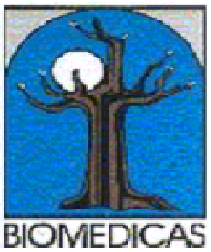

Universidad Nacional Autónoma de México

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Doctorado en Ciencias Biomédicas

Identificación de genes hormono-regulados en el cisticerco de la *Taenia crassiceps* involucrados en la proliferación y el establecimiento del parásito

Tesis que para obtener el grado de:

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Comité Tutoral

Dr. Jorge Morales Montor **Dr. Carlos Larralde** **Dr. Marco A. Cerbón**

Ciudad Universitaria, Abril de 2008

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¿Qué busco?

Es una buena pregunta.
He tratado muchas veces
de buscar a Dios
y a la justicia.

Soy un pobre diablo
que anda
entre el cielo y el infierno.

Soy una gente
que lo quiere todo
y que no ha alcanzado nada.

Durante meses o años
busco
la justicia, el pan, la comida,
la sal, la mujer
y hay momentos,
breves momentos,
en que he querido buscar a Dios...

Nunca lo he encontrado,

el día que lo encuentre

me quedo callado...

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Académicamente,

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A Mamá. Porque con tu ayuda, valor y amor he tratado de llevar al límite tu acertada demanda de excelencia. Mamá, ¡me saqué diez!

A Andrik, Arian y Abraham, mis valientes, incansables y admirables hermanos.

A Sonia, por llegar, saludar y quedarse a compartir una vida. Te amo Princesa.

ÍNDICE

RESUMEN

Durante diversas infecciones, sobre todo parasitarias se ha reportado dimorfismo sexual en la susceptibilidad y/o resistencia de las mismas. Tal es el caso de la cisticercosis experimental murina provocada por el metacéstodo de la *Taenia crassiceps*. Dicho dimorfismo se atribuye a las diferencias hormonales entre machos y hembras, diferencias que pueden incidir sobre la respuesta inmunológica específica contra el parásito o sobre el parásito mismo. Por lo tanto, el tema central de esta tesis fue determinar los efectos directos de las hormonas esteroides sexuales 17β-estradiol, progesterona, testosterona y 5α-dihidrotestosterona sobre el cisticerco de la *Taenia crassiceps in vitro*. Nuestros resultados muestran que 17β-estradiol estimula la reproducción del parásito en 400% mientras que progesterona lo hace sólo en 20%. Por el contrario, testosterona y 5α-dihidrotestosterona disminuyen la capacidad reproductiva del parásito en 70 y 85%, respectivamente. Estos efectos fueron dependientes de la concentración hormonal usada. Además de esto, la infectividad del parásito, así como la síntesis de DNA se vieron favorecidas por los tratamientos con progesterona y 17β-estradiol, mientras que testosterona y 5α-dihidrotestosterona disminuyeron ambas en el cisticerco. Interesantemente el tratamiento previo del parásito con Tamoxifen inhibió los efectos proliferativos de 17β-estradiol sobre éste. Por otra parte, RU-486 y Flutamida no previnieron los efectos de progesterona y los andrógenos testosterona y 5α-dihidrotestosterona, respectivamente. Estos hallazgos sugieren que el mecanismo a través del cual 17β-estradiol lleva a cabo sus efectos sobre el cisticerco de la *Taenia crassiceps* puede ser mediado por un receptor de estrógenos específico, sintetizado por el parásito y con capacidad de unir 17β-estradiol exógeno. Alternativamente, los efectos de este estrógeno sobre el cisticerco también pueden deberse a la acción de vías de señalización mediadas por cascadas de segundos mensajeros tales como LYN-SYK-ERK, activada en favor del tratamiento estrogénico y bloqueada mediante el uso de inhibidores específicos. Por otra parte, los efectos de progesterona y andrógenos pueden ser mediados por mecanismos alternativos aún por determinar. Los resultados de este trabajo pretenden ampliar nuestro concepto de la relación hospedero-parásito, con la finalidad de entender la dinámica molecular y evolutiva de ambos organismos en los contextos de salud y enfermedad, conocimiento que podría ayudarnos a prevenir y controlar no sólo esta, sino diversas infecciones parasitarias de importancia médica y veterinaria.

ABSTRACT

Sexual dimorphism of the immune response has been frequently reported during several parasite infections. This is the case of murine experimental cysticercosis caused by the helminth parasite *Taenia crassiceps*, where female mice are more susceptible than males to infection. These differences are strongly related to sex hormones variations between both genders, which in turn may differentially affect either host immune response or parasite itself. Under these considerations, the matter of this work was to determine the specific effects of 17β-estradiol, progesterone, testosterone y 5α-dihydrotestosterone upon *Taenia crassiceps* cysticerci *in vitro*. Our findings show that 17β-estradiol increases parasite reproduction at 400% meanwhile progesterone does it at 20%. By the opposite, testosterone y 5α-dihydrotestosterone decrease parasite reproduction at 70 and 85% respectively. All hormonal effects upon cysticerci were dependent of each sex-steroid concentration. In addition to, both infectivity and DNA synthesis were stimulated by 17β-estradiol and progesterone treatments, whilst androgens reduced both of them. Interestingly, Tamoxifen inhibited the proliferative effects of 17β-estradiol upon parasites. On the other hand, RU-486 and Flutamide did not reverse progesterone and androgen effects respectively. These results suggest that 17β-estradiol exerts its proliferative effects upon the parasite through a specific estrogen receptor from the cysticercus, able to bind exogenous sex hormones. Alternatively, 17β-estradiol effects could be related to signal transduction pathways regulation such as LYN-SYK-ERK signaling cascade, which can be activated by this estrogen and, in turn, blocked by specific inhibitors of those second messengers before mentioned. On the other side, progesterone and androgen effects are mediated by still undetermined mechanisms. Finally, these results could extend our understanding about host-parasite relationship at molecular and evolutionary levels, under health and sickness conditions not only in this infection but also in other parasite infections of medical and veterinary relevance.

INTRODUCCIÓN

Durante diversas infecciones, sobre todo parasitarias, se ha reportado dimorfismo sexual en la susceptibilidad y/o resistencia de las mismas (Lockshin, 2001). Este dimorfismo se establece cuando organismos de la misma especie, pero de sexo distinto, poseen la capacidad de responder de manera diferencial a un mismo estímulo antigénico. Tal es el caso de la cisticercosis experimental murina provocada por el metacéstodo de la *Taenia crassiceps*.

