) *ad libitum*. A partial daily exchange of 10% water was carried out by siphoning to collect feces and uneaten food. The water quality parameters were daily monitored (mean ± standard deviation, SD) with temperature (27.36 ± 0.6°C), dissolved oxygen (4.8 ± 0.4 mg/L, oximeter YSI 85; OH), pH (7.1 ± 0.3, HANNA HI 991001, Romania). In the second stage, feeding continued with (40 g/Kg DHA) and the control diet (CD) where only larvae identified as cannibals were used. In this stage, 10 larvae were used per treatment replicate (3 replicates). Weight and size were recorded at the beginning and end of the bioassay (14-24 DAH). Quantification of cannibalism and collection of samples for enzymatic activity and gene expression were collected in both stages.

2.3. Formulation and preparation of the experimental diets

The formulation of the diets was carried out by using the software MIXITWIN v.5.0. (Microsoft Windows, Redmond, WA) following Álvarez-González et al. (2001). The macronutrients were weighed and mixed, followed by the incorporation of micronutrients. This was followed by addition of liquid ingredients. To achieve an adequate mixture, water was added 400 ml/Kg per diet and mixed 15 minutes with each addition (total 60 min of mixing time per diet). The mixture was passed through a meat grinder (Torrey, M-22RI, Monterrey, N.L, México) pellets were oven dried at 55°C for 12 hr (Coriat, HC-35-D, CDMX, México). Finally, the pellets were manually ground and sieved to particles smaller than 0.5 mm (used during co-feeding), and larger than 0.7 mm (used after co-feeding). Diets were stored in hermetic plastic bags at -20°C for later use. In all diets, the proximal components (moisture, ash, lipids, and protein) were analyzed according to AOAC (2000) (Table 1), lipids (Table 2), and amino acids (Table 3).

2.4. Growth indexes and feed quality

Weight and length were measured: at the beginning of the experiment (3 DAH) and at the end of the experiment (13 DAH) as well as in the second phase (14-24 DAH). The individual weight of each organism was determined by using an analytical balance (A&D Company, Limited mod.HR-250AZ, Korea). The total length was calculated by analyzing the

photographs taken of the organisms, through a transparent container with a scale using the software ImageJ 1.51j8 (U.S. National Institutes of Health, Bethesda, MD). The following biometrics were calculated as follow: Survival (S): (final fish number/initial fish number) × 100; feed intake (FI): total feed intake per experimental unit/number of rearing days; absolute weight gain (AWG): final weight (g) - initial weight (g), specific growth rate (SGR): [(ln final weight − ln initial weight)/days] × 100, feed conversion rate (FCR): (feed intake, g dry matter)/(fish weight gain, g); condition factor (K): [(wet weight (g) × total length−3 (cm)] × 100 and protein efficiency ratio (PER): fish live weight gain (g)/dry protein fed (g). Visual deformed organisms (scoliosis, crossbite, pugheadness, lower jaw reduction, and without eyes) were identified and counted by monitoring organisms with erratic swimming, high pigmentation, and visible malnutrition. These organisms were collected after death, and the deformity was confirmed visually using a stereomicroscope (Carl Zeiss mod. Stemi DV4, Germany). The percentage of deformed organisms was calculated using the following formula: Deformity (D) (fish with deformity / initial fish number) × 100. Also, the coefficient of variation (%) (CV): (standard deviation of individual weight/ mean individual weight) × 100, and size heterogeneity (weight) (SH): (final coefficient of variation/ initial coefficient of variation) were calculated. A frequency graph (length and weight) was used to show the changes in the distribution of weight and size of the larvae, using the data of the final biometrics of each stage.

2.5. Collection of biological samples

At the end of the experiment of the first stage, 15 larvae per treatment were euthanized by thermal shock to determine the effect of DHA on growth, digestive enzymes, gene expression, and cannibalism. In the second stage nine larvae per treatment were used. For the analysis of enzymatic activity, the larvae were preserved at -80°C. Finally, to analyze gene expression, the larvae were preserved in RNAlater buffer according to manufacturer´s instructions (Invitrogen, USA) at -80°C. Whole larvae were used in all analyses.

2.6. Cannibalism

In both stages, cannibalism was measured by monitoring larvae 30 min before and 60 min after each feeding. The criteria to determine cannibalism was the following: Attack by bites (a fish attacks another fish by bites without the attacker ingesting the prey), partial

cannibalism (one fish partially eats another fish), and complete cannibalism (one fish entirely eats another fish). These three behaviors are classified as cannibalism. The formula (fish with cannibalistic behavior/initial fish number) × 100) was used to quantify cannibalism in each treatment. The attacking larvae (cannibal) and the attacked larvae (non-cannibal) were counted with weight and size registered. Five larvae were sampled for molecular analysis and five for enzymatic activity, as described earlier.

2.7. Digestive enzyme activity

For the quantification of digestive enzyme, the larvae were manually macerated inside 1.5 ml tubes on ice. For this, 100 mg of tissue were placed in a total volume 0.5 ml and centrifuged at 14,000 *g* at 4°C for 15 min. Supernatant was recovered and stored at -80°C in 30 µl aliquots. Soluble protein was determined using the Bradford assay (1976). For the quantification of acid proteases, 0.5% hemoglobin solubilized in 100 mM glycine-HCl pH 2 buffer was used as a substrate. Alkaline proteases were quantified using 0.5% casein solubilized in 50 mM Tris-HCl and 10 mM CaCl at pH 9 (Sarath et al., 1989). In both assays, the samples were incubated at 37°C, and the reaction was stopped using 0.5 ml of 20% trichloroacetic acid and centrifuged at 16,000 *g* per five minutes. Absorbance was read at 280 nm. The extinction coefficient (ε) to calculate the activity of acid and alkaline proteases was 0.005 ml/µM cm. To quantify trypsin activity, 1 mM BAPNA (Nα-Benzoyl-DL-Arginine-P-nitroanilide) dissolved in 50 mM Tris-HCl was used as a substrate, in pH 8 at 37°C. For trypsin was read at 410 nm using an ε of 8,800 ml/µM cm (Erlanger et al., 1961). The Maroux et al. (1973) method was used to determine the activity of Leucine aminopeptidase where 0.1 M leucine p-nitroanilide dissolved in DMSO with 50 mM sodium phosphate was the substrate, pH 7.2, incubated at 37°C. Absorbance was measured at 410 nm with an ε of 8,800 ml/µM cm. Lipase activity was determined using β-naphthyl acetate (100 mM) dissolved in 50 mM Tris-HCl at as substrate, at pH 7.5 with sodium taurocholate (100 mM) at 37°C. Reaction was stopped with 0.72 N TCA. Fast Blue (100 mM) and a 1:1 ethanol/ethyl acetate mixture was added, and the absorbance was quantified at 540 nm using ε d 0.02 ml/µM cm (Versaw et al., 1989). The enzyme activity was determined using the following equations: units by ml (U/ml) = [Δabs × final reaction volume (ml)] / [ε × time (min) × volume extracted (ml)] −1; specific activity (U/mg protein) = U ml/ mg of soluble protein; the molar extinction coefficient (ε).

2.8. RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from whole larvae samples using Trizol (Invitrogen, Waltham, MA) according to the manufacturer's protocol. RNA concentration and purity of were assessed by the ratio between the absorbance at 260 and 280 nm in a spectrophotometer (Jenway GenovaNano, Cole-Parmer, Staffordshire, U.K.). RNA (1 µg) was reverse-transcribed (RT) using the SuperScript II kit (Invitrogen), with a final volume of 20 µl. RT reactions were performed in a thermocycler (Mastercycler nexus GSX1, Eppendorf AG, Hamburg, Germany). The standard RT program used was as follows: 5 min at 65°C, 10 min at 25°C, 50 min at 42°C (cDNA strand extension), 15 min at 70°C (reverse transcriptase inactivation), and finally, 20 min at 37°C. Somatostatin receptor 1 (*sstr1*), tyrosine hydroxylase (*th*), histidine decarboxylase (*hdc*), corticotropin-releasing hormone (*crh*), 5-hydroxytryptamine (serotonin) receptor 1A G protein-coupled (*htr1a*), gonadotropin-releasing hormone 1 (*gnrh1*), dopamine receptor D1 (*drd1*), arginine vasopressin induced 1 (*avpi1*), and tryptophan hydroxylase 1 (*tph1*) primers for *A. tropicus* were designed from the species transcriptome (NCBI Accession: PRJNA395289) (Martínez-Burguete et al., 2021) (Table 4). These candidate genes were selected due to their influence over aggressive behaviors and interactions with specific neurotransmitters (Filby et al., 2010; de Abreu et al., 2019). The RT-qPCR was performed in a CFX96 Real-Time System (BioRad, Hercules, CA) using 5 µl of EvaGreen Supermix (BioRad), 0.5 µl primers mix (3mM), and 4 µl of cDNA (5 ng μL^{-1}) and 0.5 µl of water for a final volume of 10 µl. The RT-qPCR program was used at: 50°C for 2 min, 95°C 10 s, followed by 40 cycles at 95°C 15 s and 62°C 1 min. *β-actin* was used as the reference gene (Jimenez-Maritnez et al., 2021). Relative gene expression was calculated as fold-change compared with control and using $2-\Delta\Delta Ct$ formula (Livak and Schmittgen, 2001).

2.9. Effect of the mitigant on the ethology of cannibal larvae

To identify the effect of DHA (Algamac®) (40 g/Kg) over cannibalistic behavior and/or ethology in the second stage experiment, cannibal larvae were placed in 15 x 10 x 8 cm fish tanks. To reduce the effect of stress caused by the transference of larvae to experimental tanks, an acclimation time of 15 min was allowed. Subsequently, 15 min video recordings were made (Gopro Hero 7 Silver, USA). Two larvae per tank were evaluated under the following challenges: without shelter, with rocks, and with artificial vegetation. The videos

were analyzed with Tracker 5.1.5 (Free Software Foundation, Inc.) and BORIS 7.9.24 (Friard and Gamba, 2016), allowing to identify and measure aggressive behavior and shelter preference (rocks and artificial vegetation). The same experiment was carried out on CD fed larvae to determine the effects of DHA over cannibalism.

2.10. Statistics analysis

Growth index, survival, cannibalism, digestive enzyme activity, gene expression, and the difference between the cannibal and non-cannibal larvae were tested for normality (Kolmogorov–Smirnov) and homoscedasticity (Barttlet) tests were used. One-way ANOVA was carried out for all the analysis, and when significant differences were found, a posteriori test of unequal N HSD (Tukey) was used. A student's t test was used to compare differences between treatments where applicable. To determine significant gene expression, nonparametric Kruskal–Wallis and Nemenyi posteriori tests were used. All tests were performed using the software Prism V. 9.0 and significant difference were established at p-values of 0.05.

3. Results

3.1. Growth indexes and survival

The average final weight of *A. tropicus* larvae fed the 20 g/Kg DHA supplemented diet was the highest with 0.023 ± 0.0009 g, however, no significant differences were found compared to the rest of the treatments. Similarly, larvae fed the 20 g/Kg DHA treatment presented the largest size (1.79 ± 0.05 cm) but without statistical significance compared to the rest of treatments (Figure 1). The highest survival was registered in the larvae fed with 40 g/Kg DHA (30.5 ± 4.94 %), followed by CD (29.5 ± 2.5 %), 30 g/Kg DHA (27.75 ± 5.30 %), and 20 g/Kg DHA (24.25 ± 2.47 %). Larvae fed with 20 g/Kg DHA showed the highest CV (13.78 ± 2.06) and SH (1.54 ± 0.23) which were significantly different ($p < 0.05$) compared to the other treatments. The distribution of the weight and length of the treatments corresponds to the following way: in weight the first five groups (0.019 - 0.026 g) of nine represent an average of 88.3% of the population (CD: 96.6%; 20 g/Kg: 90%; 30 g/Kg: 83.3%; 40 g/Kg: 83.3%); in length, the first five groups (1.61-1.86 cm) of eight represent an average of 82.85% of the population (CD: 87.71%; 20 g/Kg: 80.95%; 30 g/Kg: 71.42%; 40 g/Kg: 85.71%). In

both distributions it is possible to see a positive asymmetric distribution (Figure 2). No differences were found in AWG, SGR, FCR, PER, and K (Table 5).

3.2. Cannibalism and deformities

Cannibalism occurred at 8 DAH in all treatments. The lowest percentage of cannibalism was observed in larvae fed the 40 g/Kg DHA diet (65.25 ± 2.47 %), and the highest percentage occurred in larvae fed with 20 g/DHA (72.75 ± 5.30 %) diet. There was no significant difference ($p > 0.05$) with respect to the larvae of the CD (64.75 ± 1.76) (Figure 3). The mean percentage of larvae with deformities for all the larvae used was 4.85 ± 1.00 %, and no differences were observed between treatments ($p > 0.05$) (Figure 3). The average weight of larvae identified as cannibals (0.025 ± 0.002 g) was not significantly different with respect to the prey weight (0.024 ± 0.002). The total length of the cannibals was 1.80 ± 0.09 cm, presenting a difference with the total length of the prey of 1.75 ± 0.09 cm (*t-test*, df = 88, $p = 0.0113$). The average difference between the weight of cannibal larvae and their prey was 0.002 ± 0.002 g, which corresponds to 9.18 ± 9.49 %. Regarding the total length, the average difference between the cannibal larvae and their prey was 0.119 ± 0.085 cm, corresponding to 6.74 ± 4.82% (Figure 4).

Based on these results, 40 g /Kg DHA was selected as the best apparent treatment, given the lowest percentage of cannibalism and the highest survival. The treatment continued to be administered only to the larvae identified as cannibals for another 10 days and comparison were made against CD.

3.3. Growth indexes and survival only cannibals

A higher final weight (0.179 ± 0.03 g) was observed in CD fed larvae compared to the 40 g/Kg DHA treatment (0.132 ± 0.03 g). The same trend was observed for total length in both CD (3.68 ± 0.34 cm) and 40 g/Kg DHA (3.35 ± 0.51 cm) groups. For both results, no significant differences were observed (*t-test*, $p > 0.05$) (Figure 5). Survival was significantly higher in larvae fed with 40 g/Kg DHA (35.00 ± 7.07 %) compared to CD (23.33 ± 5.77 %) (*t-test*, $p > 0.05$). The FCR value for the 40 g/Kg DHA treatment was 8.02 ± 1.99, CD recorded 5.66 ± 0.62. On the other hand, CD fed organisms presented the highest CV value (55.08 ± 6.84) with respect to 40 g/Kg DHA (39.14 ± 8.46). The CD treatment presented a significantly

higher SH value (6.07 ± 0.75, *t-test*, $p < 0.0026$), compared to the 40 g/Kg DHA (2.48 ± 0.53). AWG, SGR, FCR, PER, and K did not present significant differences (Table 6).

3.4. Cannibalism

The percentage of cannibalism was higher in the CD fed larvae (76.66 ± 5.77 %) but it was not significant compared to 40 g/Kg DHA fed larvae (68.33 ± 7.63 %) (Figure 6). The distribution of the weight and length of the treatments was as follows: in weight the first three groups (0.052 - 0.136 g) of five represent an average of 83.32% of the population (CD: 77.7%; 40 g/Kg: 88.8%); in length, the first three groups (2.11-3.45 cm) of six represent an average of 72.18% of the population (CD: 66.6%; 40 g/Kg: 77.7%), which could be an effect of cannibalism on the distribution of the population (Figure 7).

3.5. Digestive enzyme activity

At the end of the first stage, the activity of alkaline proteases was significantly higher in CD fed larvae compared to the other treatments. The activity of acid protease did not present any difference. Trypsin activity was significantly higher in the 30 g/Kg DHA fed larvae compared to CD ($p < 0.05$). The activity of leucine aminopeptidase was significantly higher in treatment CD and 30 g/Kg DHA compared to 40 g/Kg DHA that registered the lowest activity. The 30 and 40 g/Kg DHA treatments registered the highest lipase activity compared to CD.

In the second stage, cannibal larvae continued feeding with the 40 g/Kg DHA and CD treatments. The highest activity for alkaline protease was found in 40 g/Kg DHA while trypsin was higher in CD, both activities were significant ($p < 0.05$). Acid protease, leucine aminopeptidase and lipase do not show differences (Table 7).

Significant differences were observed across all treatments regarding enzymatic activity between cannibals and non-cannibals, excluding leucine aminopeptidase (Table 7). Acid protease activity was higher in non-cannibal larvae fed 40 g/Kg DHA ($p < 0.05$). Interestingly, a significant 5-fold-change increase in acid protease was observed in cannibals compared to non- cannibals. Alkaline protease and trypsin had the highest trypsin activity in all treatments, a trend that was also observed in leucine aminopeptidase. In lipases, the non-cannibals of the 20g/Kg DHA treatment had the highest activity.

3.6. Gene expression

The effect of different DHA concentrations over gene expression in cannibals and non-cannibals larvae is shown Figure 8. Non-cannibal larvae showed a decreased expression in all evaluated genes (*avpi1, crh, hdc, htr1a, th, tph1,* and *sstr1*). The response of *sstr1* and *htr1a* appeared dependent on DHA concentration. In cannibals, the increase of DHA (30 and 40 g/Kg DHA) in the diet generated an overexpression in *crh, hdc, htr1a, tph1,* and *sstr1*. In the second stage, the cannibal larvae fed with 40 g/Kg DHA showed significant overexpression of *avpi1, crh, gnrh, sstr1, htr1a,* and *th*, and a sub-expression of *hdc* and *tph1* (Figure 9).