Taenia crassiceps es un céstodo helminto del orden de los ciclofilideos (Chandler, 1955), cuyo ciclo de vida se desarrolla entre sus hospederos intermediario y definitivo. El primero se encuentra representado por diversas especies de roedores (*Mus musculus*, *Microtus arvalis*, *Peromyscus vulgaris*, etc.), donde la forma larvaria del parásito, o cisticerco, es capaz de alojarse a nivel del músculo esquelético. Complementariamente, cuando un cánido (*Canis lupus*, *Canis vulgaris*) depreda a un roedor cisticercoso se convierte en el hospedero definitivo, pues es capaz de alojar intestinalmente a la tenia, es decir, la forma adulta del parásito. Una vez establecido, este gusano hermafrodita puede autofecundarse y producir diariamente miles de huevesillos que, una vez liberados al ambiente a través de las heces, serán ingeridos por el ratón, donde se desarrollará el cisticerco, cerrando así el ciclo de vida de éste (Figura 1).

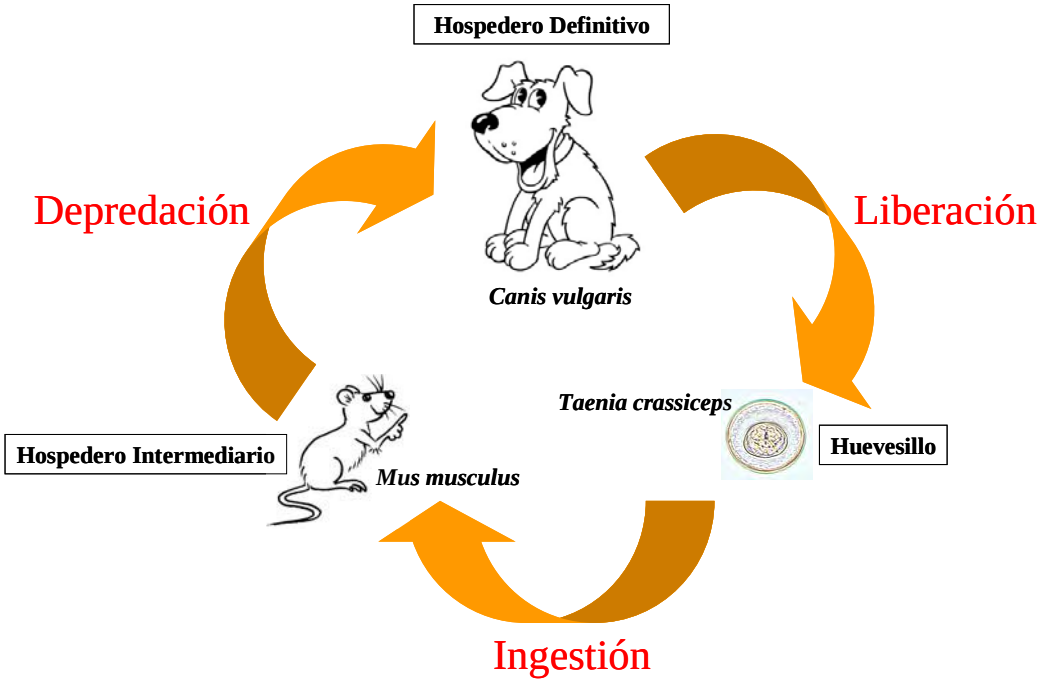

Figura 1. Ciclo de vida de la *Taenia crassiceps*.

Por otra parte, la cisticercosis murina por *Taenia crassiceps* también se puede inducir experimentalmente, tras la inoculación intraperitoneal del cisticerco en el ratón (*Mus musculus*) (Sciutto et al., 1991). Una de las características más importantes de esta infección, es la marcada susceptibilidad asociada al sexo que se presenta durante la misma, en donde las hembras desarrollan cargas parasitarias cuatro veces mayores que los machos durante el período agudo de la infección (Larralde et al., 1995). Dicho fenómeno se atribuye a las diferencias hormonales entre machos y hembras, dándose en éstas últimas un microambiente fisiológico apropiado para el establecimiento y proliferación del cisticerco de la *T. crassiceps*, como se podrá observar más adelante (Larralde et al., 1995).

Por otro lado, es importante destacar que llegado el período crónico de la infección (después de cuatro semanas), la carga parasitaria entre hembras y machos se iguala, borrando así todo indicio de dimorfismo sexual entre ambos sexos (Larralde et al., 1995). Este aumento en la carga parasitaria de los machos, coincide con un importante incremento en los niveles séricos de 17β-estradiol [E2] (hasta 500% más con respecto a sus valores normales) así como en una dramática disminución de los niveles circulantes de testosterona [T4] (hasta 90% menos de sus valores normales). Esto se debe a que la expresión testicular de la

P-450 aromatasa se ve aumentada, al mismo nivel que en el ovario de una hembra normal, dando como consecuencia la conversión desmedida de T4 a E2 en el macho parasitado (Larralde et al., 1995). Cabe mencionar que este serio desajuste hormonal afecta otras esferas biológicas del hospedero, como la conducta sexual, pues los machos parasitados pierden progresivamente la conducta de intromisión, enseguida la conducta de monta y, finalmente, la respuesta de eyaculación (Morales et al., 1996). Al mismo tiempo, los niveles de interleucina 6 (IL-6) se ven significativamente elevados en machos crónicamente infectados (Larralde et al., 1995; Morales-Montor et al., 2003). A este respecto, se sabe que algunas citocinas, como es el caso de la IL-6, pueden potenciar la expresión de la P-450 aromatasa en muchas especies de mamíferos.

Lo anterior explicaría, al menos parcialmente, porqué los niveles de expresión de esta enzima y por lo tanto los niveles de E2, se ven tan aumentados; todo recae sobre la regulación de una fina pero sumamente complicada red de interacciones inmunoendocrinas (Morales-Montor et al., 2001), véase Fig.2.

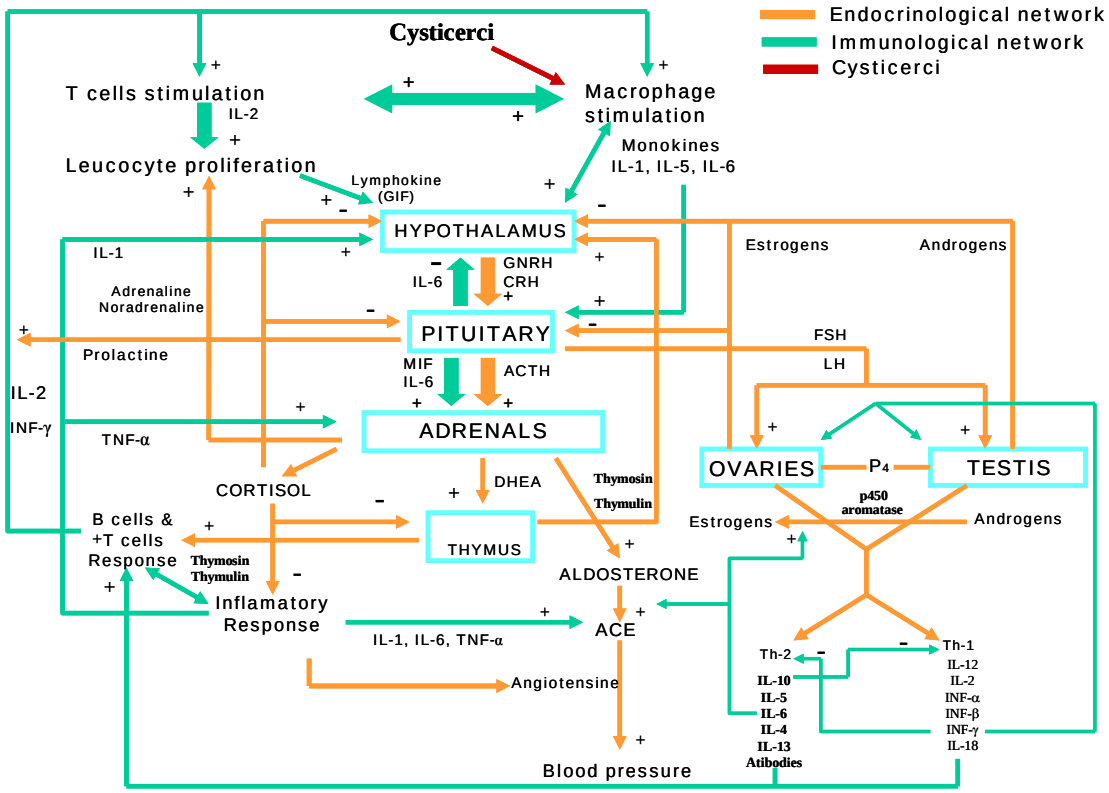

Figura 2. Red de interacciones inmunoendocrinas durante la cisticercosis experimental murina. Tomado de Morales-Montor et al., 2004.

Con base en estas evidencias, es pertinente pensar que el cisticerco borra la diferencia en susceptibilidad entre machos y hembras, porque es capaz de igualar el microambiente hormonal entre ambos sexos, particularmente el de los esteroides sexuales, aprovechando así una gama compleja de estímulos inmunoendocrinos que favorecerán su establecimiento en el macho crónicamente infectado.

Sin embargo, esta interacción bidireccional entre la respuesta inmune y las hormonas esteroides, no solo recae sobre los efectos de E2 y T4, sino también sobre otros tipos hormonales, específicamente aquellos que se producen mayoritariamente durante ciertas fases del ciclo estral y la gestación, como la progesterona [P4]. Evidencias recientes en cerdos y ratones, muestran que la frecuencia de cisticercosis se ve favorecida durante la gestación en hembras y la castración en machos (Morales et al., 2002), lo cual sugiere que factores endocrinos que se liberan durante esta etapa del ciclo reproductivo (particularmente P4 y prolactina), poseen funciones inmuno-moduladoras y por lo tanto, un papel sumamente importante durante el proceso de infección (Vargas-Villavicencio et al., 2005).

De esta manera, queda claro que el sistema inmune es directamente influenciado por factores endocrinos, y éstos a su vez retroalimentados por el sistema inmune y sus moléculas efectoras, constituyendo una fascinante pero compleja red de comunicación entre dos de los más importantes sistemas homeostáticos de los organismos superiores: el sistema inmune y el sistema endocrino.

Esta aseveración no excluye a la cisticercosis experimental murina, en donde, debido a la colaboración de múltiples grupos interdisciplinarios de trabajo, se ha avanzado importantemente en el conocimiento de esta parasitosis y los múltiples diálogos que se suscitan entre los sistemas antes mencionados durante la misma (Sciutto et al., 1991; Larralde et al., 1995; Terrazas et al., 1999; Morales-

Montor et al., 2001; Rodríguez-Sosa et al., 2002; Morales-Montor y Larralde, 2005; Vargas-Villavicencio et al., 2006).

Sin embargo, y a pesar de que se ha avanzado mucho en el conocimiento de ésta infección experimental, aún quedan diversas preguntas por contestar. Por ejemplo, ¿la sola influencia del sistema endocrino sobre el sistema inmune es capaz de determinar el curso de una infección parasitaria?, ¿el parásito es capaz de responder al microambiente hormonal del hospedero?, ¿pueden los esteroides sexuales afectar directamente, y sin mediación del sistema inmune, el establecimiento, crecimiento y/o reproducción parasitaria?, ¿las hormonas esteroides sexuales del hospedero, tales como E2, P4, T4 y 5α-dihidrotestosterona (DHT), actúan directamente sobre el cisticerco de la *Taenia crassiceps*? El planteamiento de estas preguntas es la materia de este trabajo, y pretende incidir no sólo en el entendimiento de la cisticercosis experimental murina, la cisticercosis porcina o la neurocisticercosis humana, sino en nuestra concepción de la relación hospedero-parásito, a niveles como el molecular, el evolutivo o el terapéutico, y no solo en ésta, sino también en otras infecciones (Fig. 3).

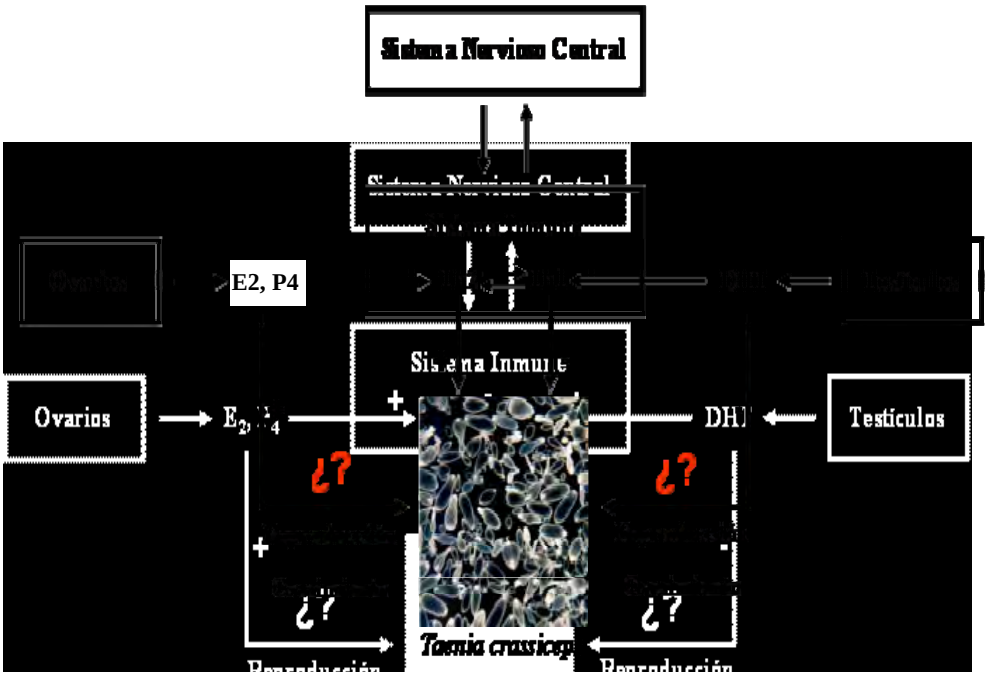

Figura 3. ¿Pueden las hormonas del hospedero afectar directamente la fisiología parasitaria?