3.7. Behavior

A total of 277 minutes of recording were obtained across 18 videos where a total of a total of 26 cannibalistic events were registered. From these, 17 were defensive behaviors (escapes) while the remaining nine were direct attacks (six in 40 g/Kg DHA and three in CD). Larvae from tanks with rocks as refuge presented 12 events, followed by nine events in the tanks without refuges. Finally, only five events were recorded amongst in tanks with artificial vegetation as a refuge. No attack between larvae was recorded when artificial vegetation was used. Among the attacks, the lateral was the most common (77.77%), the remaining percentage comprised frontal attack (Table 8).

4. Discussion

4.1. DHA and its nutritional contributions

Fatty acids are essential for the optimal development of fish larvae as it contributes to fulfill the energetic requirements demanded by different morphological changes and continuous growth. DHA it is used as an energy source, and it is necessary for various physiological processes (Watanabe, 1993). Our first stage results showed a positive tendency in weight with the larvae fed with DHA treatments. Physiologically it is assumed that *A. tropicus* larvae have the capacity to biosynthesize ARA, EPA, and DHA from fatty acyl desaturases (*fads*) and by elongation of very long-chain fatty acid (*elovl*) that allows fish to meet energy requirements (de la Cruz-Alvarado et al., 2021). During the ontogeny of *A. tropicus* (0-30 DAH), a greater expression of acetyl-CoA carboxylase (*acc1*), fatty acid synthase (*fas*), and carnitine palmitoyl transferase 1 (*cpt1c*) occurs from 0 to 15 DAH while a

decrease in the expression of these genes occurs between 5 and 10 DAH (Jiménez-Martínez et al., 2018). It is at this stage (8 DAH) where cannibalism starts. Furthermore, diet supplementation of vegetable oils (soybean, corn, canola, and olive) as lipid source increased expression of *cpt1c* that leads to fatty acid beta oxidation in *A. tropicus*. This has also promoted a decrease in expression of *fas* and *acc1* that are related to lipogenesis (Jiménez-Martínez et al., 2020). Although our diets were manufactured with optimal lipid concentrations as per *A. tropicus* requirements (Huerta-Ortiz et al., 2018), no significant growth indexes results were observed by the addition of DHA during the first and second stages. This is perhaps explained by the short period of DHA administration, the physiological condition of the organism to biosynthesize DHA or due to cannibalism stress.

The use of DHA as an additive in diets for fish larvae has been widely used, either administered by enrichment of live food (*Artemia*, rotifer) or directly in the diet, These feeding approaches stimulate better growth, enhance specific growth rate (SGR), survival and in some cases pigmentation changes as observed in yellow perch (*Perca flavescens*), meagre (*Argyrosomus regius*), California halibut (*Paralichthys californicus*) (Grayson and Dabrowski, 2022; Vallés and Estévez, 2015; Vizcaíno-Ochoa et al., 2010). Likewise, the importance of administering the adequate percentage of lipids in the diet is essential for optimal growth.

4.2. DHA and its effects on cannibalism

Regarding cannibalism, administration of 2% BL10 (*Aurantiochytrium*) with high DHA content promotes the presence and abundance of HUFAs and 5-HT in the brain of orange-spotted grouper (*Epinephelus coioides*) that associated to cannibalism reduction. Furthermore, it was shown that cannibalism reduction was related to 5-HT levels in brain and serum cortisol while the ratio of n-3/n-6 fatty acids in the brain correlated with 5-HT (Chang et al., 2019). On the other hand, DHA administration did not decrease cannibalism in *Argyrosomus regius*, affecting survival considerably (14%) (Campoverde and Estevez, 2017). In the second stage of the bioassay, the 40 g/Kg DHA fed cohort showed higher survival and cannibalism reduction compared to the CD treatment. Additionally, CV was lower, and SH showed a difference with respect to CD indicating that larvae without DHA presented greater dispersion in size, which could be explained by the higher cannibalism observed in CD. This difference in CV and SH can also be explained by the "jumper" effect,

wherein a cannibal larva rapidly increases its weight and size after ingesting other larvae contributing to size heterogeneity of the culture (Baras et al., 2011). Difference in weight or size between larvae can trigger cannibalism. For example, in Atlantic cod (*Gadus morhua*) cannibalism was triggered by a 1:1.5 body length ratio (Folkvord, 1992), in African catfish (*Heterobranchus longifili*), cannibals attacked preys of inferior size (Baras et al., 2014), in larvae of giant grouper (*Epinephelus lanceolatus*) a difference greater of 30% in length increased the probability of cannibalism (Hseu et al., 2004). For black rockfish (*Sebastes schlegelii*), fry showed a relationship between the cannibalism rate and difference in length, cannibalism increased when the length difference was greater than 35 mm. Contrarily, if the difference is less than 25 mm, the cannibalism rate decreases (Xi et al., 2017). Comparatively, our study showed a low percentage of the difference between cannibal and non-cannibal with regards to weight and size, a condition that can limit the strategy of selecting by size as a preventive strategy of cannibalism.

4.3. DHA and its effect on digestive enzyme activity

Higher activity of digestive enzymes increases the hydrolysis of macronutrients, that facilitates greater bioavailability of micronutrients (Bone and Moores, 2008). Althought *A. tropicus* larvae presents exogenous feeding from 3 DAH, the digestive tract (esophagus, stomach, and intestine) develops between day 7 and 9 DAH which allows them to absorb and use nutrients (Frías-Quintana et al., 2015). This morphological change becomes relevant since the onset of cannibalism in *A. tropicus* larvae is around 8 DAH (Palma-Cancino et al., 2019) and the presence of a higher mouth depth angle (85.63 ± 6.41°) occurs at 10 DAH (Sepúlveda-Quiroz et al., 2023) which is an anatomical characteristic that facilitates swallowing congeners of their own size and weight (intracohort cannibalism).

In our results, DHA treatments influenced enzyme activity compared to CD in the first stage. On the other hand, cannibal larvae only presented a significant difference in alkaline protease and trypsin compared to CD. Similarly, cannibal, and non-cannibal larvae present significant differences in enzymatic activities, except for leucine aminopeptidase.

In our study, cannibals showed greater trypsin activity compared to non-cannibals. Trypsin acts on peptide bonds favoring the hydrolysis of proteins (Moyano et al., 1996). Cannibals fed the highest concentration of DHA presented higher lipase activity compared to

the CD and 20 g/Kg DHA. Lipases facilitate digestion of lipids by denaturation of triacylglycerol to diacylglycerol and, subsequently to monoacylglycerol, (Cahu and Zambonino, 2001). Although the relationship between digestive enzyme components and cannibalism is unknown, this work demonstrated that there are significant differences in enzymatic activities between cannibals and non-cannibals with and without DHA dietary inclusion. This demonstrates that cannibals and non-cannibals have different enzymatic machinery to hydrolyze macronutrients and therefore differential absorption of micronutrients. On the other hand, if cannibalism is considered a stress promoter with similar molecular pathway as aggressiveness, via neurotransmitters (Sahu et al., 2020), *A. tropicus* larvae would present a state of alertness and constant stress due to continuous attacks, persecutions and/or unwanted interactions. These factors could be leading to homeostasis imbalance with consequent alterations in the enzymatic or digestive activity as it occurs with any other stressor factor (Yang et al., 2021).

4.4. Gene expression and metabolic pathways related to aggression

Neuropharmacological research has contributed to identifying the bases of aggressiveness with the purpose of developing interventional strategies to mitigate it (de Abreu et al., 2019). Analysis of different brain regions (hypothalamus, hindbrain, telencephalon, and optic tectum) in dominant-subordinate males-females of zebra fish (*Danio rerio*), identified genes associated to aggressiveness neurological pathways: HNS, 5-HT, somatostatin, dopamine, HPI, HPG, nitric oxide and histamine with greater expression in the hypothalamus and telencephalon. Also, notorious changes in the expression of said genes was due to sexual and hierarchical nature within the species (Filby et al., 2010). Our results indicated that there is a significant difference in gene expression between cannibalistic and non-cannibalistic larvae of *A. tropicus*. Some of the genes are components of the somatostatin, dopamine, histamine, HPI, HNS and 5-HT pathways. Somatostatin is a brain neuromodulator, linked to the regulation of mobility and reproductive aspects. In dominant males of the cichlid (*Astatotilapia burtoni*), somatostatin high expression appeared to regulate aggression (Trainor and Hofmann, 2006), like our results, *sstr1* gene expression was higher in cannibal larvae. On the other hand, arginine vasotocin (AVT) *avpl* (arginine vasopressin-like) in HNS pathway is related to aggression and social interactions (Caldwell et al., 2008). The neurotransmitters

dopamine, norepinephrine and epinephrine are catecholamines that share the same biosynthesis pathway (Beaver and Walsh, 2011). Dopamine (3-hidroxitiamina) is related to biological processes such as movement control, learning, reward system, and long-term memory (Rossato et al., 2009; Arias-Carrión et al., 2010). Regarding aggression, dopamine biosynthesis is activated when the organism is under threat (Ferrari et al., 2003). Histamine is synthesized from the amino acid histidine and produced in cells of neuroepithelial origin. Histamine participates in gastric acid secretion, immunomodulation, muscle contraction, vasodilatation, control of the epithelial and endothelial barrier, as well as being a regulator of behavior (regulation of temperature, anxiety, learning, perception of stress and pain) (Brown et al., 2001; Nuutinen and Panula 2010). Histamine (H3) receptors were identified in aggressive behaviors and anxiety in zebrafish (Reichmann et al., 2020). Its importance lies in the fact that it is a neurotransmitter that regulates serotonin, norepinephrine, dopamine, and acetylcholine (Haas et al., 2008). For example, in dominant male and female zebrafish, HSN, 5-HT, somatostatin, dopamine, histamine, HPI, HPG-related genes were overexpressed, in this case histamine H1 receptors may inhibit serotonin synthesis (5-HT) (Filby et al., 2010). Our results are consistent with gene expression observed during first and second stages (with exception *hdc* and *tph1*) between cannibal and non-cannibal *A. tropicus* larvae. The HPI pathway is related to behavioral and cognitive processes through hormones and neurotransmitters (cortisol and serotonin), in addition, it has a connection with the gut-brain axis (GBA) in fish, wherein cortisol can modify intestinal integrity as a secondary effect after a stress response (Rosengren et al., 2018). The 5-HT is a neurotransmitter responsible for appetite control, reproduction and physiological processes related to the immune system and intestinal homeostasis (Akiyama et al., 1996; Smith and Seddon, 1998; Comai et al. 2020). Additionally, 5-HT has been linked to aggression, reaction to stress, eating, maturation, and sexual behavior (Sahu et al., 2020). In the second stage in our study, cannibalistic larvae fed the 40 g/Kg DHA diet presented an overexpression of *avp1*(HNS), *crh* (HPI), *gnrh* (HPG), *sstr1* (somatostatin), *htr1* (5-HT), and *th* (dopamine), and a subexpression in *hdc* (histamine) and *tph1* (5-HT). These genes are related to aggressive behavior in fish, mice, and humans (de Abreu et al., 2019). The absence of these type of study in fish larvae aggressivity, complicates comparing our results in other fish species. Most of previous fish research addressing behavior and gene expression were carried out using adult fish in reproductive stages, where behavior is

linked to hormonal changes. Our work reports significant gene expression of the same genes but at larval stage, allowing us to understand aggressive behavior in perhaps a hormone free environment to better understand the relation of gene expression and cannibalism at early stages. On the other hand, a possible way to understand cannibalistic behavior in *A. tropicus* larvae is to analyze whether there is any social order or hierarchy in this stage, which may be also linked to cannibalistic behavior and expression of specific genes.

4.5. Changes in aggressive-cannibal behavior on *A. tropicus* larvae

Regarding the use of rocks and artificial vegetation as shelters, no attacks on *A. tropicus* were detected in any of the treatments when artificial vegetation was used. Likewise, Sepúlveda et al. (2023), identified that using artificial vegetation in *A. tropicus* larvae decreased aggressive behaviors when compared to other type of refuge. The decrease of aggressive behavior is most likely influenced by the replication of their habitat zones and reproduction in the natural environment with the use of artificial vegetation. Zhang et al. (2020) used artificial vegetation in juvenile black rockfish in the same way (*Sebastes schlegelii*) and reported a decrease in aggressive behavior and basal cortisol levels, in turn improving the stress response. It has been shown that implementation of enriched environments (replicating conditions of the natural environment) reduced fish stress in laboratory conditions that translated into a reduction of aggressiveness, cannibalism, energy expenditure, injuries, or diseases (Hecht and Appelbaum, 1988; Qin et al., 2004; Näslund and Johnsson, 2014).

5. Conclusion

The inclusion of DHA in the diet of tropical gar (*A. tropicus*) larvae did not influence growth parameters. However, DHA influenced the dispersion of growth and total length, which was lower in larvae fed DHA diets. A high concentration of DHA favored a tendency to increase survival and decrease cannibalism. On the other hand, the enzymatic activity between cannibalistic and non-cannibalistic larvae presented significant differences. In addition, cannibalistic larvae fed only with 40 g/Kg DHA registered an over expression of *avp1*(HNS), *crh* (HPI), *gnrh* (HPG), *sstr1* (somatostatin), *htr1* (5-HT), and *th* (dopamine), and a sub-expression of *hdc* (histamine) and *tph1* (5-HT). These genes are actively involved in metabolic pathways and neurotransmission highly associated to aggressive behavior. Additionally, the use of artificial vegetation combined

with a dose of DHA resulted in no attack among the larvae. Although there was a trend in decreasing cannibalism only with DHA supplementation, combining other mitigating strategies (refuge implementation) it is recommended to further improve survival and decrease cannibalism in larvae of *A. tropicus* with the purpose of establishing its aquaculture as a thriving industry.

Availability of data and material

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Ethics approval

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (NOM-062-ZOO- 1999, 2001).

Authors' contributions statement

Each member of the authors team made a significant contribution to this study. All the authors are aware of and agree with the content of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Table 1.- Composition of experimental diets with different concentration of DHA and control diet (CD) (Algamac 3050®).

Ingredients	DHA (Algamac 3050®) (g/Kg)			
	CD	**20**	**30**	**40**
Fish meal[a]	305.4	305.4	305.4	305.4
Renderer meal[a]	300.0	300.0	300.0	300.0
Soy meal[a]	150.0	150.0	150.0	150.0
Corn starch[b]	67.1	58.3	53.9	49.5
Algamac 3050®[c]	0.0	20.0	30.0	40.0
Oil soy[d]	116.5	105.3	99.7	94.1
Cellulose[e]	30.0	30.0	30.0	30.0
Premix vit-min[f]	15.0	15.0	15.0	15.0
Grenetine[g]	10.0	10.0	10.0	10.0
Vit C[h]	5.0	5.0	5.0	5.0
Vit E[i]	1.0	1.0	1.0	1.0
Proximate composition (g/100g dry matter), except energy				
Energy (Kj/g)	17.91	17.86	17.94	17.97
Protein	44.00	44.35	44.53	44.70
Ether extract	16.38	18.31	19.24	19.45
Fibre	1.05	1.16	1.03	1.09
Ahs	13.43	13.28	13.30	14.05
NFE[1]	25.14	22.9	21.9	20.71

[a]Marine and agricultural proteins S.A. de C.V., Guadalajara, Jalisco; [b]IMSA Corn Industrializer S.A de C.V. Guadalajara, Jalisco, México; [c]Aquafauna Bio-Marine, Inc. Hawthorne, California 90250, USA; [d]Ragasa industries S.A. de C.V. ; [e]Sigma-Aldrich Quimica S. de R.L. de C.V.; [f]Vitamin premix composition g, mg or International Units per kg of diet: Vitamin A, 10,000,000 IU; Vitamin D3, 2,000,000 IU; Vitamin E, 100,000 IU; Vitamin K3, 4.0 g; Thiamine B1, 8.0 g; Riboflavin B2, 8.7 g; Pyridoxine B6, 7.3 g; Vitamin B12, 20.0 mg; Niacin, 50.0 g; Pantothenic acid, 22.2 g; Inositol, 0.15 mg; Nicotinic Acid, 0.16 mg; Folic Acid, 4.0 g; Biotin, 500 mg; Vitamin C, 10.0 g; Choline 0.3 mg, Excipient q.s. 2 g; Manganese, 10 g; Magnesium, 4.5 g; Zinc, 1.6 g; Iron, 0.2 g; Copper, 0.2 g; Iodine, 0.5 g; Selenium, 40 mg; Cobalt 60 mg. Excipient q.s. 1.5 g.; [g]D'gari, food and diet products relámpago S.A. de C.V.; [h]ROVIMIX® STAY-C® 35-DSM, Guadalajara, México; [i]GELPHARMA, S.A. de C.V. NFE[1] = nitrogen-free extract: 100-(%protein-%ethereal extract-%ash-%fibre); [f]Trouw Nutrition México S.A. de C.V. (by courtesy).

Table 2.- Analysis of total fatty acids in experimental diets used for *A. tropicus* larvae.

Fatty acids (%)	DHA (Algamac 3050®) (g/Kg)			
	CD	**20**	**30**	**40**
C13:0	7.0	9.3	9.7	9.2
C14:0	1.2	2.0	2.5	3.0
C16:0	16.7	17.0	17.7	17.8
C17:0	ND	0.3	ND	ND
C18:0	5.8	5.6	5.7	5.6
C23:0	ND	0.6	0.9	1.1
ΣSFA	**30.7**	**34.9**	**36.4**	**36.6**
C16:1n7	2.0	2.0	2.1	2.1
C18:1n9	21.0	19.9	19.7	19.2
C18:1n7	1.6	1.6	1.6	1.6
ΣMUFAS	**24.6**	**23.5**	**23.4**	**22.9**
C18:2n6	34.9	31.5	29.5	28.6
C18:3n3	0.3	0.3	3.8	3.8
C18:4n3	4.7	4.1	ND	ND
C20:3n3	0.5	0.6	0.6	0.6
C20:4n6	0.3	ND	ND	ND
C20:5n3	1.6	1.5	1.6	1.7
C22:5n3	0.4	0.4	0.4	0.4
C22:6n3	1.9	3.2	4.2	5.3
ΣPUFAS	**44.6**	**41.6**	**40.2**	**40.5**

Table 3. Analysis of total amino acid in experimental diets used for *A. tropicus* larvae.