Por otra parte, no sería descabellado suponer que los esteroides sexuales del hospedero pudieran regular diversos procesos moleculares y celulares en el parásito, ya que es bien conocido que las hormonas, tanto proteicas cómo esteroides, regulan y coordinan una gran variedad de funciones celulares y fisiológicas dentro de un organismo (Fig. 3). Estas funciones incluyen el crecimiento, reproducción y diferenciación, además de procesos como balance y aprovechamiento nutricional, fenómenos excretorios, respuesta corta y prolongada al estrés, mantenimiento del medio interno del organismo y, como ya se ha mencionado, la respuesta inmunológica dirigida contra un agente patógeno (Akmaev, 1996).

En relación a esto, estudios recientes sugieren que el hospedero, a través de las hormonas y factores de crecimiento que él sintetiza, puede modificar diversos aspectos de la fisiología parasitaria, tales como infectividad, diferenciación y crecimiento (Mendoca et al., 2000; Charder et al., 1992; Escobedo et al., 2005). De manera específica, se ha reportado que el tratamiento con el andrógeno adrenal dehidroepiandrosterona (DHEA) de ratones infectados con cercarias de *Schistosoma mansoni*, reduce considerablemente la intensidad parasitaria (Mendoca et al., 2000). En el mismo sentido, se ha observado que el crecimiento de *Heterakis spumosa* se ve afectado por el tratamiento con T4 (Charder et al., 1992). Por otra parte, en la infección murina provocada por *Nippostrongylus brasilensis*, existe evidencia experimental que apoya que los esteroides sexuales modulan de manera directa el establecimiento parasitario (León et al., 1986).

Adicionalmente, en merozoitos de *Plasmodium falciparum*, el tratamiento con cortisol aumenta el número y tamaño de gametocitos en este parásito (Lingnau et al., 1993). De manera similar, merozoitos tratados con insulina, E2, P4 y T4, incrementan considerablemente el número de gametocitos, aumentando además el crecimiento y la reproducción del parásito en este estadio. Además, cuando estos parásitos son tratados con 16-α-bromoepiandrosterona, un análogo de la DHEA, su crecimiento se ve disminuido hasta en un 25% (Freilich et al., 2000).

De manera contrastante, la exposición a E2, P4 y T4 *in vitro* de trofozoítos de *Entamoeba histolytica*, no afecta al parásito en ninguna función. Sin embargo, el tratamiento con cortisol incrementa la síntesis de DNA y la proliferación de los trofozoítos, mientras que los parásitos tratados con DHEA, muestran una disminución en los mismos parámetros, además de pérdida progresiva de adherencia y motilidad, culminando con lisis parasitaria (Carrero et al., 2006).

Por otro lado, el tratamiento de la forma adulta de *Schistosoma haematobium* con T4 disminuye la fecundidad y, por lo tanto, la capacidad reproductiva de este parásito (Remoue et al., 2002). Adicionalmente, amastigotes de *Trypanosoma cruzi,* tratados con factor de crecimiento epidermal murino (EGFm), incrementan considerablemente la síntesis de DNA, crecimiento y actividad metabólica, induciendo receptores con actividad de cinasas de tirosina, tales como la proteína cinasa C (PKC) y diversos miembros de la cascada de activación de las MAP cinasas (Ghansah, 2002). De modo similar, se ha descrito un efecto estimulante del EGFm sobre el desarrollo y la maduración de diversas filarias, particularmente la de *Brugia malayi* (Dissanayake, 2000). También se ha demostrado que micofilarias de *Onchocerca volvulus* y *Oncocerca lienalis* incrementan su actividad metabólica cuando se les trata con 20-hidroxiecdisona (Townson y Tagboto, 1996). Finalmente, el factor estimulante de la colonia de macrófagos-granulocitos (MG-CSF) promueve el crecimiento de promastigotes de *Leishmania mexicana* (Charlab et al., 1990). Toda esta evidencia sugiere que las hormonas, particularmente las esteroides, pueden modificar el curso de una infección a través de ejercer sus efectos directamente sobre el parásito, no de manera aleatoria o inespecífica, sino regulando en éste procesos tan importantes como la expresión génica, la síntesis de proteínas y/o la activación de cascadas de segundos mensajeros. Sorprendentemente, los mecanismos a través de los cuales las hormonas del hospedero o exógenas llevan a cabo sus funciones sobre el parásito, han sido escasamente estudiados (Maswoswe et al., 1985; Escobedo et al., 2005).

En términos endocrinológicos, dos de los mecanismos más importantes a través de los cuales las hormonas esteroides ejercen sus múltiples funciones en el

organismo son, en primer término, aquellos que mediados por un receptor nuclear o citoplásmico específico, son capaces de activar la transcripción de diversos genes con funciones proliferativas o de regulación, encontrando sitios de anclaje directos sobre el DNA (Nilsson et al., 2001). A dichos mecanismos se les conoce como mecanismos de acción genómica o clásica (Figura 4).