Amino acid	CD	20	30	40
Essential amino acids				
HIS	1.1	1.1	1.0	1.2
ARG	5.2	5.4	5.6	5.4
THR	1.7	1.8	1.7	1.7
VAL	1.7	1.7	1.5	1.6
MET	0.5	0.30	0.5	0.9
LYS	5.7	4.8	4.8	5.0
ILE	1.3	1.3	1.1	1.3
LEU	3.5	3.4	3.2	3.4
PHE	1.4	1.4	1.2	1.4
Subtotal	**22.1**	**21.2**	**20.7**	**21.8**
Non-essential amino acids				
ASP	2.4	2.5	2.2	2.3
SER	2.3	2.4	2.4	2.3
GLU	5.8	6.1	5.5	5.8
GLY	7.0	7.2	8.5	7.2
ALA	3.4	3.6	3.6	3.5
TYR	1.5	1.4	1.3	1.4
Subtotal	**22.3**	**23.2**	**23.7**	**22.6**
TAU	0.6	0.6	0.7	0.6
Tryptophan (mg/g)	10.60	10.16	10.10	14.39

Table 4. Oligonucleotide design for real-time polymerase chain reaction (qPCR) of aggressive genes in *A. tropicus* larvae.

Protein	Gen	Primers (5'-3')	Alignment temperature (°C)
Somatostatin receptor 1	*sstr1*	FW: CCTCAGCATTGACCGCTACA RV: AATACCGCCATCCACTGACG	60
Tyrosine hydroxylase	*th*	FW: GGACCAGATGTACCAGCCAG RV: GCAGTTCATCCCTCGCAGAT	59
Histidine decarboxylase	*hdc*	FW: GCATTTCGACTGCACTGCTT RV: CTTCGGCTGAGTGGGATCTG	59
Corticotropin releasing hormone	*crh*	FW: AACGTCAACAGGGCTTTCCA RV: TCTTCCCGTCAGGTCTTCCA	60
5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled	*htr1a*	FW: AAGCGCAGTGTGGAACCTAA RV: GCTGTCGGGGTATTAGGCAG	60
Gonadotropin releasing hormone 1	*gnrh1*	FW: AGTCAGCACTGGTCATACGG RV: CTCACCTCCTCCGCAATGTC	59
Dopamine receptor D1	*drd1*	FW: TTTTTGGCCCTTTGGCTCATT RV: AAGTTCAAAATGGAGGCTGTGG	59
Arginine vasopressin induced 1	*avpi1*	FW: AGGGAGGACCACTGAAGATGA RV: CCAGCAGAGGACAAGTCTGC	60
Tryptophan hydroxylase 1	*tph1*	FW: CCCCCGTATCGAGTTCACAG RV: AGGGGCAGGTTCTTGAGGTA	60

Table 5. Growth performance and feed utilization indexes in first stage of *A. tropicus* larvae fed different concentration of DHA and control diet (CD) (Algamac 3050®) (mean ± standard deviation, *SD*).

Growth indexes	DHA (Algamac 3050®) (g/Kg)			
	CD	**20**	**30**	**40**
Initial weight (g)	0.018 ± 0.001	0.018 ± 0.001	0.018 ± 0.001	0.018 ± 0.001
Final weight (g)	0.022 ± 0.0003	0.023 ± 0.0009	0.022 ± 0.0004	0.022 ± 0.0005
Initial total length (cm)	1.28 ± 0.09	1.28 ± 0.09	1.28 ± 0.09	1.28 ± 0.09
Final total length (cm)	1.78 ± 0.05	1.79 ± 0.05	1.78 ± 0.01	1.78 ± 0.02
S (%)	28.25 ± 1.76	24.25 ± 2.47	27.75 ± 5.30	30.5 ± 4.94
FI (g/d)	0.031 ± 0.001	0.031 ± 0.003	0.031 ± 0.003	0.031 ± 0.001
AWG (g/fish)	0.004 ± 0.0003	0.005 ± 0.0001	0.004 ± 0.0001	0.005 ± 0.0001
SGR (%/d)	5.07 ± 0.52	5.18 ± 0.51	5.02 ± 0.18	5.08 ± 0.20
FCR	7.23 ± 0.85	6.50 ± 0.76	6.70 ± 0.89	6.53 ± 0.09
PER	0.13 ± 0.01	0.14 ± 0.02	0.15 ± 0.02	0.15 ± 0.002
K	0.39 ± 0.02	0.38 ± 0.01	0.41 ± 0.03	0.41 ± 0.05
CV (%)	6.43 ± 2.98^{b}	13.78 ± 2.06^{a}	11.14 ± 0.95^{b}	9.26 ± 0.67^{b}
SH	0.71 ± 0.33^{b}	1.54 ± 0.23^{a}	1.24 ± 0.10^{b}	1.03 ± 0.07^{b}

Significant differences within the diets are indicated by different letters ($p < 0.05$). FI: feed intake; AWG: absolute weight gain; SGR: specific growth rate; S: Survival; FCR: feed conversion rate; PER: protein efficiency rate; K: condition factor, CV: Coefficient of variation, SH: Size heterogeneity.

Table 6.- Growth performance and feed utilization indexes in second stage of *A. tropicus* cannibal larvae fed with 40 g/Kg DHA (Algamac 3050®) and control diet (CD) (mean ± standard deviation, *SD*).

Growth indexes	CD	40 g/Kg DHA (Algamac 3050®)
Initial weight (g)	0.049 ± 0.004	0.042 ± 0.0001
Final weight (g)	0.179 ± 0.03	0.132 ± 0.03
Initial total length (cm)	2.16 ± 0.11	2.08 ± 0.07
Final total length (cm)	3.68 ± 0.34	3.35 ± 0.51
S (%)	30.00 ± 17.32	35.00 ± 7.07
FI (g/d)	0.72 ± 0.07	0.79 ± 0.02
AWG (g/fish)	0.13 ± 0.03	0.09 ± 0.03
SGR (%/d)	14.62 ± 2.87	12.21 ± 5.09
FCR	5.66 ± 0.62	8.02 ± 1.99
PER	0.17 ± 0.01	0.11 ± 0.004
K	0.35 ± 0.02	0.35 ± 0.05
CV (%)	55.64 ± 6.84	39.14 ± 8.46
SH	6.07 ± 0.75*	2.48 ± 0.53

Significant differences within the diets are indicated by different letters ($p < 0.05$). FI: feed intake; AWG: absolute weight gain; SGR: specific growth rate; S: Survival; FCR: feed conversion rate; PER: protein efficiency rate; K: condition factor, CV: Coefficient of variation, SH: Size heterogeneity.

Table 7. Digestive enzymatic activities (mean ± standard deviation, *SD*) of *A. tropicus* larvae fed with different concentration of DHA and control diet (CD) (Algamac 3050®).

	Digestive enzyme activities (U/mg protein)		DHA (Algamac 3050®) (g/Kg)			
			CD	**20**	**30**	**40**
First stage	Acid protease		12.233 ±0.924	12.438±1.788	10.766±0.410	16.671±0.848
	Alkaline protease		17.374±1.550^{a}	8.429±0.697^{b}	9.791±0.463^{b}	9.626±1.137^{b}
	Trypsin		0.213±0.002^{b}	0.196±0.011^{b}	0.364±0.034^{a}	0.160±0.001^{b}
	Leucine aminopeptidase		0.073±0.002^{a}	0.034±0.004^{ab}	0.066±0.003^{a}	0.026±0.005^{b}
	Lipases		1.711±0.654^{b}	3.531±0.242^{ab}	4.374±0.244^{a}	4.233±0.471^{a}
			CD		**40**	
Second stage	Acid protease		20.492±4.162		14.959±3.336	
	Alkaline protease		7.043±0.835		11.395±0.402*	
	Trypsin		0.181±0.020*		0.046±0.041	
	Leucine aminopeptidase		0.086±0.005		0.083±0.001	
	Lipases		3.178±0.640		4.354±0.139	
			CD	**20**	**30**	**40**
Cannibals vs Non cannibals	Acid protease	C	28.261±0.028^{b*}	25.505±0.049^{c*}	11.133±0.115^{d}	29.616±0.050^{a}
		N	23.576±0.062^{b}	5.708±0.026^{d}	17.074±0.037^{c*}	34.693±0.087^{a*}
	Alkaline protease	C	15.887±0.016^{a}	13.337±0.035^{c*}	14.657±0.0328^{b*}	11.552±0.027^{d}
		N	18.021±0.128^{a*}	12.912±0.068^{c}	13.798±0.018^{b}	12.813±0.095^{c*}
	Trypsin	C	0.417±0.035^{a*}	0.220±0.012^{c*}	0.151±0.006^{d*}	0.314±0.012^{b*}
		N	0.325±0.008^{a}	0.131±0.006^{c}	0.130±0.004^{c}	0.210±0.008^{b}
	Leucine aminopeptidase	C	0.056±0.008	0.068±0.042	0.139±0.061	0.143±0.060
		N	0.036 ±0.017	0.065±0.021	0.128±0.031	0.078±0.021
	Lipases	C	0.900±0.011^{d}	2.622±0.008^{b}	1.466±0.007^{c*}	3.826±0.023^{a*}
		N	3.324±0.019^{c*}	6.896±0.014^{a*}	0.749±0.005^{d}	3.411±0.020^{b}

Significant differences within the diets are indicated by different letters ($p < 0.05$). Significant differences within the cannibal (C) and non-cannibal (N) are indicated by * ($p < 0.05$).

Table 8. Attack types and cannibalism behavior of *A. tropicus* larvae fed with 40 g/ Kg DHA and control diet (CD) in combination with different shelters.

	Behaviors						
		Lateral attack			**Frontal attack**		
	Escape	**Head**	**Middle**	**Tail**	**Head**	**Middle**	**Total**
				Without shelter			
Control	2	2	0	0	0	0	4
40 g/ Kg DHA	3	0	0	2	0	0	5
Total	5	2	0	2	0	0	**9**
				Rocks			
Control	2	0	1	0	0	0	3
40 g/ Kg DHA	5	1	1	0	1	1	9
Total	7	1	2	0	1	1	**12**
				Vegetation			
Control	2	0	0	0	0	0	2
40 g/ Kg DHA	3	0	0	0	0	0	3
Total	5	0	0	0	0	0	**5**
Cumulative	17	3	2	2	1	1	**26**

Figure legends

Figure 1. Growth in weight (g) and total length (cm) in first stage of *A. tropicus* larvae fed different DHA concentration and control diet (CD). Values are mean ± *SD*.

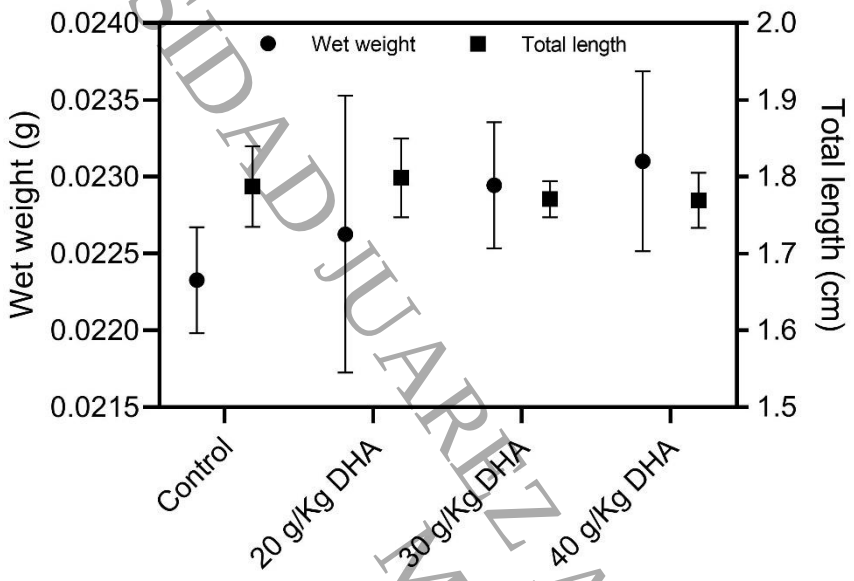


Figure 2. Wet weight (g) and total length (cm) groups distribution during first stage by cannibalisms effect in *A. tropicus* larvae fed with DHA and control diet (CD).

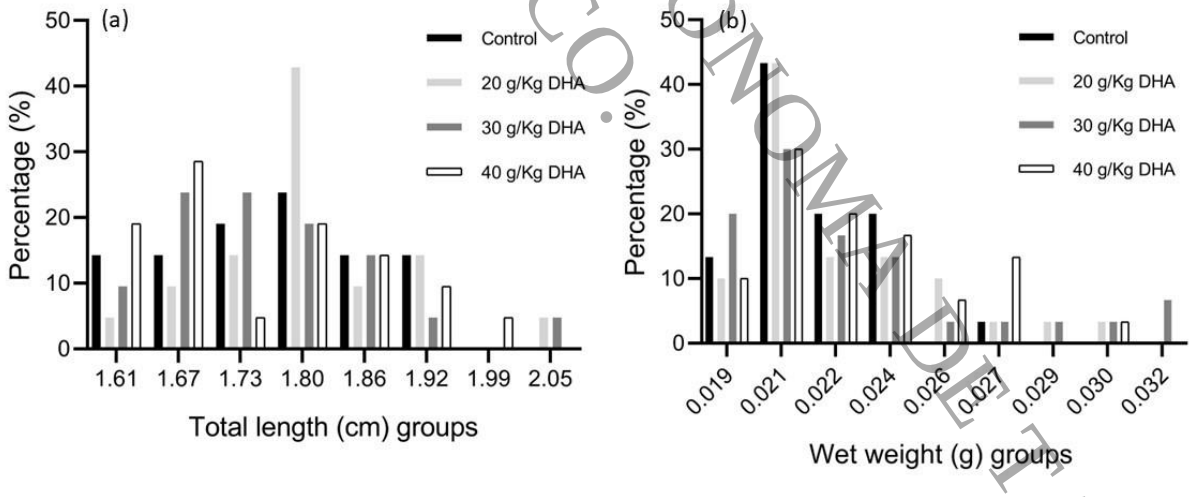


Figure 3. Survival, deformity, and cannibalism percentage in first stage in *A. tropicus* larvae fed with different DHA concentration. Values are mean ± *SD*.

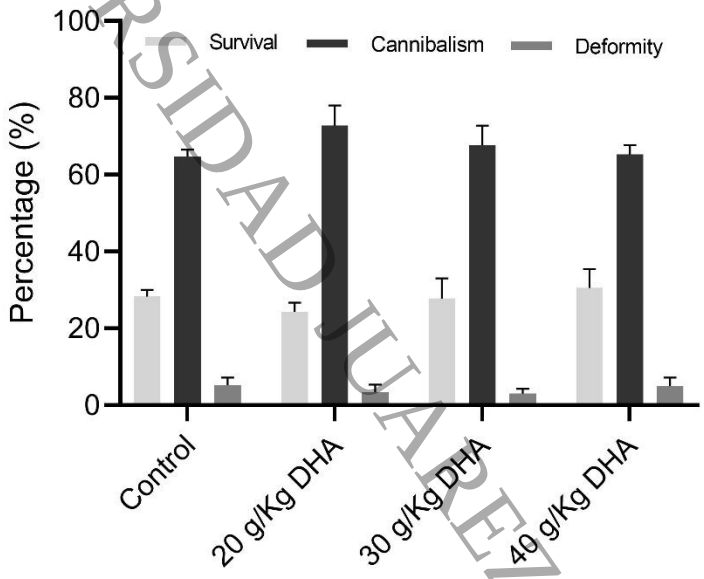


Figure 4. Wet weight (g) (*n=60*) and total length (cm) (*n=45*) of *A. tropicus* cannibal and non-cannibal larvae. Values are mean ± *SD*.

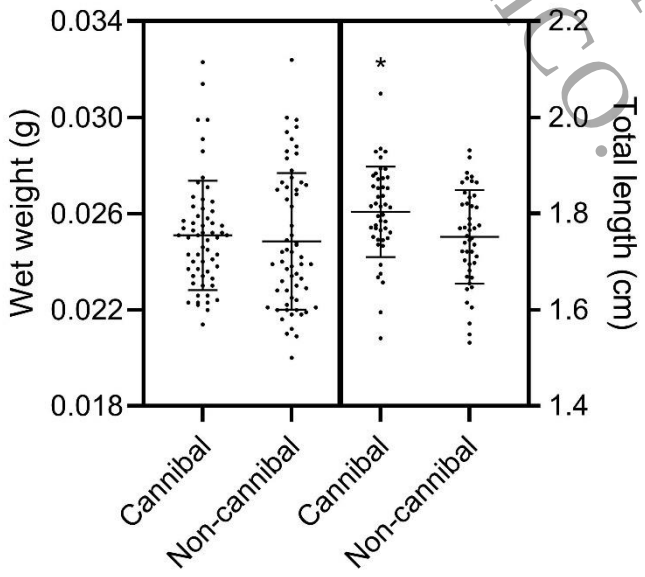


Figure 5. Growth in weight (g) and total length (cm) in second stage of *A. tropicus* cannibals' larvae fed with 40 g/Kg DHA, and control diet (CD). Values are mean ± *SD*.

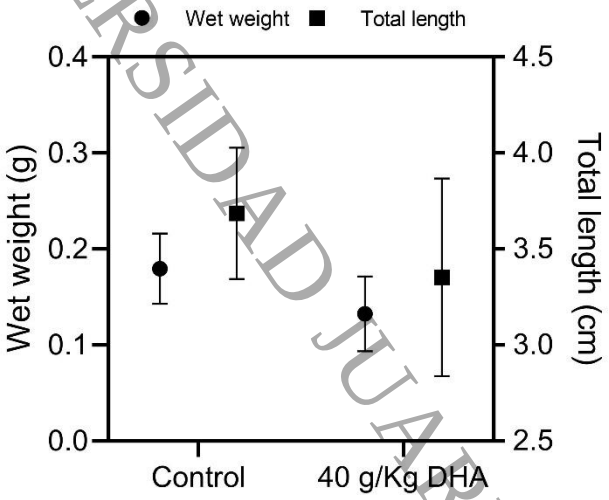


Figure 6. Survival and cannibalism of *A. tropicus* larvae cannibals fed with 40 g/Kg DHA, and control diet (CD). Values are mean ± *SD*.

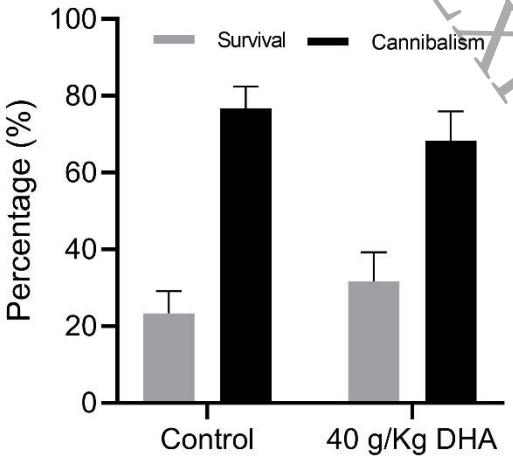


Figure 7. Wet weight (g) and total length (cm) class distribution by cannibalisms effect in *A. tropicus* larvae cannibals fed 40 g/Kg DHA and control diet (CD). Values are mean ± *SD*.

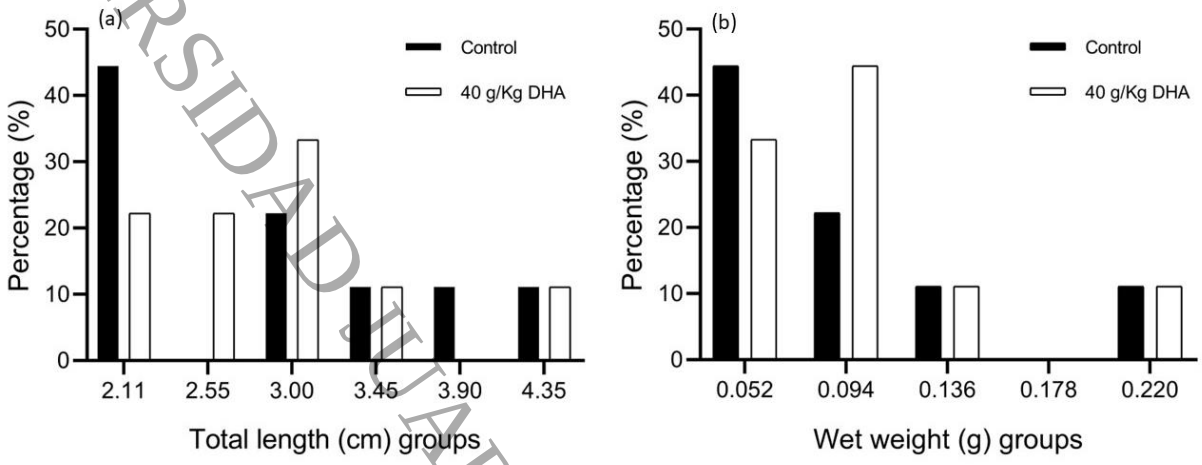


Figure 8. Relative gene expression of *sstr1*, *th*, *hdc*, *crh*, *htr1a*, *drd1*, *avpi1*, and *tph1* in *A. tropicus* larvae fed different DHA concentration (first stage). Relative mRNA levels of the indicated genes were measured by RT-qPCR using β-actin as the reference gen. Data are presented as fold-changes in the mRNA levels, in comparison with sample with no DHA (CD) (dotted line) (n = 3, mean ± *SD*). Significant differences with respect to control (dotted line) are indicated by different letters ($p < 0.05$) Significant differences within the cannibal (C) and non-cannibal (N) are indicated by * ($p < 0.05$).

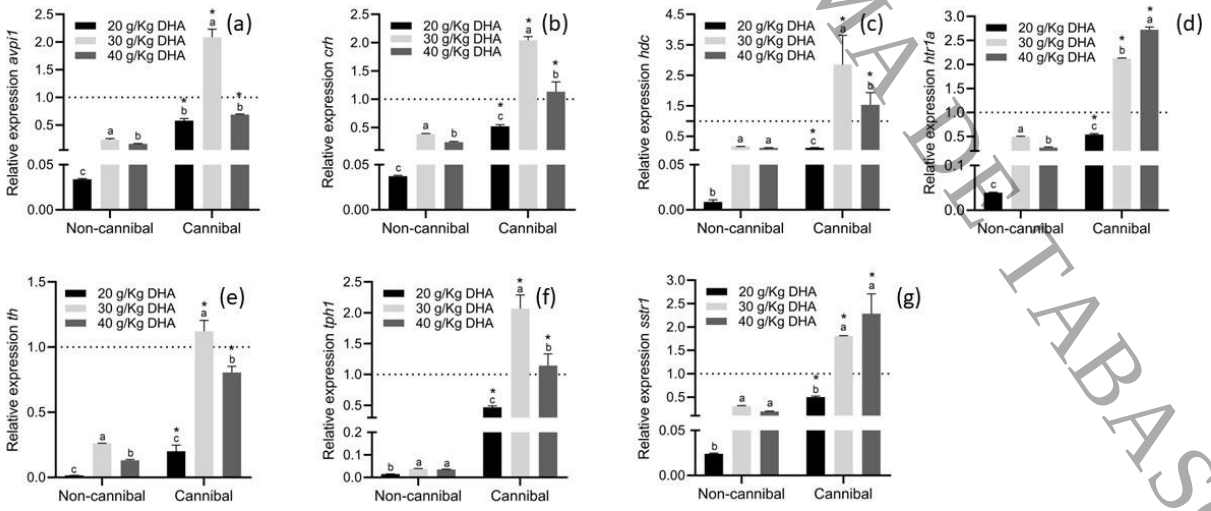
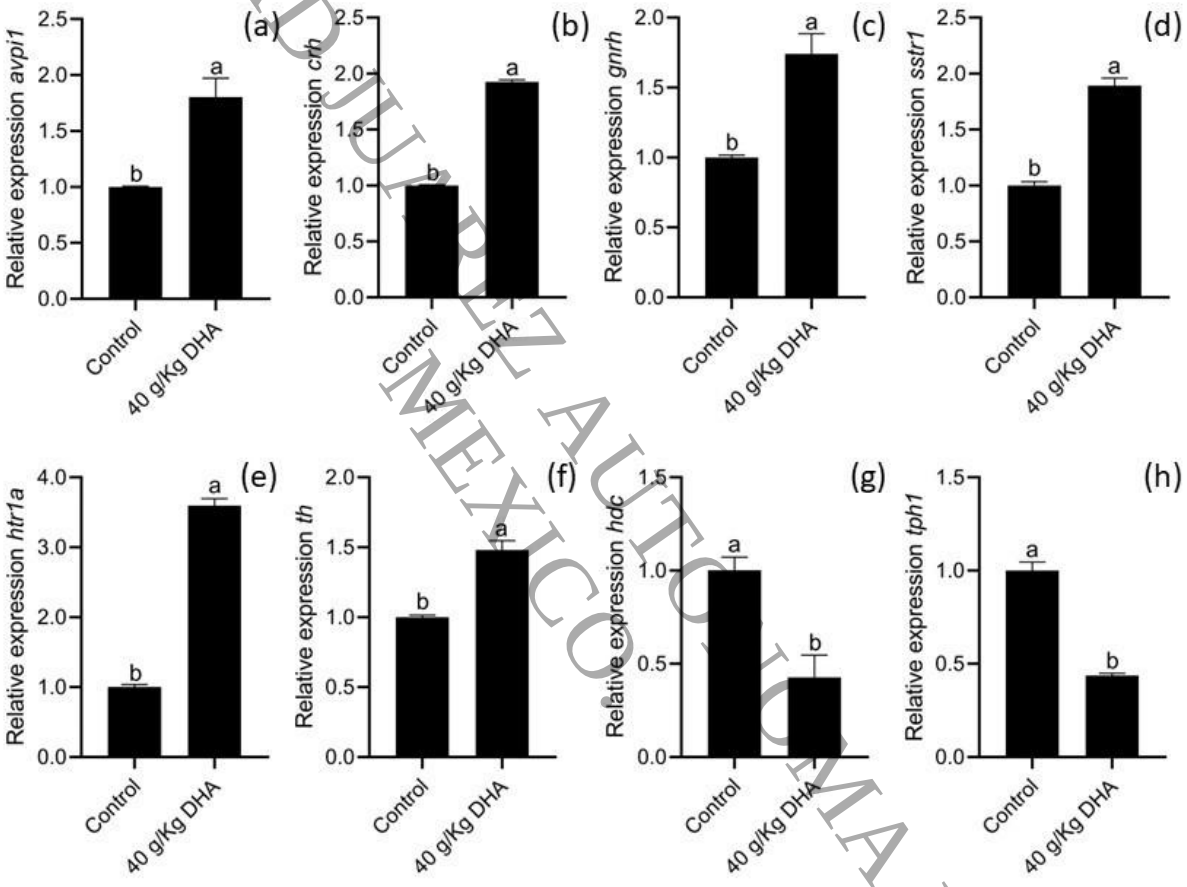


Figure 9. Relative gene expression of *sstr1, th, hdc, crh, htr1a, gnrh1, drd1, avpi1,* and *tph1* in *A. tropicus* cannibals' larvae fed whit 40 g/Kg DHA (second stage). Relative mRNA levels of the indicated genes were measured by RT-qPCR using β-actin as the reference gen. Data are presented as fold-changes in the mRNA levels, in comparison with sample with no DHA (CD) (dotted line) (n = 3, mean ± *SD*). Significant differences with respect to CD are indicated by different letters ($p < 0.05$).



Tryptophan as a mitigating of intracohort cannibalism behavior on Tropical gar (*Atractosteus tropicus*) larvae.

Tryptophan as a mitigating of intracohort cannibalism behavior on Tropical gar (*Atractosteus tropicus*) larvae.

Highlights

- El uso de 10 g/Kg Trp en la dieta mejora la supervivencia y reduce el canibalismo en larvas de *A. tropicus.*
- La diferencia promedio entre el peso y talla de una larva caníbal y su presa fue de 16.12 ± 13.44% y 8.96 ± 3.72% respectivamente.
- Las larvas caníbales de *A. tropicus* presentan un mayor crecimiento debido al efecto "jumper" a diferencia de larvas no caníbales.
- Larvas caníbales y no caníbales de *A. tropicus* muestran una diferencia en su actividad enzimática y expresión de genes.
- El empleo de vegetación artificial como refugio y Trp puede ser una buena estrategia para controlar el canibalismo en larvas de *A. tropicus*.

Abstract

El canibalismo intracohorte presente en larvas de tropical gar (*A. tropicus*) genera grandes problemas en su cultivo, siendo este un inconveniente en su escalabilidad de cultivo como pez nativo en el sur de México. El objetivo de este trabajo fue evaluar en larvas de *A. tropicus* la adición de triptófano (Trp) (10, 20, 30 g/Kg) y una dieta control (CD) sin Trp sobre crecimiento supervivencia, canibalismo, comportamiento, actividad enzimática digestiva y genes relacionados a la agresividad y/o canibalismo en dos etapas (0-13 días post eclosión (DAH); solo caníbales (14-24 DAH). En la primera etapa, no se observaron diferencias en parámetros de crecimiento, sin embargo, los tratamientos con Trp presentaron una tendencia a mejor supervivencia siendo el mejor tratamiento 10 g/Kg Trp (34.75 ± 5.30 %). De igual manera, el canibalismo fue menor con el uso de Trp, siendo el menor porcentaje el tratamiento

10 g/Kg Trp (56.75 ± 2.47 %) con diferencias con CD (64.75 ± 1.76 %). La diferencia promedio entre el peso de una larva caníbal y su presa fue de 0.003 ± 0.003 g (16.12 ± 13.44 %), en talla la diferencia de 0.15 ± 0.06 cm (8.96 ± 3.72 %). En la segunda etapa, el mayor peso y talla fue obtenido fue CD (0.179 ± 0.03 g; 3.68 ± 0.34 cm) con diferencia con 10 g/Kg Trp (0.080 ± 0.01 g; 2.72 ± 0.18 cm) derivado del efecto "jumper" por el canibalismo. La supervivencia fue mayor en 10 g/Kg Trp (75.00 ± 7.07 %) que en CD (23.33 ± 5.77 %) originado por una disminución del canibalismo con el uso de 10 g/Kg Trp (20.0 ± 10.0 %) a diferencia de CD (76.66 ± 5.77 %). AWG, SGR, FCR, PER, CV y SH fueron mejores en CD que en 10 g/Kg Trp originado por el canibalismo. Larvas caníbales y no caníbales muestran una diferencia en su actividad enzimática y expresión de genes. Las larvas caníbales alimentadas con 10 g/Kg Trp presentan mayor actividad enzimática en proteasas acidas y alcalinas, y leucina aminopeptidasa, así como sobreexpresión de *avp1, crh,* y *htr1* y una sub expresión de *tph1, th, sstr1* y *hdc* ($p < 0.05$). Por otro lado, el comportamiento entre larvas caníbales no se registraron conductas de agresión en el tratamiento 10 g/Kg Trp a diferencia de CD. El uso de vegetación artificial también registro cero comportamientos agresivos en ambos tratamientos. El uso de 10 g/Kg Trp mejora la supervivencia y reduce/mitiga el canibalismo en larvas de *A. tropicus*.

Keywords: Intracohort cannibalism, Mitigation, Survival, Fish larvae, Behavior.

1. Introduction

En peces se han documentado alrededor de 390 especies que muestran algún tipo de canibalismo, este comportamiento se presenta en la mayoría de las veces durante toda su vida y en otros exclusivamente en las primeras etapas de desarrollo (larval y/o juvenil) (Pereira et al., 2017). En el sureste de México, el tropical gar (*Atractosteus tropicus*) presenta canibalismo en la etapa larval (10 días post eclosión) y juvenil en organismos tanto del medio natural como en cautiverio (Aguilera et al., 2012; Márquez, 2000). Esta especie considerada de alta importancia económica debido a su cultivo como por su importancia ecológica y cultural (Márquez-Couturier and Vázquez-Navarrete, 2015; Nelson, 2006). Sin embargo, la presencia de canibalismo en las primeras etapas de desarrollo limita mucho los valores de crecimiento, supervivencia y rentabilidad del cultivo. Por ejemplo, Frías-Quintana et al. (2017) reportaron en condiciones de laboratorio, valores de supervivencia y canibalismo de

24% y 33% respectivamente. Lo que puede influir en los acuicultores locales a la elección de otras especies, dejando de lado esta especie nativa ancestral. En un estudio, Palma-Cancino et al. (2019) implementaron una co-alimentación (dieta comercial y *Artemia*) en larvas de *A. tropicus*; el canibalismo se registró con ingesta completa (a los 11 días post-eclosión) e incompleta (al día 21 post eclosión), obteniendo valores de supervivencia de 1 al 33%. El problema se magnifica ya que las larvas no consumidas pero que si fueron heridas por los ataques por lo general morían. Por otra parte, Jiménez-Martínez et al. (2020), al modificar la dieta con una baja concentración de polyunsaturated fatty acid se observó un canibalismo del 40%, y valores de 15 a 30% de supervivencia en larvas de *A. tropicus*.

A raíz de este problema, en diversas especies se han probado diferentes estrategias para mitigar el canibalismo en peces, una de ellas ha sido la incorporación de Trp en la dieta. El Trp, es un aminoácido esencial, el cual es el precursor de 5-hydroxytryptamine (5-HT) (serotonine) (Leathwood, 1987), neurotransmisor encargado del control del apetito (Smith and Seddon, 1998), reproducción (Alkiyama et al. 1996), y procesos fisiológicos relacionados con la inmunidad y la homeostasis intestinal (Comai et al. 2020). Se ha observado que la administración del Trp en los peces *Aequidens pulcher* y *Apteronotus leptorhynchus*, redujo su agresividad (Maler and Ellis, 1987; Munro, 1986). Mientras que, la administración del inhibidor de 5-HT (p-chlorophenylalanine), aumentó la agresividad en *Cichlasoma meeki* (Adams et al., 1996). Así mismo, la incorporación de Trp en concentraciones de 2, 4, and 6 ppm and 2 and 3% en la dieta, indujo una disminución significativa del 50% en el canibalismo de larvas de Pabda (*Ompok bimaculatus*) (Biswas et al., 2018, 2019).

Por lo tanto, el objetivo de este trabajo es evaluar el efecto de la administración de Trp en la dieta de larvas de *A. tropicus*, como posibles mitigantes del comportamiento caníbal. Así mismo, se analizó la tasa de canibalismo, la supervivencia, crecimiento, actividad enzimática, expresión de genes relacionados al canibalismo y comportamiento.

2. Material and Methods

2.1. Biological material

La obtención de las larvas de *A. tropicus* para este experimento se llevó a cabo mediante la inducción al desove utilizando la hormona LHRHa (30 **µ**g/Kg) en la hembra,

posteriormente, en un tanque circular de 2000 L de capacidad se colocaron tres machos y una hembra para promover el desove y posterior fertilización de los huevos. Lo anterior se realizó con el lote de reproductores del Laboratorio de Fisiología en Recursos Acuáticos (LAFIRA) de la DACBiol-UJAT.