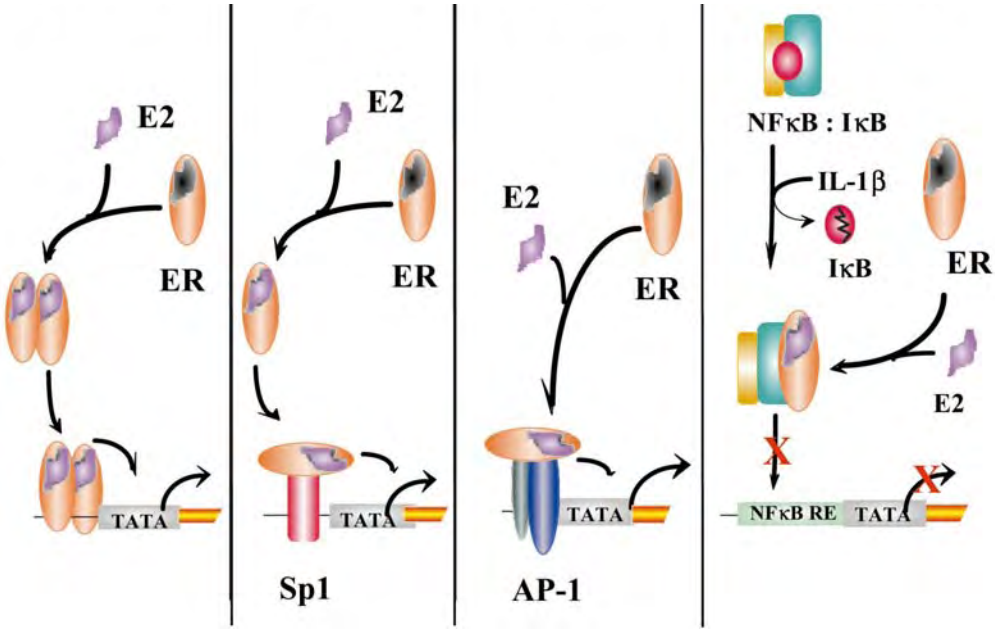

Figura 4. Mecanismo de acción genómica o clásica. Las hormonas esteroides llevan a cabo sus funciones a través de la unión a sus receptores nucleares o citoplásmicos específicos y el reconocimiento de sitios de regulación de la transcripción en el DNA. Tomado de Cheskis, 2004.

Por otra parte, existen también los mecanismos de acción rápida de las hormonas esteroides, dentro de los cuales se encuentran aquellos capaces de activar distintas vías de señalización intracelular mediadas por cascadas de segundos mensajeros (Cheskis, 2004; Curtis-Hewitt et al., 2005), véase figura 5.

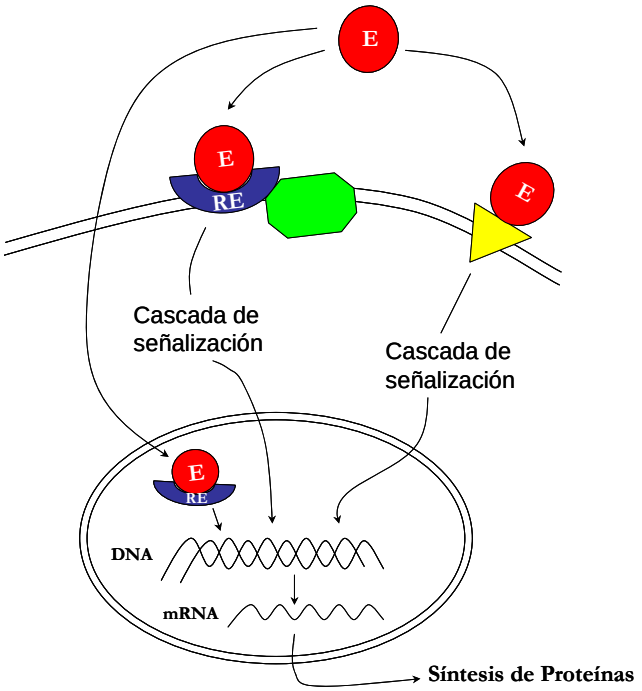

Figura 5. Mecanismos de acción rápida metdiados por segundos mensajeros. Los efectos rápidos de las hormonas esteroides (E) mediados por segundos mensajeros pueden llevarse a cabo mediante, al menos, dos mecanismos: A) el receptor de hormonas esteroides (RE) actúa coordinadamente con otras proteínas de membrana (verde) activando cascadas de señalización. B) Alternativamente, proteínas asociadas a la membrana (amarillo), pero no al RE, con capacidad de unir hormonas esteroides pueden también conducir a una respuesta intracelular rápida. Modificado de Curtis-Hewitt et al., 2005.

En relación a esto, más de una línea de investigación ha sido establecida con el fin de determinar si es posible encontrar receptores específicos de hormonas esteroides en parásitos, y aún más, si dichos receptores poseen la capacidad de reconocer hormonas esteroides exógenas y dirigir los eventos transcripcionales subsecuentes a dicha unión.

En este sentido, Sani y colaboradores (1985) caracterizaron proteínas de unión específica para retinol y ácido retinoico en *Onchocerca volvulus*, *Oncocerca gibsoni*, *Dipetalonema viteae*, *Brugia pahangi* y *Dirofilaria immitis*. El papel que se le atribuyó a estas proteínas fue el de mediar, uniéndose a hormonas del hospedero, los efectos biológicos de los retinoides sobre la fisiología parasitaria.

Por su parte, Unnasch y su grupo de trabajo (1999) reportaron que el genoma de *Onchocerca volvulus* codifica al menos para tres receptores nucleares

de esteroides. De éstos, se han caracterizado dos receptores similares a los receptores a retinoides en vertebrados, y a la proteína EiP78c de *Drosophila melanogaster*, denominadas OvNR-1 y OvNR-2. Estudios de modelaje computacional sugieren que estas moléculas poseen una cavidad de unión al ligando que por su forma y tamaño corresponde a un esteroide.

Por otro lado, en *Schistosoma mansoni* se ha sugerido la existencia de receptores que pueden unir a E2 (de Mendoca et al., 2000). Se propuso entonces que dicho receptor podría mediar el efecto protector que esta hormona posee en el ratón infectado con este parásito. A partir de diversas secuencias de DNA, en este mismo tremátodo se han modelado computacionalmente receptores nucleares de esteroides, hormonas tiroideas y ecdiesteroides. La homología que estos receptores poseen con los descritos en *Drosophila melanogaster*, ratón y humano se encuentra entre el 70 y el 95%; de ahí su enorme capacidad para unirse a las hormonas esteroides del hospedero y afectar de este modo diversos procesos del desarrollo de *Schistosoma sp* (de Mendoca et al., 2000).

Hasta la fecha, tan sólo en nueve especies parasitarias se ha sugerido la existencia de proteínas capaces de unirse a hormonas del hospedero y mediar de esta manera el proceso de transcripción, cuyos productos favorecerán en última instancia el establecimiento parasitario y la continuidad del ciclo infeccioso. Ver Tabla 1.

Parásito	Hormona	Estadio del parásito	Efecto	Mecanismo molecular
Plasmodium falciparum	Csol, E_2, P_4, T_4, Ins	Merozoito	↑Crecimiento, ↑Reproducción	Mecanismo desconocido
	DHEA	Merozoito	↓Crecimiento	Efecto citotóxico sobre el parásito
Schistosoma mansoni	Csol DHEA	Cercaria, esquistosomula, gusano adulto	↓Reproducción, ↓Viabilidad ↓Oviposición	Unión a un NR clásico y posterior inhibición de genes del metabolismo de la glucosa
Schistosoma haematobium	T_4	Cercaria	↓Reproducción	Inhibición de *SmND5* y su función mitocondrial
Trypanosoma cruzi	EGF	Amastigotes	↑Crecimiento, ↑Reproducción, ↑Actividad metabólica	Activación de las vías PKC y MAPK
Brugia malayi	EGF	Microfilarias	↑Crecimiento, ↑Diferenciación	Incremento en la transcripción de Raf y Ran
Onchocerca volvulus, O. lienalis	Hidroxiecdisona	Microfilarias	↑Actividad metabólica	Unión del esteroide a NR parasitario y posterior regulación de la transcripción
Leishmania mexicana	MG-CSF	Promastigotes	↑Crecimiento	Estimulación directa por mecanismos aun por describir
Entamoeba histolytica	Csol	Trofozoítos	↑Reproducción, ↑Actividad metabólica	Posible estimulación de la actividad de alguna enzima del metabolismo de la glucosa
	DHEA	Trofozoítos	↓Reproducción, ↓Viabilidad, ↓Infectividad, ↓Actividad metabólica	Posible inhibición de la actividad de alguna enzima del metabolismo de la glucosa
Taenia crassiceps	E_2, P_4	Cisticerco	↑Crecimiento, ↑Reproducción, ↑Viabilidad, ↑infectividad	Unión del esteroide a su receptor específico y activación de la transcripción de *c-fos* y *c-jun*
	T_4, DHT	Cisticerco	↓Crecimiento, ↓Reproducción, ↓Viabilidad, ↓Infectividad	Unión del esteroide a su receptor específico e inhibición de la transcripción de *c-fos* y *c-jun*

Tabla 1. Efectos directos de hormonas del hospedero sobre la fisiología parasitaria y sus mecanismos moleculares propuestos. Csol=Cortisol, Ins=Insulina, EGF=Factor de Crecimiento Epidermal. Modificado de Escobedo et al., 2005.

Por otra parte, evidencia experimental reciente sugiere que las hormonas esteroides pueden ejercer sus efectos sobre el parásito, a través de mecanismos mediados por cascadas de señalización. Interesantemente, estos mecanismos de acción rápida han sido un poco más explorados en parásitos que los mecanismos genómicos (Escobedo et al., 2005). Sin embargo, esta aparente ventaja es sólo relativa, pues los primeros reportes que indican que hormonas del hospedero pueden activar cascadas de segundos mensajeros en parásitos aparecieron entre finales del siglo pasado y principios de este (Ghansah et al., 2002; Townson y Tagboto, 1996). Por ejemplo, una molécula que puede activar distintas vías de señalización en parásitos y que ha sido sumamente estudiada es el factor de crecimiento epidermal (EGF, por sus siglas en inglés).

En *Brugia malayi* se ha determinado la presencia de toda una cascada de señalización en la que participa importantemente Raf, un segundo mensajero involucrado en regular funciones del citoesqueleto y la expresión génica

(Dissanayake, 2000). Interesantemente, el EGF de origen murino incrementa en este parásito la transcripción de dicha cinasa y de Ran, una GTPasa nuclear. Además, aumenta la interacción física entre Ran y otras proteínas aún por definir y potencia la fosforilación de algunas proteínas de origen microfilarial (Dissanayake, 2000).

Por otro lado, en *Toxoplasma gondii* se han caracterizado cuatro proteínas transmembranales (TgMIC6, TgMIC7, TgMIC8 y TgMIC9) que poseen múltiples dominios de unión al EGF. Estas proteínas poseen un dominio externo de unión a este factor, una región transmembranal y un pequeño dominio citoplásmico, semejando estructural y funcionalmente a los receptores de EGF descritos previamente en mamíferos (Meissner et al., 2002).

De manera similar, los amastigotes de *Trypanosoma cruzi* sintetizan un receptor capaz de unir al EGF de origen humano, que induce en el parásito la actividad de las cinasas MAP y PKC en una forma concentración y tiempo dependiente. Como los mismos autores describen, estos resultados sugieren la existencia de un mecanismo que regula el crecimiento y la proliferación parasitaria mediante distintas vías de señalización dependientes del EGF humano (Ghansah et al., 2002).

En *Plasmodium falciparum,* se han descrito, a la fecha cuatro proteínas de superficie de los merozoitos que contienen dominios de unión al EGF (MSP-1, MSP-4, MSP-5 y MSP-8). La propuesta es que estas proteínas juegan un papel crítico en la invasión a los eritrocitos humanos (Marshall et al., 1997). De la misma manera, la presencia de un receptor (SmRTK-1) con actividad de cinasa de tirosina en *Schistosoma mansoni* ha sido descrita. Los autores sugieren que la localización preferencial de SmRTK-1 en esporocitos y ovocitos podría favorecer procesos de diferenciación y crecimiento en este parásito (Vicogne et al., 2003). Finalmente, estudios recientes sugieren que *Schistosoma haematobium* sintetiza una proteína de 28 KDa (Sh28GST) capaz de unirse a T4 y facilitar su transporte, metabolismo y acción fisiológica en el parásito (Remoue et al., 2002).

PLANTEAMIENTO DEL PROBLEMA

Toda la evidencia antes descrita, sugiere fuertemente que las hormonas, tanto esteroides como proteicas, juegan un papel sumamente importante durante el establecimiento y desarrollo de una infección parasitaria. Esto podría deberse no solo a sus acciones sobre el sistema inmune, sino también por sus posibles efectos sobre el parásito, modificando directamente en éste procesos tan importantes como reproducción, crecimiento, diferenciación, e infectividad, por medio de diversos mecanismos moleculares.

Por esta razón, consideramos importante determinar el efecto de las hormonas esteroides sexuales sobre la fisiología del cisticerco de la *Taenia crassiceps*, adentrándonos en el mecanismo molecular de las mismas y estudiando la relevancia que esto tiene en el desarrollo no solo de la cisticercosis murina, sino de otras infecciones de importancia médica y veterinaria, como la provocada por el metacéstodo de la *Taenia solium*. Además, el entendimiento de estos mecanismos puede ampliar nuestro concepto de la relación hospedero-parásito, integrándolo en una compleja red dinámica en la que dos organismos, el parásito y el hospedero, interaccionan de manera bidireccional, repercutiendo sobre el curso fisiológico, patológico y evolutivo de la infección.

HIPÓTESIS

Las hormonas esteroides sexuales 17β-estradiol, progesterona, testosterona y 5α-dihidrotestosterona, afectan directamente la reproducción del cisticerco de la *Taenia crassiceps in vitro*, a través de un receptor específico de hormonas esteroides, expresado por el parásito, o por medio de la activación de distintas vías de señalización en las células del cisticerco, ambos mecanismos con capacidad de modificar la expresión de genes relacionados con el establecimiento y la proliferación parasitaria.