2.2. Experimental design

Este trabajo se realizó al mismo tiempo que el reportado por Sepúlveda-Quiroz et al. (202X) usando las mismas instalaciones, el mismo desove y control. De igual manera este trabajo se efectuó en dos etapas, en la primera, tres concentraciones de Trp (10, 20, 30 g/Kg) fueron comparadas con una dieta control (CD) sin Trp. Se utilizaron 200 larvas de *A. tropicus* (0.018 ± 0.001 g and 1.28 ± 0.09 cm) por cada tanque, con forma circular y con capacidad de 70 L. Cada tratamiento se realizó por triplicado. Los tanques de cultivo estaban conectados a un sistema de recirculación by a 0.5-HP water pump (Jacuzzi, JWPA5D-230A, Delavan, WI) and a 1,500 L reservoir for solids deposition and biological filter. Las larvas fueron co-alimentadas durante cinco días posterior a la absorción de su saco vitelino correspondiente al día tres post eclosión (DAH), utilizando la dieta experimental y nauplios de *Artemia*. Al finalizar la co-alimentación, se alimentó únicamente con el alimento formulado de manera manual cuatro veces por día (8:00, 12:00, 16:00, 20:00) *ad libitum*. La duración de este experimento fue del día 0 al 13 DAH. En la segunda etapa, se identificaron y se seleccionaron únicamente las larvas caníbales y se continuo con el tratamiento de Trp (10 g/Kg) y la dieta control (CD). El número de larvas fue de 30 por tratamiento (10 por replica). Se calculó el peso y talla al inicio y al final de cada etapa y se cuantifico el canibalismo. En ambos casos, se realizó un recambio diario parcial del agua del 10% mediante sifoneo con la finalidad de recolectar heces y alimento no consumido. Los parámetros de calidad del agua fueron cuantificados diariamente (promedio ± desviación estándar, SD), temperatura promedio (27.36 ± 0.6°C), el oxígeno disuelto (4.8 ± 0.4 mg/L) determinada por un oxímetro (YSI 85; OH), el pH (7.1 ± 0.3) con un potenciómetro (HANNA HI 991001, Romania).

2.3. Formulation and preparation of the experimental diets

La formulación de las dietas se llevó mediante el uso del software MIXITWIN v.5.0. (Microsoft Windows, Redmond, WA) siguiendo el protocolo de Álvarez-González et al. (2001). Macronutrientes fueron pesados y mezclados, seguido por la incorporación de los

micronutrientes, posteriormente, los ingredientes líquidos fueron agregados, para conseguir una mezcla adecuada se agregó agua 400 ml/Kg per diet. Se mezcló por 15 minutos en cada incorporación (total 60 min de tiempo de mezcla por dieta). Al conseguir la mezcla, fue pasada por un molino (Torrey, M-22RI, Monterrey, N.L, México) generando pellets los cuales fueron secados a 55°C por 12 hr en un horno de convección (Coriat, HC-35-D, CDMX, México). Finalmente, los pellets fueron molidos manualmente por medio de de tamices. Se consiguieron partículas menores de 0.5 mm (co-alimentación), y mayores de 0.7 mm (posterior a la co-alimentación). Las dietas fueron guardadas en bolsas plásticas herméticas a -20°C para su posterior utilización. En todas las dietas se analizaron sus componentes proximales (humedad, ceniza, lípidos y proteína) de acuerdo con AOAC (2000) (Table 1), lípidos (Table 2) y aminoácidos (Table 3).

2.4. Growth indexes and feed quality

Se realizaron dos biometrías, una al inicio del experimento (3 DAH), y diez días después de la alimentación con las dietas con mitigantes (13 DAH). Se determinó el peso individual de cada organismo mediante el uso de una balanza analítica (A&D Company, Limited mod.HR-250AZ, Korea). La talla total se calculó por medio del análisis de las fotografías tomadas a los organismos, a través de un recipiente transparente con escala, utilizando el software ImageJ 1.51j8 (U.S. National Institutes of Health, Bethesda, MD). Además, se calculó la survival (S): (final fish number/initial fish number) × 100; feed intake (FI): total feed intake per experimental unit/number of rearing days; absolute weight gain (AWG): final weight (g) - initial weight (g), specific growth rate (SGR): [(ln final weight − ln initial weight)/days] × 100, feed conversion rate (FCR): (feed intake, g dry matter)/(fish weight gain, g); condition factor (K): [(wet weight (g) × total length−3 (cm)] × 100 and protein efficiency ratio (PER): fish live weight gain (g)/dry protein fed (g). El conteo de organismos deformes se realizó mediante el seguimiento de organismos con nado errático, alta pigmentación obscura, y desnutrición visible. Estos mismos organismos fueron colectados una vez muertos de cada tanque y se confirmó visualmente la deformidad usando un microscopio estereoscopio (Carl Zeiss mod. Stemi DV4, Germany). El porcentaje de organismos deformes se calculó mediante la siguiente formula: Deformidad (D) (peces con deformidad / initial fish number) × 100. Besides the coefficient of variation (%) (CV): (standard deviation of

individual weight/ mean individual weight) × 100, and size heterogeneity (weight) (SH): (final coefficient of variation/ initial coefficient of variation) were calculated.

2.5. Collection of biological samples

Para conocer el efecto del Trp sobre el crecimiento, enzimas digestivas, expresión de genes y el canibalismo en las larvas de *A. tropicus*, en la primer etapa 15 peces por tratamiento fueron sacrificados en la biometría final por medio de shock térmico. En la segunda etapa nueve larvas por tratamiento fueron usadas. Para los análisis de la actividad enzimática las larvas fueron preservadas a -80°C. Finalmente, para analizar expresión de genes las larvas fueron preservados en RNAlater a -80°C. En todos los estudios se utilizaron larvas completas.

2.6. Cannibalism

El canibalismo se cuantifico durante todo el bioensayo mediante la observación durante 30 min antes y una hora después de cada alimentación. Los criterios para determinar el canibalismo fue el siguiente: Ataque por mordidas (un pez ataca a otro pez por mordidas sin que el atacante ingiera a la presa), canibalismo parcial (un pez ingiere parcialmente a otro pez) y canibalismo completo (un pez ingiere completamente a otro pez). Estos tres comportamientos son catalogados como canibalismo. Se utilizó la formula (peces con comportamiento caníbal / initial fish number) x 100) para cuantificar el canibalismo en cada tratamiento. El pez atacante (caníbal) y el pez afectado (no caníbal) fueron contabilizados, separados, y se obtuvieron su peso y talla. Posteriormente, solo durante la primera etapa se recolectaron y sacrificaron por shock térmico, cinco organismos se utilizaron para los análisis moleculares, y cinco organismos más para la evaluación de la actividad enzimática; siguiendo el criterio de preservación de las muestras descrito en el apartado de 2.5.

2.7. Digestive Enzyme activity

Para la cuantificación de enzimas digestivas, las larvas fueron maceradas manualmente dentro de tubos de 1.5 ml con hielo para evitar daño en la muestra, para este proceso se utilizó 100 mg de tejido y se agregó agua destilada a un volumen total de 500 ml. Posteriormente se centrifugo a 12000 rpm a 4°C durante 15 min recuperando el sobrenadante. De este sobrenadante se tomaron alícuotas de 30 µl y se preservaron a -80°C. Se determino la proteína soluble mediante la técnica de Bradford (1976). La cuantificación de proteasas acidas se usó

hemoglobina 0.5% como sustrato solubilizado en 100 mM glicina-HCl pH 2. Proteasas alcalinas fue cuantificado usando caseína al 0.5% solubilizado en 50 mM Tris-HCl y 10 mM CaCl a pH 9 (Sarath, de La Motte, & Wagner, 1989). En ambas técnicas, las muestras fueron incubados a 37°C y se detuvo la reacción con 0.5 ml de 20% ácido tricloroacético y se centrifugo a 16,000 g por minuto, la absorbancia fue leída a 280 nm. El coeficiente de extinción (ε) para calcular la actividad de proteasas acidas y alcalinas fue 0.005 ml/μM cm. Para cuantificar la actividad de Tripsina se utilizó 1 mM BAPNA (Nα-Benzoyl-DL-Arginine-P-nitroanilide) como sustrato disuelto en 50 mM Tris-HCl, con pH 8 a 37°C, y se cuantifico la absorbancia a 410 nm usando un ε de 8,800 ml/μM cm (Erlanger, Kokowsky, and Cohen,1961). Se utilizo el método de Maroux, Louvard, and Barath (1973) para determinar la actividad de Leucina aminopeptidasa, con 0.1 M leucine p-nitroanilide como sustrato disuelto en DMSO con 50 mM sodium phosphate, pH 7.2, incubated at 37°C, se cuantifico la absorbancia a 410 nm con un ε of 8,800 ml/μM cm. La actividad de lipasas fue determinada usando β-naphthyl acetate (100 mM) como sustrato disuelto en 50 mM Tris-HCl at pH 7.5 con sodium taurocholate (100 mM) a 37°C deteniendo reacción con 0.72 N TCA. Fast blue (100 mM) y una mezcla de 1:1 ethanol/ethyl acetate fue agregado, la absorbancia fue cuantificada a 540 nm usando ε d 0.02 ml/μM cm (Versaw, Cuppett, Winters, & Williams, 1989). The enzyme activity was determined using the following equations: units by ml (U/ml) = [Δabs × final reaction volume (ml)] / [ε × time (min) × extract volume (ml)] −1; specific activity (U/mg protein) = U ml/ mg of soluble protein; the molar extinction coefficient (ε). The enzyme activity was determined using the following equations: units by ml (U/ml) = [Δabs × final reaction volume (ml)] / [ε × time (min) × extract volume (ml)] −1; specific activity (U/mg protein) = U ml/ mg of soluble protein; the molar extinction coefficient (ε).

2.8. RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from complete larvae samples using Trizol (Invitrogen, Waltham, MA) according to the manufacturer protocol. The concentration and purity of RNA samples were assessed by the ratio between the absorbance at 260 and 280 nm in a spectrophotometer (Jenway GenovaNano, Cole-Parmer, Staffordshire, UK). RNA (1 μg) was reverse-transcribed (RT) using the SuperScript II kit (Invitrogen), with a final volume of 20 μl. RT reactions were performed in a thermocycler (Mastercycler nexus GSX1, Eppendorf AG,

Hamburg, Germany). The standard RT program used was as follows: 5 min at 65°C, 10 min at 25°C, 50 min at 42°C (cDNA strand extension), 15 min at 70°C (reverse transcriptase inactivation) and finally, 20 min at 37°C. Somatostatin receptor 1 (*sstr1*), tyrosine hydroxylase (*th*), histidine decarboxylase (*hdc*), corticotropin releasing hormone (*crh*), 5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled (*htr1a*), gonadotropin releasing hormone 1 (*gnrh1*), arginine vasopressin induced 1 (*avpi1*), and tryptophan hydroxylase 1 (*tph1*) for *A. tropicus* were designed from the species transcriptome (NCBI Accession: PRJNA395289) (Table 4). The RT-qPCR was performed in a CFX96 Real-Time System (BioRad, Hercules, CA) using 5 µl of EvaGreen Supermix (BioRad), 0.5 µl primers mix and 4.5 µl of cDNA for a final volume of 10 µl. The RT-qPCR program was used at: 50°C for 2 min, 95°C 10 s, followed by 40 cycles at 95°C 15 s and 62°C 1 min. *β-actin* were used as reference gene. Relative gene expression was calculated as fold-change compared with control and using −ΔΔCt formula (Livak & Schmittgen, 2001).

2.9. Efecto de los mitigantes en la etología de las larvas caníbales

Para identificar el efecto en el comportamiento y/o etología en las larvas caníbales por acción del Trp (10 g/Kg) con respecto a las larvas alimentadas con la dieta control (CD) (segunda etapa), dos larvas por pecera (15×10×8 cm) fueron utilizadas por triplicado. Para omitir el efecto de estrés causado por el traslado de los organismos, se estableció un tiempo de adecuación de 15 min previo a cada grabación (15 min) (Gopro Hero 7 Silver, USA). Se evaluó los siguientes escenarios en las peceras: sin refugio, con rocas, y vegetación artificial. Los videos fueron analizados en los softwares Tracker 5.1.5 (Free Software Foundation, Inc.) y BORIS 7.9.24 (Friard & Gamba, 2016), permitiendo cuantificar conductas agresivas y efecto de los refugios (rocas y vegetación) sobre el canibalismo.

2.10. Statistics analysis

Normality (Kolmogorov–Smirnov) and homoscedasticity (Barttlet) tests were tested. One-way ANOVA was carried out for all the analysis, and in case of finding differences, a posteriori test of unequal N HSD (Tukey) was used. A student's t test was used to compare between treatments where applicable. To gene expression data nonparametric Kruskal–Wallis and Nemenyi posteriori tests were used. All tests were performed using the software Prism V. 9.0 with a significance value of 0.05.

3. RESULTS

3.1. Growth indexes and survival

En la primera etapa, el peso promedio final de las larvas de *A. tropicus* alimentadas con las dietas con Trp fue de 0.023 ± 0.0001 g. No se observaron diferencias significativas con las larvas alimentadas con CD (0.022 ± 0.0003 g). Los peces alimentados con 20 g/Kg de Trp obtuvieron la talla total mayor (1.83 ± 0.03 cm), sin presentar diferencias significativas con CD (1.78 ± 0.05 cm) (Fig. 1). Los tratamientos con Trp presentaron una mejor supervivencia siendo el mejor tratamiento 10 g/Kg Trp (34.75 ± 5.30 %), sin diferencias con CD (28.25 ± 1.76). No se encontraron diferencias en AWG, SGR, FCR, PER, K, CV and SH ($p < 0.05$) (Table 5). Al finalizar esta etapa, la distribución de los pesos y tallas de los peces se comportaron sin presentar diferencia significativa entre tratamientos (Fig. 2).

3.2. Cannibalism and deformity

En todos los tratamientos donde se administró Trp el porcentaje de canibalismo fue menor (56.75-64.25 %) que CD. El menor porcentaje de canibalismo se observó en 10 g/Kg Trp (56.75 ± 2.47 %) y el valor más alto se presentó en las larvas del tratamiento CD (64.75 ± 1.76 %), presentando diferencia significativa ($p < 0.05$). El porcentaje de peces con deformidad fue de 5.75 ± 0.43 %, y no se observaron diferencias entre los tratamientos ($p > 0.05$) (Fig. 3).

Las larvas identificadas como caníbales y no caníbales presentaron un peso promedio de 0.025 ± 0.002 g y de 0.023 ± 0.003 g respectivamente (*t* test, $p = 0.007$). Las larvas identificadas como caníbales presentaron una talla total de 1.83 ± 0.09 cm, las larvas identificadas como no caníbales una talla total de 1.72 ± 0.10 cm (Fig. 4) (test, $p < 0.0001$). La diferencia promedio registrada entre el peso de un pez caníbal y su presa fue de 0.003 ± 0.003 g (16.12 ± 13.44 %). En talla total, la diferencia promedio entre el pez caníbal y su presa fue de 0.15 ± 0.06 cm (8.96 ± 3.72 %).

Para continuar con la etapa dos, en base a los resultados obtenidos, se seleccionó la dieta 10 g/Kg Trp donde se presentó el menor porcentaje de canibalismo y mayor supervivencia para seguir con su administración solo a larvas identificados como caníbales por 10 días más.

3.3. Growth indexes and survival of cannibals

Las larvas caníbales del tratamiento CD obtuvieron un mayor peso final (0.179 ± 0.03 g), a diferencia con el tratamiento 10 g/Kg Trp (0.080 ± 0.01 g) (*t* test, *p*= 0.0224), lo misma tendencia se observó con la talla total de 3.68 ± 0.34 cm (CD), y 2.72 ± 0.18 cm (10 g/Kg Trp) (Fig. 5). La mayor supervivencia se registró en 10 g/Kg Trp con 75.00 ± 7.07%, presentando diferencias significativas con CD (30.00 ± 17. 32 %) (*t* test, *p*= 0.0044). El AWG fue mayor para CD (0.13 ± 0.03) (*t* test, *p*= 0.019). SGR fue mayor en CD (14.62 ± 2.87) (*t* test, *p*= 0.029) El valor de FCR para el tratamiento 10 g/Kg Trp fue de 12.43 ± 1.60, con diferencia significativa con CD (5.66 ± 0.62) (*t* test, *p*= 0.030). El valor de PER fue mayor en CD con 0.17 ± 0.01 (*t* test, *p*= 0.041). Por otro lado, CD presentó el mayor valor de CV (55.64 ± 6.84) con diferencia con el tratamiento 10 g/Kg Trp (26.74 ± 14.20) (*t* test, *p*= 0.015). De igual manera, CD presentó el mayor valor de SH (6.07 ± 0.75), con diferencias con 10 g/Kg Trp (*t* test, *p*= 0.012). FI and K no presentaron diferencias significativas (Table 6).

3.4. Cannibalism

El porcentaje de canibalismo fue del 20.0 ± 10.0 % en los peces alimentados con 10 g/Kg Trp, con diferencia significativa con CD (76.66 ± 5.77 %) (*t* test, *p*= 0.0011) (Fig. 6). Al finalizar el bioensayo, se observó la modificación de la distribución de los pesos y tallas de los peces por tratamientos, lo que corresponde al efecto del canibalismo sobre la distribución de la población (Fig. 7).

3.5. Digestive enzyme activity

En la primera etapa, las larvas del tratamiento CD presentaron mayor actividad en acid protease activitie ($p < 0.05$) con 10 y 20 g/Kg Trp. De igual manera CD obtuvo mayor actividad en proteasa alcalina con diferencia con todos los tratamientos con Trp ($p < 0.05$). El valor de tripsina fue mayor en CD siendo únicamente diferente con 20 g/Kg Trp ($p < 0.05$). El tratamiento 10 y 20 g/Kg Trp obtuvieron la mayor actividad de lipasas que el resto de los tratamientos ($p < 0.05$). Leucine aminopeptidase no mostro diferencias significativas entre los tratamientos ($p > 0.05$).

Al comparar la actividad enzimática digestiva entre caníbales y no caníbales, la actividad de proteasa acida fue mayor para caníbales y no caníbales del tratamiento 10 g/Kg

Trp ($p < 0.05$). Larvas caníbales y no caníbales de CD presentaron mayor actividad de proteasas alcalinas ($p < 0.05$). La actividad enzimática de tripsina entre caníbales y no caníbales fue mayor en los caníbales en CD ($p < 0.05$). Todos los tratamientos con Trp presentan mayor actividad de leucine aminopeptidase que CD, sin embargo, no se registra diferencia entre caníbales y no caníbales. El tratamiento 10 g/Kg Trp registró la mayor actividad de lipasas ($p < 0.05$), las larvas no caníbales presentan mayor actividad que los caníbales (*t* test, $p < 0.05$).

En la segunda etapa, las larvas del tratamiento con Trp registraron mayor actividad en proteasas acidas y alcalinas, y leucina aminopeptidasa (*t* test, $p < 0.05$). CD registró mayor actividad en tripsina (*t* test, $p < 0.05$). No se observa diferencias en lipasas entre CD y 10 g/Kg Trp (Table 7).

3.6. Gene expresión

Al comparar larvas caníbales y no caníbales, todos los caníbales del tratamiento 20 g/Kg Trp presentaron sobre expresión en todos los genes, seguido 30 g/Kg Trp con sobre expresión solo en *avp1, crh, htr1a, sstr1, th* y *tph1*. Entre las larvas no caníbales, el tratamiento 10 g/Kg Trp presento mayor expresión, con diferencia significativa con el resto de las concentraciones de Trp y con su mismo tratamiento en larvas caníbales (*avp1, crh, hdc, htr1a, sstr1*, y *th*). Solo en la expresión de *tph1*, la sobre expresión en todos los tratamientos con larvas caníbales fueron diferentes a los no caníbales (Fig. 8).

Con respecto a la segunda etapa, las larvas del tratamiento 10 g/Kg Trp presentaron sobreexpresión de *avp1, crh*, y *htr1a* ($p < 0.05$) con respecto a CD. Misma tendencia se observa en *gnrh* ($p > 0.05$). Se registró una sub expresión de los genes *tph1, th, sstr1* y *hdc* ($p < 0.05$) en larvas alimentadas con 10 g/Kg Trp (Fig. 9).

3.7. Behavior

En total se obtuvieron 12 videos con una suma total de 185.8 minutos de grabación. Se registraron un total de 12 eventos de comportamiento, de los cuales 9 fueron comportamientos defensivos (huidas) por parte de uno de los peces sin existir contacto entre ellos, los 3 restantes eventos fueron ataques directos (con contactos). Los organismos con rocas como refugios presentaron 3 eventos, seguido por sin refugio con 6 eventos, finalmente, solo 3

eventos se registraron en los organismos con vegetación como refugio. Con respecto al efecto de la dieta, las larvas con 10 g/Kg Trp registraron el 25% de los comportamientos y CD el 75%. Con respecto al número de ataques, los caníbales alimentados 10 g/Kg Trp no registraron conductas de agresión a diferencia de CD. El uso de vegetación artificial también registro cero comportamientos agresivos en ambos tratamientos (Table 8).

4. **Discussion**

4.1. Crecimiento

El Trp al ser un aminoácido (AA) esencial en peces ha generado conocer su requerimiento nutricional en gran diversidad de especies (*e.g. Salmo gairdneri*, *Oncorhynchus mykiss*, *Rhamdia quelen*), valor que ronda alrededor de 0.1% to 0.5% (Mai et al., 2022). En este sentido, la formulación de la dieta CD utilizada contiene un 1.06% de Trp de acuerdo con el análisis de aminoácidos totales realizado, lo que nos permite saber la concentración real de las dietas experimentales con Trp con respecto a CD. Por otro lado, la relación de las funciones del Trp en el desarrollo de las larvas de peces ha sido muy estudiado; se ha demostrado que la baja administración de Trp está relacionada con la presencia de deformidades (scoliosis, lordosis, and eye cataracts) (Walton et al., 1984). Además, su administración a sido relacionada con la modulación de la agresividad, feed intake, sistema inmune, estrés, oxidative damage, feed efficiency ratio (Coloso et al., 2004; Hoseini et al., 2016; Hseu et al., 2003; Jiang et al., 2015), por lo que se debe de buscar la administración de su nivel óptimo en cada especie.

El efecto de la administración de Trp en el crecimiento en otras especies de peces ha sido bien reportado, en juveniles de Asian seabass (*Lates calcarifer*), el Trp redujo el crecimiento y consumo de alimento, aunque aumento los niveles de serotonina en el cerebro (Chi et al., 2018). En juveniles de grouper (*Epinephelus coioides*) al administrar 0.25%, 0.5% and 1% of triptofano en la dieta presentaron menor talla y peso que el control (Hseu et al., 2003). De la misma manera, en pikeperch (*Sander lucioperca*) post-larvae al usar 5, 10 and 20 g Trp per kg, el peso y talla final de los peces fue menor que el tratamiento control (Król and Zakęś, 2015). En Pabda, (*Ompok bimaculatus*) fry el crecimiento de los peces con Trp (2, 4, 6 ppm) obtuvieron menor crecimiento que el control (Biswas et al., 2019). En el caso particular de Indian major carp (*Cirrhinus mrigala*) y Indian catfish, (*Heteropneustes fossilis*)

una alta concentración de Trp en la formulación de la dieta se ve reflejado en una disminución del crecimiento (Ahmed, 2012; Ahmed and Khan, 2005). Las larvas de *A. tropicus* no presenta diferencia significativa en su crecimiento en la primera etapa, solo una ligera tendencia de un mejor crecimiento y mayor longitud total con respecto a CD. Sin embargo, en la segunda etapa, las larvas caníbales del tratamiento con Trp (10 g/Kg) presentaron un menor crecimiento y talla final que las larvas de CD notándose diferencias en los parámetros productivos. Esta diferencia entre ambos tratamientos en el crecimiento se atribuye al canibalismo mediante el efecto "jumper", el cual contempla una ganancia de peso y talla de forma rápida cundo una larva caníbal consume a otra larva (Baras et al., 2011), lo que además puede propiciar un aumento en la heterogeneidad de tallas y peso. Además, tanto los valores de CV y SH fueron mayores para CD, comprobando que se le atribuye el alto canibalismo de este tratamiento (76.66 ± 5.77 %) la baja supervivencia (23.33 ± 5.77 %) y la alta heterogeneidad de la población de CD, a diferencia de las larvas con Trp (canibalismo: 20.0 ± 10.0 %; survival: 75.0 ± 7.07%). Resultados similares se han registrado en larvas de Asian seabass (*Lates calcarifer*), donde el coefficient of size variation (%) and size heterogeneity results decreased with increased level of Trp supplementation (Kumar et al., 2017). Esta diferencia en la heterogeneidad de la talla puede también ser un detonante del canibalismo en el Atlantic cod (*Gadus morhua*), African catfish (*Heterobranchus longifili*), giant grouper (*Epinephelus lanceolatus*), and, black rockfish (*Sebastes schlegelii*) (Baras et al., 2014; Folkvord, 1992; Hseu et al., 2004; Xi et al., 2017).

4.2. Canibalismo

Aunque se aprecia una tendencia en la disminución del canibalismo por la administración de Trp en la primera etapa, es en la segunda donde fue significativamente más claro. Estos resultados coinciden con Asian seabass (*Lates calcarifer*) fry que al utilizar 0.5, 1.0, 1.5 and 2% de Trp disminuyó el canibalismo y aumentó la supervivencia con respecto al control, siendo el menor porcentaje (0.5) el mejor tratamiento (Kumar et al. 2017). En juveniles de grouper (*Epinephelus coioides*) el uso de 0.25%, 0.5% and 1% of triptofan en la dieta disminuyó el canibalismo, aumentando la concentración de serotonina (5-HT) in the brain of groupers (Hseu et al., 2003). En pikeperch (*Sander lucioperca*) post-larvae al usar 5, 10 and 20 g Trp per kilogram, incrementa los niveles de 5-HT en el tejido de los peces,

disminuyendo el canibalismo (Król, & Zakęś, 2015). En Pabda, (*Ompok bimaculatus*) fry al usar 2, 4, 6 ppm de Trp se disminuye el canibalismo y aumenta la supervivencia con respecto al control (Biswas et al., 2019) Por otro lado, la suplementación de Trp en el alimento (28g/K) administrado a juveniles de Atlantic cod (*Gadus morhua*) permitió que redujeran la agresividad (Höglund et al., 2005). De igual manera, en rainbow trout (*Oncorhynchus mykiss*) el Trp al aumentar su concentración en plasma y cerebro, se disminuyó el comportamiento agresivo en peces dominantes (Lepage et al., 2003, 2005), relacionado con el neurotransmisor de 5-HT. La serotonina (5-HT), es un neurotransmisor que se ha relacionado en comportamientos como la agresión, la reacción al estrés, la alimentación, la maduración y comportamiento sexual (Sahu et al., 2020). La síntesis de 5-HT se lleva a cabo en las neuronas serotoninérgicas, donde el Trp sirve como precursor. La enzima triptófano hidrolasa hidroxila el triptófano pasando a L-5-hidroxitriptófano, posteriormente se descarboxila por medio de la enzima L- aminoácido descarboxilasa generando 5-hidroxitriptamina (5-HT). Continuando con el proceso, la 5-HT es degradada por la enzima monoamino oxidasa transformándolo en 5-hidroxiindol acetaldehído, al final de la reacción la enzima aldehído deshidrogenasa produce ácido 5-hidroxiindolacético (5-HIAA) (Höglund et al., 2019; Sahu et al., 2020; Winberg and Nilsson, 1993). La biodisponibilidad de Trp en el cerebro de los organismos es importante ya que permite que la síntesis de se lleve a cabo. En ese sentido, el Trp compite con otros aminoácidos (AA) (valina, isoleucina, leucina, tirosina, fenilalanina y metionina) para ingresar al cerebro de los organismos, lo que hace importante el óptimo balance entre Trp, el resto de AA y los carbohidratos (ya que estos promueven la captación de AA exceptuando al Trp) para propiciar las concentraciones adecuadas de AA en el plasma permitiendo el flujo de Trp al cerebro (Höglund et al., 2019), y con ello la síntesis de 5-HT. El uso del Trp como mitigador del canibalismo en peces se basa en el principio de incrementar la biodisponibilidad de este aminoácido que funciona como sustrato indispensable para la síntesis de 5-HT y a su vez reduce la agresividad en peces promoviendo la diminución del canibalismo. Por otra parte, la diferencia entre el peso y talla total de un pez caníbal y su presa fue 16.12 ± 13.44 % y 8.96 ± 3.72 % respectivamente, porcentajes similares a los reportados por Sepúlveda-Quiroz et al. (20XX, 202X).

4.3. Enzimas digestivas

La interacción entre el Trp y las enzimas digestivas ha sido estudiada en diversos trabajos, sin embargo, esta interacción relacionada con el canibalismo aún no ha sido abordado. Previamente se ha reportado en las larvas de *A. tropicus* que la funcionalidad de su sistema digestivo y la diferenciación de sus órganos está completamente desarrollada al 9 DAH (Frías-Quintana et al., 2015) y sumado a las condiciones anatómicas que presentan (mouth width and Depth, Length upper and lower jaw, and mouth depth angle) les permite capturar e ingerir a sus propios congéneres (canibalismo intracohorte) (Sepúlveda-Quiroz et al. 20XX), y esto favorece a su rápido crecimiento. Con la administración de Trp las enzimas acid and alcaline protease, trypsin, and, leucine aminopeptidase registraron una menor actividad que CD, a excepción de lipases. En amylase, lipase, and trypsin se ha demostrado en forma *in vitro* que el Trp puede ser un activador de estas enzimas (Svatos, 1994). Un aumento en la actividad enzimática se refleja en una mayor hidrolisis de macronutrientes, liberando un mayor número de micronutrientes (Bone & Moores, 2008), lo que es aprovechado por el organismo obteniendo una mayor absorción de estos microelementos. Además, un óptimo crecimiento en los peces está relacionado con la actividad enzimática digestiva y borde de cepillo (Hakim et al., 2006). De igual manera larvas caníbales y no caníbales presentaron diferencias significativas en su actividad, principalmente la actividad de tripsina fue mayor en todos los caníbales, a su vez, las lipasas fueron mayores en larvas no caníbales. Estos resultados concuerdan con Sepúlveda-Quiroz et al. (202X), en donde la tripsina fue mayor en larvas caníbales que en las no caníbales independientemente del tratamiento de DHA, lo mismo sucede parcialmente con lipasas donde los no caníbales obtienen mayor actividad (CD y 20 g/Kg DHA). La función de las tripsinas es la hidrolisis de las proteínas mediante el rompimiento de los enlaces peptídicos (Moyano et al., 1996). A su vez, las lipasas favorecen la digestión de los lípidos, participando en la desnaturalización del triacilglicerol a diacilglicerol posteriormente pasando a monoacilglicerol (Cahu and Zambonino, 2001). En el caso particular de la segunda etapa, las larvas caníbales con la administración de Trp (10 g/Kg) mostraron mayor actividad enzimática (acid and alkaline protease, leucine aminopeptidase) que las larvas caníbales CD. La tripsina continúa siendo mayor en larvas caníbales CD. En juveniles de Jian carp (*Cyprinus carpio*) al usar una concentración de 3.8 g/kg de Trp en la dieta, los organismos incrementaron su crecimiento y obtuvieron una mayor actividad de enzimas digestivas (trypsin, lipase and α-amylase), de enzimas borde de cepillo, y

un incremento en la altura de pliegues intestinales (Tang et al., 2013). Por otro lado, se ha relacionado al Trp como un componente que puede mejorar la actividad enzimática digestiva por medio de dos componentes, la melatonina (metabolito del Trp) y la cholecystokinin (hormona reguladora), ambos actúan sobre la secreción de enzimas pancreáticas (Aldman et al., 1992; Jaworek, 2006), tales como trypsin, chymotrypsin, lipase and amylase (Zambonino-Infante and Cahu, 2001). Por otro lado, la administración de Trp en juveniles de silver catfish (*Rhamdia quelen*) no presentó diferencia en tripsina y quimotripsina, sin embargo, mediante la realización de una regresión polinomial se identificó un aumento en la actividad de proteasa acida con respecto al aumento del Trp administrado (1-3.1 g/kg) (Pianesso et al., 2015).

4.4. Gene expresión

Los resultados de la expresión de genes muestran una diferencia significativa entre larvas caníbales y no caníbales, donde los caníbales expuestos a un tratamiento con alto Trp prestan una mayor sobre expresión. El Trp funciona como sustrato en la síntesis de 5-HT, neurotransmisor que regula entre otros aspectos el comportamiento agresivo (Sahu et al., 2020; Winberg & Nilsson, 1993). En pez cebra (*Danio rerio*), se ha reportado una variación de los niveles de expresión de genes de acuerdo a las regiones específicas del cerebro (hypothalamus, hindbrain, telencephalon, and, optic tectum), así como también, a las variables biológicas como el sexo y la jerarquía social que modifican la expresión, además, se identificaron siete rutas neurológicas (hypothalamo-neurohypophysial-system (HNS), serotonin (5-HT), somatostatin, dopamine, hypothalamo-pituitary-interrenal (HPI), hypothalamo-pituitary-gonadal (HPG) and histamine) con relación a los genes expresados y la agresividad (Filby et al., 2010). Entre los genes que participan en estas rutas metabólicas son los utilizados en este estudio (*sstr1, th, hdc, crh, htr1a, gnrh1, avpi1, tph1*), y cuya relación con el comportamiento agresivo ya ha sido reportado en humanos y animales como en ratones y peces (de Abreu et al., 2019).