OBJETIVO GENERAL

Determinar los efectos de 17β-estradiol, progesterona, testosterona y 5α-dihidrotestosterona sobre el cisticerco de la *Taenia crassiceps in vitro*, identificando genes parasitarios que pudieran ser regulados por estos esteroides sexuales, estudiando los posibles mecanismos de acción de estos últimos sobre el cisticerco.

OBJETIVOS ESPECÍFICOS

1) Precisar el efecto de las hormonas esteroides 17β-estradiol, progesterona, testosterona y 5α-dihidrotestosterona sobre la viabilidad y la reproducción del cisticerco de la *Taenia crassiceps in vitro*.

2) Determinar si los cisticercos tratados *in vitro* con los esteroides antes mencionados, presentan algún cambio en su capacidad de establecimiento y reproducción al ser reinoculados en ratones completos.

3) Esclarecer si los cisticercos de la *Taenia crassiceps* expresan receptores de hormonas esteroides sexuales, y si no, proponer un mecanismo alternativo que explique cómo se producen los efectos de dichas hormonas.

4) Identificar genes del cisticerco de la *Taenia crassiceps* que pudieran ser regulados por esteroides sexuales, evaluando su patrón de expresión en respuesta al tratamiento hormonal.

MATERIALES Y MÉTODOS

Obtención de los cisticercos

Los cisticercos de la *Taenia crassiceps* (cepa ORF) fueron obtenidos a partir de ratones hembras (*Mus musculus*) donadoras de 16 semanas de infección, y lavados cinco veces con PBS 1X estéril, suplementado con 100 U/mL de antibióticos y 100 U/mL de antimicóticos (Gibco, Grand Island) (Esch and Smith, 1976). Una vez concluidos los lavados manuales, los cisticercos mantenidos en PBS 1X estéril fueron colocados en tubos Falcon de 50 mL y centrifugados por 10 minutos a 1000 rpm. El sobrenadante fue descartado y este proceso se repitió cinco veces. Después del lavado final, el número de cisticercos viables fue determinado por conteo directo utilizando un microscopio estereoscópico con los objetivos de 1 y 4X. Utilizando placas de cultivo de 24 pozos (Falcon, Becton Dickinson Labware, Franklin Lakes, New Jersey), diez cisticercos fueron colocados, por pozo, en 1 mL de DMEM (Serum Free-Medium. Gibco, BRL) suplementado con 3% de HEPES, 2% de aminoácidos no esenciales, 2% de L-glutamina y 2% de penicilina-estreptomicina, e incubados a 37ºC bajo una atmósfera constante de 5% de CO_2.

Tratamiento de los cisticercos con esteroides sexuales

Las hormonas esteroides 17β-estradiol [(8R,9S,13S,14S,17S)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta [a]phenanthrene-3,17-diol], progesterona [(8S,9S,10R,13S,14S,17S)-17-acetyl-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one], Testosterona [(8R,9S,10R,13S,14S,17S)-17-hydroxy-10,13-dimethyl-1,2,6,7,8,9,11,12,14, 15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one] y 5α-dihidrotestosterona [(5S,8R,9S,10S,13S,14S,17S)-17-hydroxy-10,13-dimethyl-1,2,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydrocyclopenta [a]phenanthren-3-one] grado de cultivo, fueron obtenidas de Sigma-Aldrich. Para los ensayos in vitro, E2 y P4 hidrosolubles, fueron disueltas en medio de cultivo DMEM (Dulbecco's Modified Eagle's Medium) sin suplementar, mientras que T4 y DHT fueron disueltas en etanol absoluto; todas las hormonas fueron llevadas hasta una concentración final de 10nM, 1μM y 10μM, y esterilizadas por filtración (0.2 mm millipore filter, Gibco, BRL). El diseño experimental para todas las hormonas usadas se describe a continuación: en una placa de cultivo

de 24 pozos, 6 pozos fueron usados como controles sin adición de hormona, 6 pozos fueron adicionados con el vehículo en el cual las hormonas fueron disueltas, 6 pozos fueron tratados con concentraciones crecientes de E2 (0.00064, 0.001, 0.32, 3.18 y 63.60μM), 6 pozos fueron tratados con concentraciones crecientes de P4 (0.07, 0.29, 2.93, 3.67 y 146.85μM), 6 pozos fueron tratados con concentraciones crecientes de T4 (0.07, 0.35, 1.38, 3.46 y 34.67μM) y 6 pozos fueron tratados con concentraciones crecientes de DHT (0.07, 0.34, 1.37, 3.44 y 34.43μM). Cada hormona fue adicionada, de acuerdo a su concentración, en 1mL de medio por pozo. Las concentraciones hormonales usadas en este trabajo fueron determinadas por medio de los valores séricos reportados en la literatura y de estudios previos de los niveles circulantes de esteroides sexuales encontrados en ratones feminizados (Larralde et al., 1995; Morales et al., 1996; Morales-Montor et al., 1999; Morales-Montor et al., 2001). Todas las curvas concentración-respuesta y temporales fueron realizadas por triplicado, en un total de diez experimentos. La reproducción fue medida como número de gemas en cada cisticerco (producidas en respuesta al tratamiento hormonal) y contadas directamente empleando un microscopio estereoscópico en los objetivos de 1 y 4X.

Pruebas de viabilidad y reproducción

La viabilidad de los cisticercos fue determinada microscópicamente. La morbilidad de los cisticercos fue reconocida por una desorganización interna progresiva, desarrollo de áreas opacas en el líquido vesicular y pérdida de la motilidad. La viabilidad fue determinada de acuerdo al tamaño, motilidad e incorporación de colorantes orgánicos (azul tripano). Los cisticercos teñidos, con desarrollo de áreas opacas y no motiles fueron considerados muertos. Todos los ensayos de viabilidad fueron realizados microscópicamente. La reproducción fue medida como número de gemas producidas en cada cisticerco en respuesta al tratamiento hormonal. Las gemas fueron contadas directamente con ayuda de un microscopio estereoscópico en los objetivos de 1 y 4X. Se obtuvieron las micrografias de los cisticercos tratados con los distintos esteroides sexuales.

Ensayos de proliferación

Los cisticercos fueron cultivados como ya se describió anteriormente. Al tercer día de cultivo, éstos recibieron un pulso de Timidina-3H (10 μCi/mL de medio) (methyl-3H TDR, sp act. 247.9 GBq/mmol, NEN, Boston, MA) y fueron incubados por un tiempo restante de 56 horas. Enseguida, el DNA de estos parásitos se purificó como se mencionó anteriormente y la radiactividad emitida por éste se midió por Sistema Betaplate (Wallac Mod 1205, Turku, Finland).