En la segunda etapa, las larvas caníbales de *A. tropicus* alimentados con Trp presentaron una sobre expresión de *avp1*(HNS), *crh* (HPI), y *htr1a* (5-HT), así como una sub expresión de *tph1*(5-HT), *th* (dopamine), *sstr1* (somatostatin), *hdc* (histamine), con respecto a larvas caníbales alimentadas con CD. La expresion de *avpl* (arginine vasopressin-like) está relacionado a comportamientos como la agresión e interacciones sociales, en caso particular se

ha detectado una sobre expresión en machos dominantes de zebrafish (*Danio rerio*) (Caldwell et al., 2008; Filby et al., 2010). Por otro lado, la ruta HPI es la encargada de diferentes procesos como la respuesta al estrés (Wendelaar Bonga 1997), en este sentido, la hormona liberadora de corticotropina (CRH; or corticotropin-releasing factor (CRF)) funciona como un activador de esta ruta (Conrad et al., 2011). En Rainbow trout (*Oncorhynchus mykiss*) la administración de CRF por medio de inyección disminuyó el número de ataques, aumentó su locomoción y movimientos de cabeza, además, se incrementó concentración de serotonina, 5-HIAA y dopamina (Carpenter et al., 2007, 2009). Un aumento en los receptores agonistas 5-HT1A y 5-HT1B disminuye el comportamiento agresivo en ratones (Nelson and Trainor, 2007). Al compara machos dominantes y subordinados de *Astatotilapia burtoni*, la abundancia relativa de mRNA de los transportadores de 5-HT como *htr1a*, *htr2a* en el telencéfalo fueron más altas en subordinados que en machos dominantes, relacionado a un aumento en la producción de 5-HT (Loveland et al., 2014). En este sentido, el uso de un agonista específico (8-OH-DPAT) en receptores HTR1A disminuye la agresividad en fighting fish (*Betta splendens*) (Clotfelter et al., 2007). En el caso de Sepúlveda-Quiroz et al., 202X, bajo el mismo diseño experimental, pero con DHA (40 g/Kg) como mitigante, las larvas caníbales de *A. tropicus* presentaron una sobre expresión de *avp1*, *crh*, *gnrh*, *sstr1*, *htr1a,* y *th*, y una sub-expresión de *hdc* y *tph1*. Comparando ambos trabajos, los genes *th y sstr1* presentaron una sub-expresión por parte del Trp a diferencia del DHA. En machos de zebrafish (*Danio rerio*), se identificó una diferenciación entre dominantes y subordinados con respecto a genes involucrados a genes sexuales (*cyp19a1b*, *cyp17*, *hsd11b2*, *hsd17b3*, *ar*) y agresividad (*avplr1b*, *tph1b*, *htr1a*, *sst1*, *sstr1*, *th*, *slc6a3*), siendo en machos dominantes los de mayor expresión (Filby et al., 2012). Aunque en larvas de *A. tropicus* aún no se ha estudiado los componentes sociales, los resultados obtenidos indican que las rutas HNS, 5-HT, y HPI fueron modificadas por la utilización de Trp, y con ello la disminución del canibalismo.

4.5. Behaviour

El efecto de la administración de Trp se logra apreciar en el nulo comportamiento agresivo de las larvas de *A. tropicus* bajo la influencia de posibles refugios. En donde sin importar la presencia o no de cualquier tipo de refugio (rocas y vegetación artificial) las larvas con administración de Trp (10g/K) no presentaron ningún ataque a diferencia de las larvas con

CD. De los tres escenarios empleados, la vegetación artificial no presento ataques entre las larvas en CD, al igual que lo reportado por Sepúlveda-Quiroz et al. (20XX, 202X). La utilización de ambientes enriquecidos son aquellos que intentan replicar condiciones de medio natural dentro de los estanques de cultivos, lo que en gran medida mejora los niveles de estrés disminuyendo la agresividad, el canibalismo, el gasto energético, las lesiones y enfermedades (Hecht and Appelbaum, 1988; Näslund and Johnsson, 2014; Qin et al., 2004; Zhang et al. (2020).

5. Conclusion

El uso 10 g/Kg de Trp en la dieta en larvas de *A. tropicus* disminuye el canibalismo mejorado la supervivencia, comprobado específicamente en larvas con comportamiento caníbal. Tanto larvas caníbales como no caníbales demostraron una diferencia en actividad enzimática digestiva y expresión de genes de agresividad. La inclusión de triptófano genera la activación de las rutas HNS, 5-HT y HPI demostrado por la sobre expresión de los genes *avp1*, *crh*, y *htr1a.* Además, etológicamente no se presentó comportamientos caníbales con el uso de Trp sin importar el tipo de refugio, aunque la vegetación artificial fue mejor que otros refugios. Se recomienda que se continúen con los trabajos enfocados en explicar otros efectos del Trp en *A. tropicus*.

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Availability of data and material

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Ethics approval

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (NOM-062-ZOO-1999, 2001).

Authors' contributions statement

Each member of the authors team made a significant contribution to this study. All the authors are aware of and agree with the content of the manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Figure legends:

Figure 1. Growth in weight (g) and total length (cm) of *A. tropicus* larvae fed different Trp concentration and control diet (CD). Values are mean ± *SD*.

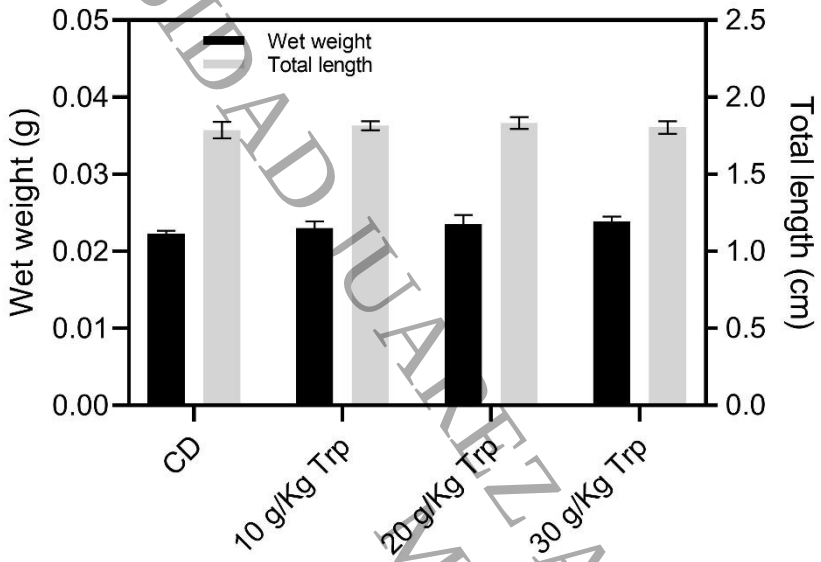

Figure 2. Wet weight (g) and total length (cm) class distribution by cannibalisms effect in *A. tropicus* larvae fed with Trp and CD.

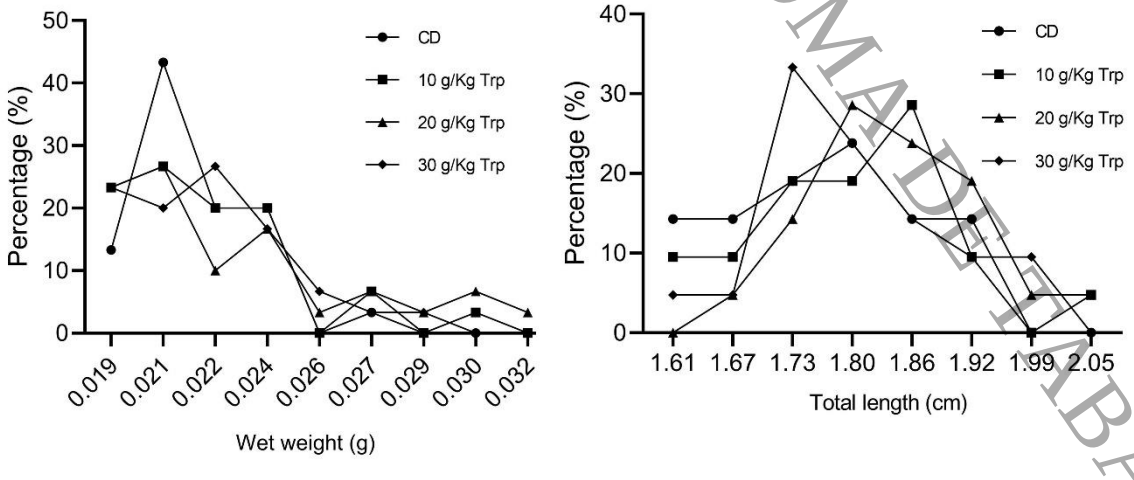

Figure 3. Survival, deformity, and cannibalism percentage in *A. tropicus* larvae fed with different Trp concentration. Values are mean ± *SD*.

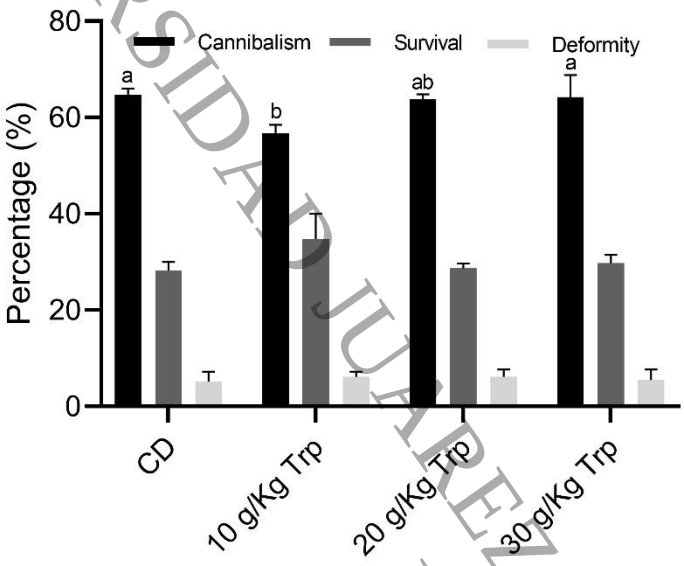

Figure 4. Wet weight (g) (*n=60*) and total length (cm) (*n=45*) of *A. tropicus* cannibal and non-cannibal larvae. Values are mean ± *SD*.

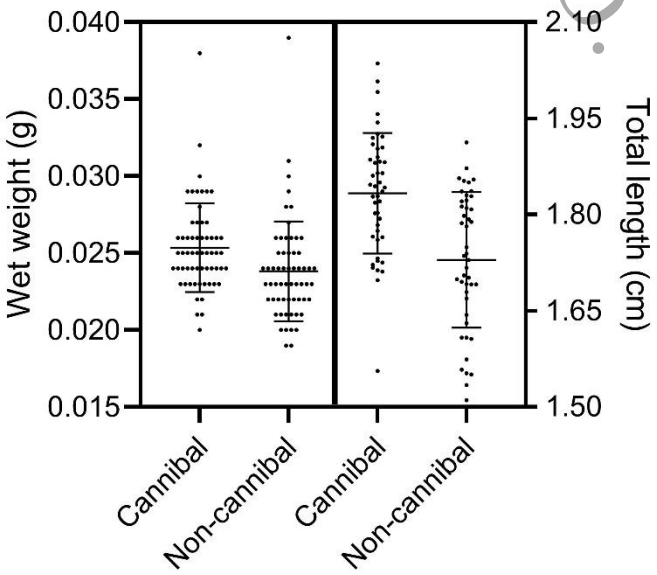

Figure 5. Growth in weight (g) and total length (cm) of *A. tropicus* cannibals' larvae fed with 10 g/Kg Trp and CD. Values are mean ± *SD*.

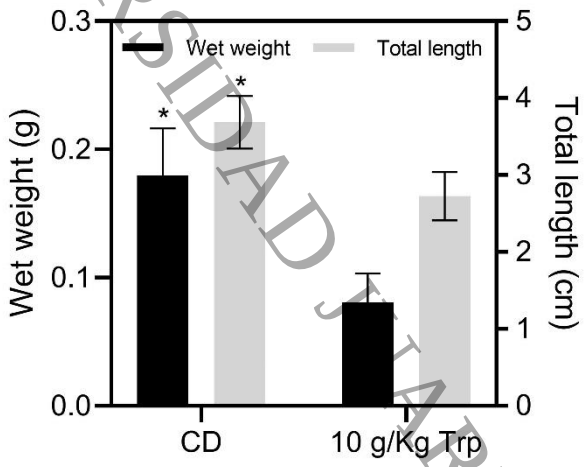

Figure 6. Survival and cannibalism of *A. tropicus* larvae cannibals fed with 10 g/Kg Trp and CD. Values are mean ± *SD*.

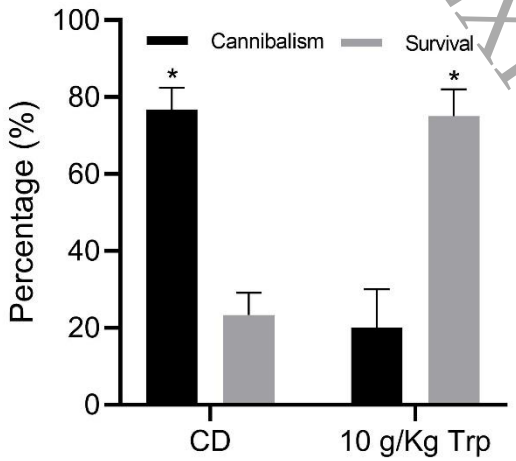

Figure 7. Wet weight (g) and total length (cm) class distribution by cannibalisms effect in *A. tropicus* larvae cannibals fed 10 g/Kg Trp and CD. Values are mean ± *SD*.

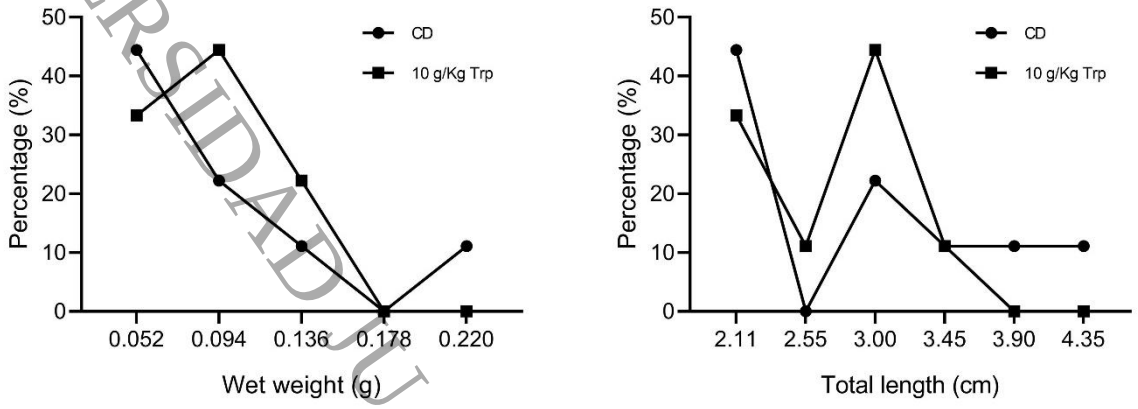

Figure 8. Relative gene expression of *sstr1, th, hdc, crh, htr1a, drd1, avpi1,* and *tph1* in *A. tropicus* larvae fed different Trp concentration. Relative mRNA levels of the indicated genes were measured by RT-qPCR using β-actin as the reference gen. Data are presented as fold-changes in the mRNA levels, in comparison with sample with CD (dotted line) (n = 3, mean ± *SD*). Significant differences with respect to control (dotted line) are indicated by different letters ($p < 0.05$) Significant differences within the cannibal (C) and non-cannibal (N) are indicated by * ($p < 0.05$).

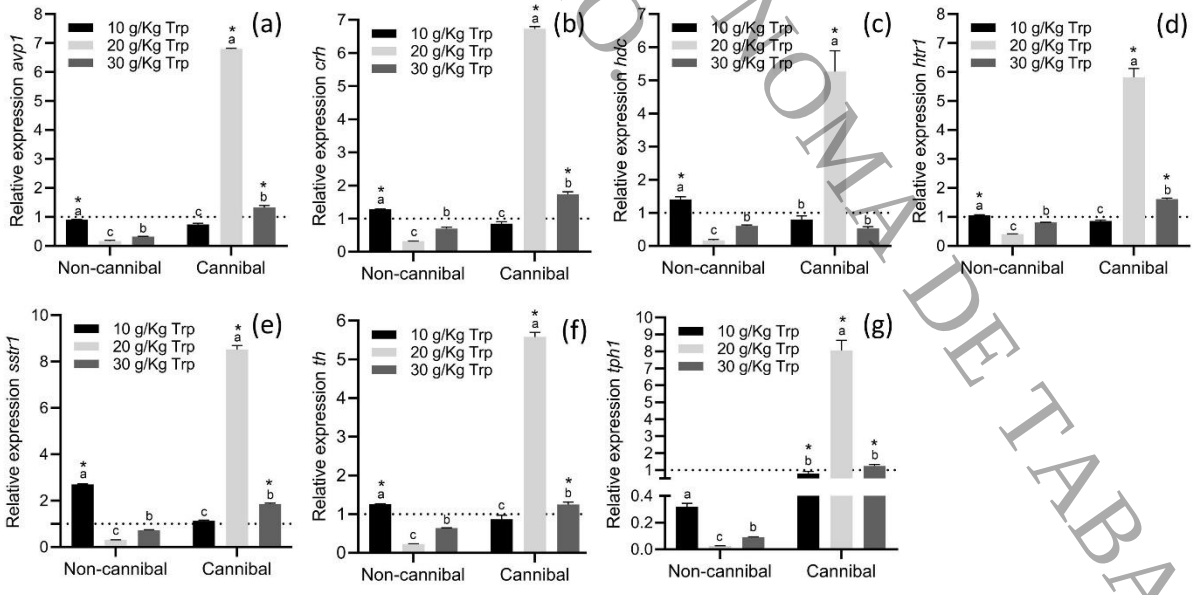

Figure 9. Relative gene expression of *sstr1, th, hdc, crh, htr1a, gnrh1, drd1, avpi1,* and *tph1* in *A. tropicus* cannibals' larvae fed whit 10 g/Kg Trp. Relative mRNA levels of the indicated

genes were measured by RT-qPCR using β-actin as the reference gen. Data are presented as fold-changes in the mRNA levels, in comparison with sample with CD (dotted line) (n = 3, mean ± *SD*). Significant differences with respect to CD are indicated by * ($p < 0.05$).

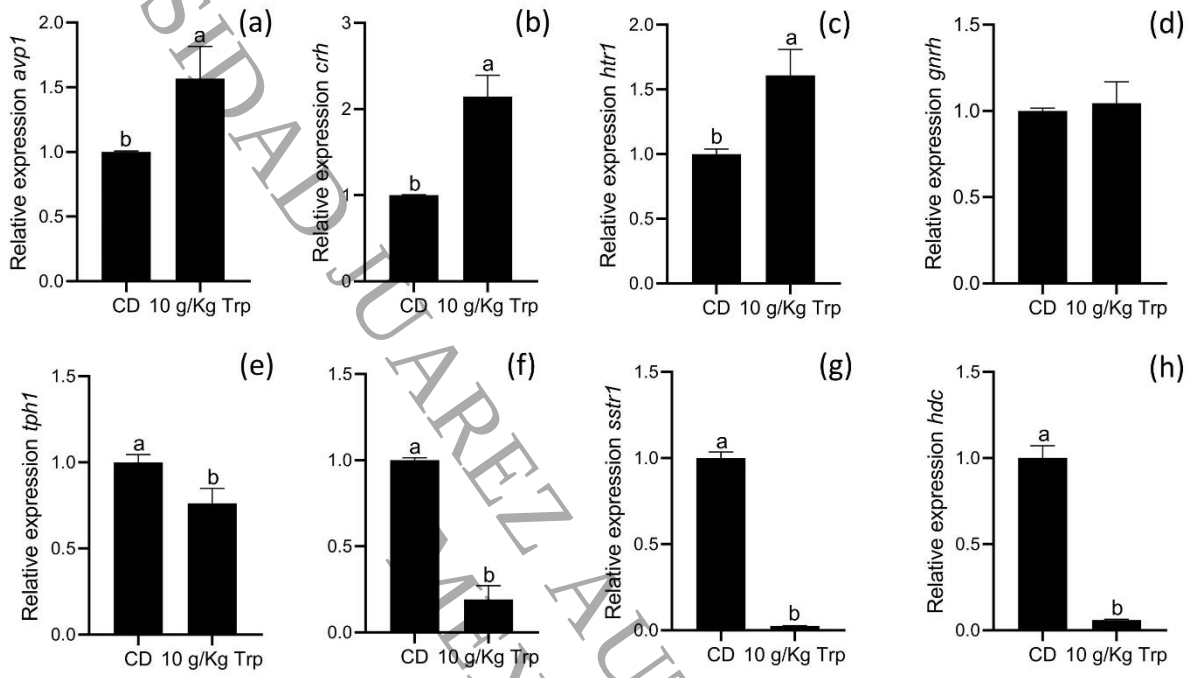

Table 1.- Composition of experimental diets with different concentration of Trp and CD.

	Trp (g/Kg)			
Ingredients	**CD**	**10**	**20**	**30**
Fish meal[a]	305.4	305.4	305.4	305.4
Renderer meal[a]	300.0	300.0	300.0	300.0
Soy meal[a]	150.0	150.0	150.0	150.0
Corn starch[b]	67.1	67.1	67.1	67.1
Oil soy[c]	116.5	116.5	116.5	116.5
Cellulose[d]	30.0	20.0	10.0	0.0
Tryptophan[d]	0.0	10.0	20.0	30.0
Premix vit-min[e]	15.0	15.0	15.0	15.0
Grenetine[f]	10.0	10.0	10.0	10.0
Vit C[g]	5.0	5.0	5.0	5.0
Vit E[h]	1.0	1.0	1.0	1.0
Proximate composition (g/100g dry matter), except energy				
Energy (Kj/g)	17.91	17.53	17.39	17.48
Protein	44.00	45.00	46.00	47.00
Ether extract	16.38	16.59	16.41	16.48
Fibre	1.05	1.12	1.07	1.01
Ahs	13.43	13.23	13.02	12.93
NFE[1]	25.14	24.06	23.5	22.58

[a]Marine and agricultural proteins S.A. de C.V., Guadalajara, Jalisco; [b]IMSA Corn Industrializer S.A de C.V. Guadalajara, Jalisco, México; [c]Ragasa industries S.A. de C.V. ; [d]Sigma-Aldrich Quimica S. de R.L. de C.V.; [e]Vitamin premix composition g, mg or International Units per kg of diet: Vitamin A, 10,000,000 IU; Vitamin D3, 2,000,000 IU; Vitamin E, 100,000 IU; Vitamin K3, 4.0 g; Thiamine B1, 8.0 g; Riboflavin B2, 8.7 g; Pyridoxine

B6, 7.3 g; Vitamin B12, 20.0 mg; Niacin, 50.0 g; Pantothenic acid, 22.2 g; Inositol, 0.15 mg; Nicotinic Acid, 0.16 mg; Folic Acid, 4.0 g; Biotin, 500 mg; Vitamin C, 10.0 g; Choline 0.3 mg, Excipient q.s. 2 g; Manganese, 10 g; Magnesium, 4.5 g; Zinc, 1.6 g; Iron, 0.2 g; Copper, 0.2 g; Iodine, 0.5 g; Selenium, 40 mg; Cobalt 60 mg. Excipient q.s. 1.5 g.; [e]D'gari, food and diet products relámpago S.A. de C.V.; [g]ROVIMIX® STAY-C® 35-DSM, Guadalajara, México; [h]GELPHARMA, S.A. de C.V. NFE[1] = nitrogen-free extract: 100-(%protein-%ethereal extract-%ash-%fibre); [e]Trouw Nutrition México S.A. de C.V. (by courtesy).

Table 2.- Analysis of total fatty acids in experimental diets used for *A. tropicus* larvae.

		Trp (g/Kg)		
Fatty acids (%)	**CD**	**10**	**20**	**30**
C13:0	7.0	10.2	9.7	7.9
C14:0	1.2	1.3	1.2	1.2
C16:0	16.7	17.0	17.0	17.4
C17:0	ND	ND	ND	ND
C18:0	5.8	6.0	6.0	6.1
C23:0	ND	ND	ND	ND
ΣSFA	**30.7**	**34.4**	**33.9**	**32.7**
C16:1n7	2.0	2.1	2.1	2.2
C18:1n9	21.0	20.4	20.5	21.1
C18:1n7	1.6	1.8	1.8	1.8
ΣMUFAS	**24.6**	**24.3**	**24.5**	**25.1**
C18:2n6	34.9	32.0	32.3	32.6
C18:3n3	0.3	4.3	4.3	4.3
C18:4n3	4.7	ND	ND	ND
C20:3n3	0.5	0.6	0.7	0.7
C20:4n6	0.3	ND	ND	ND
C20:5n3	1.6	1.7	1.8	1.7
C22:5n3	0.4	0.4	0.4	0.4
C22:6n3	1.9	2.2	2.2	2.4
ΣPUFAS	**44.6**	**41.3**	**41.6**	**42.2**
NID	**0.0**	0.0	0.0	0.0
	100.0	100.0	100.0	100.0

Table 3. Analysis of total amino acid in experimental diets used for *A. tropicus* larvae.

		Trp (g/Kg)		
Amino acid	**CD**	**10**	**20**	**30**
Essential amino acids				
HIS	1.1	1.0	1.2	1.0
ARG	5.2	5.6	5.4	5.3
THR	1.7	1.6	1.7	1.7
VAL	1.7	1.6	1.6	1.6
MET	0.5	0.5	0.2	0.2
LYS	5.7	5.0	5.3	5.5
ILE	1.3	1.2	1.3	1.3
LEU	3.5	3.2	3.5	3.5
PHE	1.4	1.2	1.4	1.4
subtotal	**22.1**	**20.9**	**21.5**	**21.7**
Non-essential amino acids				
ASP	2.4	2.2	2.4	2.4
SER	2.3	2.2	2.4	2.3
GLU	5.8	5.7	6.0	6.0
GLY	7.0	8.2	7.1	6.9
ALA	3.4	3.7	3.6	3.5
TYR	1.5	1.3	1.4	1.5
subtotal	**22.3**	**23.4**	**22.9**	**22.7**
Others				
TAU	0.6	0.7	0.6	0.6
Total	45.0	45.0	45.0	45.0
Tryptophan (mg/g)	10.60	19.74	32.42	44.21

Table 4. Oligonucleotide design for real-time polymerase chain reaction (qPCR) of aggressive genes in *A. tropicus* larvae.

Protein	**Gen**	**Primers (5'-3')**	**Alignment temperature (°C)**
Somatostatin receptor 1	*sstr1*	FW: CCTCAGCATTGACCGCTACA RV: AATACCGCCATCCACTGACG	60
Tyrosine hydroxylase	*th*	FW: GGACCAGATGTACCAGCCAG RV: GCAGTTCATCCCTCGCAGAT	59
Histidine decarboxylase	*hdc*	FW: GCATTTCGACTGCACTGCTT RV: CTTCGGCTGAGTGGGATCTG	59
Corticotropin releasing hormone	*crh*	FW: AACGTCAACAGGGCTTTCCA RV: TCTTCCCGTCAGGTCTTCCA	60
5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled	*htr1a*	FW: AAGCGCAGTGTGGAACCTAA RV: GCTGTCGGGGTATTAGGCAG	60
Gonadotropin releasing hormone 1	*gnrh1*	FW: AGTCAGCACTGGTCATACGG RV: CTCACCTCCTCCGCAATGTC	59
Dopamine receptor D1	*drd1*	FW: TTTTTGGCCCTTTGGCTCATT RV: AAGTTCAAAATGGAGGCTGTGG	59
Arginine vasopressin induced 1	*avpi1*	FW: AGGGAGGACCACTGAAGATGA RV: CCAGCAGAGGACAAGTCTGC	60
Tryptophan hydroxylase 1	*tph1*	FW: CCCCCGTATCGAGTTCACAG RV: AGGGGCAGGTTCTTGAGGTA	60

Table 5.- Growth performance and feed utilization indexes of *A. tropicus* larve fed different concentration of Trp and CD (mean ± standard deviation, *SD*).

		Trp (g/Kg)		
	CD	**10**	**20**	**30**
Initial weight (g)	0.018 ± 0.001	0.018 ± 0.001	0.018 ± 0.001	0.018 ± 0.001
Final weight (g)	0.022 ± 0.0003	0.023 ± 0.001	0.023 ± 0.001	0.023 ± 0.001
Initial total length (cm)	1.28 ± 0.09	1.28 ± 0.09	1.28 ± 0.09	1.28 ± 0.09
Final total length (cm)	1.78 ± 0.05	1.81 ± 0.02	1.83 ± 0.03	1.80 ± 0.03
S (%)	28.25 ± 1.76	34.75 ± 5.30	28.66 ± 1.04	29.75 ± 1.76
FI (g/d)	0.031 ± 0.001	0.031 ± 0.003	0.031 ± 0.003	0.031 ± 0.001
AWG (g/fish)	0.004 ± 0.0003	0.005 ± 0.0008	0.005 ± 0.001	0.005 ± 0.00004
SGR (%/d)	5.07 ± 0.52	5.35 ± 0.29	5.53 ± 0.38	5.24 ± 0.39
FCR	7.23 ± 0.85	6.68 ± 0.53	5.77 ± 0.46	5.64 ± 0.02
PER	0.13 ± 0.01	0.15 ± 0.1	0.15 ± 0.04	0.15 ± 0.04
K	0.39 ± 0.02	0.39± 0.01	0.37± 0.01	0.38± 0.02
CV (%)	6.43 ± 2.98	8.63 ± 2.23	8.20 ± 3.05	6.95 ± 0.05
SH	0.71 ± 0.33	0.96 ± 0.25	0.91 ± 0.34	0.77 ± 0.006

Significant differences within the diets are indicated by different letters ($p < 0.05$). FI: feed intake; AWG: absolute weight gain; SGR: specific growth rate; S: Survival; FCR: feed conversion rate; PER: protein efficiency rate; K: condition factor, CV: Coefficient of variation, SH: Size heterogeneity.

Table 6.- Growth performance and feed utilization indexes of *A. tropicus* cannibal larvae fed with 10 g/Kg Trp and CD (mean ± standard deviation, *SD*).

	CD	**10 g/Kg Trp**
Initial weight (g)	0.049 ± 0.004	0.048 ± 0.006
Final weight (g)	0.179 ± 0.03*	0.080 ± 0.02
Initial total length (cm)	2.16 ± 0.11	2.19 ± 0.08
Final total length (cm)	3.68 ± 0.34*	2.72 ± 0.18
S (%)	23.33 ± 5.77	75.0 ± 7.07*
FI (g/d)	0.72 ± 0.07	0.76 ± 0.08
AWG (g/fish)	0.13 ± 0.03*	0.03 ± 0.01
SGR (%/d)	14.62 ± 2.87*	5.81 ± 3.18
FCR	5.66 ± 0.62	12.43 ± 1.60*
PER	0.17 ± 0.01*	0.05 ± 0.03
K	0.35 ± 0.02	0.39 ± 0.01
CV (%)	55.64 ± 6.84*	26.74 ± 14.20
SH	6.07 ± 0.75*	1.90 ± 1.02

Significant differences within the diets are indicated by different letters ($p < 0.05$). FI: feed intake; AWG: absolute weight gain; SGR: specific growth rate; S: Survival; FCR: feed conversion rate; PER: protein efficiency rate; K: condition factor, CV: Coefficient of variation, SH: Size heterogeneity.

Table 7. Digestive enzymatic activities (mean ± standard deviation, *SD*) of *A. tropicus* larvae fed with different concentration of Trp and CD.

Activities			Trp (g/Kg)		
(u/mg protein)		**CD**	**10**	**20**	**30**
Acid protease		12.233 ±0.924[a]	2.505 ± 2.272[b]	3.546 ± 1.207[b]	7.645 ± 0.792[ab]
Alkaline protease		17.374±1.550[a]	8.160 ± 2.257[b]	7.388 ± 0.852[b]	7.784 ± 0.859[b]
Trypsin		1.334±0.088[a]	0.682±0.067[ab]	0.640±0.018[b]	0.645±0.236[ab]
Leucine aminopeptidase		0.366±0.014	0.290±0.007	0.317±0.057	0.337±0.107
Lipases		1.711±0.654[b]	4.529 ± 0.318[a]	4.514 ± 0.099[a]	2.413 ± 0.079[b]
		CD	**10**		
Acid protease		20.492±4.162	35.869 ± 3.670 * 0.033		
Alkaline protease		7.043±0.835	14.395 ± 2.342 * 0.028		
Trypsin		0.755±0.079	1.032 ± 0.086 *0.0151		
Leucine aminopeptidase		0.432±0.017	1.333 ± 0.203 * 0.0167		
Lipases		3.178±0.640	3.072 ± 0.400		
		CD	**10**	**20**	**30**
Acid protease	C	28.261±0.028[b*]	43.110±0.045[a*]	18.780±0.102[d]	25.451±0.017[c*]
	N	23.576±0.062[b]	39.280±0.049[a]	19.469±0.031[d*]	23.228±0.020[d]
Alkaline protease	C	15.887±0.016[a]	10.241± 0.218[c*]	14.263±0.035[b*]	9.963±0.072[c*]
	N	18.021±0.128[a*]	9.722±0.186[b]	8.393 ± 0.166[c]	9.762 ± 0.016[b]
Trypsin	C	1.919±0.318[a*]	1.491±0.143[b*]	1.203±0.082[b*]	1.154±0.144[b*]
	N	1.493±0.232[a]	1.170±0.078[b]	0.851±0.087[c]	0.863±0.069[c]
Leucine aminopeptidase	C	0.284±0.044[c]	0.527±0.079[a]	0.684 ±0.164[ab]	0.382±0.051[bc]
	N	0.184 ±0.086[c]	0.494±0.045[ab]	0.514±0.053[a]	0.379±0.042[b]
Lipases	C	0.900±0.011[b]	3.288±0.038[a]	0.481± 0.025[c]	0.588± 0.032[c]
	N	3.324±0.019[b*]	3.889±0.001[a*]	1.759±0.016[c*]	1.502±0.012[d*]

Significant differences within the diets are indicated by different letters ($p < 0.05$). Significant differences within the cannibal and non-cannibal are indicated by * ($p < 0.05$)

Table 8. Attacks types and cannibalism behavior of *A. tropicus* larvae fed with 10 g/Kg Trp and CD in combination with different shelters.

		Lateral attack			Frontal attack		
	Escape	**Head**	**Middle**	**Tail**	**Head**	**Middle**	**Total**
Without shelter							
CD	2	2	0	0	0	0	4
10 g/Kg Trp	2	0	0	0	0	0	2
Total	4	2	0	0	0	0	**6**
Rocks							
CD	2	0	1	0	0	0	3
10 g/Kg Trp	0	0	0	0	0	0	0
Total	2	0	1	0	0	0	**3**
Artificial Vegetation							
CD	2	0	0	0	0	0	2
10 g/Kg Trp	1	0	0	0	0	0	1
Total	3	0	0	0	0	0	**3**
SUMA total	9	2	1	0	0	0	*12*

CONCLUSIONES

En conclusión, las larvas de *A. tropicus* muestran canibalismo tipo I, II y dos variantes del tipo III. Se sugiere que esta especie es un caníbal más eficiente que un depredador interespecífico. La relación del mayor número de ataques se presentó en el tanque de color blanco lo cual podría estar relacionado con el contraste con el fondo y el color de las larvas, y a su vez, hay una menor preferencia por este color. El uso de vegetación artificial como refugio disminuye el número de ataques, y tiene un mayor porcentaje de preferencia que el utilizar rocas. Los eventos caníbales coinciden con los cambios morfométricos que les permite capturar e ingerir presas de su propio tamaño, eliminándose la conducta cuanto las larvas crecen y las relaciones morfométricas del cuerpo de *A. tropicus* cambia. El triptófano disminuye el canibalismo significativamente en larvas de *A. tropicus*. Por otro lado, las larvas caníbales y no caníbales presentan diferencia en la actividad enzimática, expresión de genes, peso y talla, en esta dos últimas variables presentándose una diferencia entre caníbal y su presa de 16.39 ± 10.86 % y 15.23 ± 5.68 %, respectivamente. La suma de toda esta información obtenida es fundamental para realizar una clasificación más eficiente de las larvas durante su cultivo e implementar estrategias para reducir el canibalismo en estado larvario en *A. tropicus* de manera más eficiente. Además, los resultados de este estudio podrían ser útiles para empezar a comprender este comportamiento en otras especies y sirva como modelo de estudio.