Aislamiento y cuantificación de DNA

Se tomaron para cada caso 200mg de tejido de cisticercos tratados con E2, P4, T4 y DHT. Posteriormente, el tejido fue homogeneizado por sonicación a 30 decibeles durante 45 segundos y lisado con Proteinasa K (100 u/mL) durante 2 horas a 50ºC. Una vez que la fracción de proteínas fue eliminada con fenol/cloroformo, el DNA total fue precipitado con etanol frío y resuspendido en buffer TE 10% libre de DNAsas. La cantidad y pureza del DNA fue determinada por lecturas de absorbancia a 260/280nm.

Inoculación en ratones después del tratamiento in vitro

Se usaron ratones (*Mus musculus*) de ambos sexos de la cepa BALB c/AnN de seis semanas de edad. Los ratones fueron alimentados con Purina Diet 5015 y agua *ad libitum*. Después de haber sido tratados in vitro con E2, P4, T4 y DHT, los cisticercos de la *Taenia crassiceps* fueron reinoculados en la cavidad peritoneal del ratón, en orden de diez parásitos por ratón. Después de ocho semanas de infección, los ratones fueron humanamente sacrificados en una cámara de $C0_2$ y los parásitos recuperados para su conteo y el registro microscópico de sus características morfológicas.

Tratamiento de los cisticercos con antihormonas e inhibidores competitivos

Con la finalidad de dilucidar el posible mecanismo a través del cual las hormonas esteroides llevan a cabo sus efectos sobre el parásito, se diseñaron cultivos *in vitro* utilizando dos tipos de inhibidores. Por un lado, tamoxifén, RU-486 y flutamida, antihormonas capaces de modular selectivamente diversos tipos de receptores de hormonas esteroides bloqueando su actividad transcripcional, fueron usados. Alternativamente, AG18, genisteína, picetanol y el inhibidor de ERK, inhibidores de proteínas con actividad de cinasa de tirosina, capaces de inhibir la fosforilación y activación de segundos

mensajeros, fueron también empleados durante este trabajo. E2, P4, tamoxifén (antihormona que se une específicamente a los receptores nucleares de estrógenos), RU-486 (antihormona que se une específicamente a los receptores nucleares de progesterona) y flutamida (antihormona que se une específicamente a los receptores nucleares de andrógenos) fueron obtenidos de Sigma-Aldrich. T4 y DHT fueron obtenidos de Sigma-Aldrich-Fluka. Los inhibidores de proteínas con actividad de cinasa de tirosina AG18 (inhibidor general de todos los receptores de superficie con actividad de cinasa de tirosina, Frasor et al., 2001) y genisteina (inhibidor de la familia de las SRC-cinasas, Wong and Leong, 2004) fueron obtenidos de ICN Biomedicals, INC, mientras que picetanol (inhibidor de Syk, Takada and Aggarwal, 2004) y el inhibidor de ERK (inhibidor de la vía de señalización de cinasas reguladas por señales extracelulares, Kelemen et al., 2002) fueron obtenidos de Calbiochem. Las antihormonas así como los inhibidores de segundos mensajeros fueron primeramente disueltos en etanol absoluto y posteriormente en medio de cultivo DMEM sin suplementar, hasta obtener la concentración de 1μM. Las antihormonas y los inhibidores fueron esterilizados por filtración (0.2mm, Millipore). Posteriormente, en una placa de cultivo de 24 pozos, 6 pozos fueron usados como controles sin adición hormonal; 6 pozos fueron adicionados con el vehículo en el cual las hormonas, antihormonas e inhibidores fueron disueltos; 6 pozos fueron tratados con 318.01nM de E2; 6 pozos fueron tratados con 2.93μM de P4; 6 pozos fueron tratados con 1.38μM de T4; 6 pozos fueron tratados con 1.37μM de DHT; 6 pozos fueron tratados con 1.77μM de tamoxifén y 318.01nM de E2; 6 pozos fueron tratados con 8μM de AG18 y 318.01nM de E2; 6 pozos fueron tratados con 4μM de genisteína y 318.01nM de E2; 6 pozos fueron tratados con 2μM de picetanol y 318.01nM de E2; 6 pozos fueron tratados con 4μM de ERK-Inh y 318.01nM de E2; 6 pozos fueron tratados con 2.32μM de RU-486 y 2.93μM de P4; 6 pozos fueron tratados con 8μM de AG18 y 2.93μM de P4; 6 pozos fueron tratados con 4μM de genisteína y 2.93μM de P4; 6 pozos fueron tratados con 2μM de picetanol y 2.93μM de P4; 6 pozos fueron tratados con 4μM de ERK-Inh y 2.93μM de P4;6 pozos fueron tratados con 3.62μM de flutamida y 1.38μM de T4; y finalmente, 6 pozos fueron tratados con 3.62μM de flutamida y 1.37μM de DHT. Todas las

curvas farmacológicas fueron realizadas por triplicado, en un total de diez experimentos. La reproducción fue medida como número de gemas en cada cisticerco (producidas en respuesta al tratamiento hormonal y/o farmacológico) y contadas directamente empleando un microscopio estereoscópico en los objetivos de 1 y 4X.

Extracción de RNA total

El RNA total fue obtenido a partir de cisticercos de la *Taenia crassiceps* tratados diferencialmente con E2, P4, T4 y DHT, mediante el método simple de extracción con Isotiocinato de Guanidina/Fenol/Cloroformo usando Trizol (Trizol Reagent-Invitrogen, Carlsbad, CA). De forma breve, los cisticercos fueron homogeneizados en reactivo de trizol (1mL trizol/100mg tejido) y por cada mililitro de homogeneizado se adicionó 0.2mL de cloroformo (Sigma). La fase acuosa fue recuperada después de 15 minutos de centrifugación a 13,000rpm. El RNA fue precipitado con Isopropanol (Sigma) y lavado con etanol al 75, 85 y 100%. Finalmente la pastilla de RNA fue resuspendida en H2O-DEPC libre de RNAsas (Sigma). La concentración total de RNA fue determinada por absorbancia a 260 nm y su pureza fue verificada mediante geles desnaturalizantes de agarosa en presencia de formaldehído 2.2 M.

Amplificación de genes por RT-PCR

El RNA total, obtenido a partir de cisticercos de la *Taenia crassiceps* tratados diferencialmente con E2, P4, T4 y DHT, fue retrotranscrito y distintos genes fueron amplificados utilizando oligonucleótidos específicos de secuencia por PCR. Los genes candidatos a ser amplificados, fueron aquellos que cumplieran las características de ser regulados por hormonas esteroides, implicados en procesos proliferativos y altamente conservados a lo largo de la escala filogenética. A partir de este análisis se diseñaron los oligonucleótidos con base en las secuencias más conservadas de genes previamente reportados para humano, rata y ratón en el Gene Data Bank. De esta manera llegamos a los miembros del complejo AP-1, *c-fos* y *c-jun*, las moléculas de señalización Lyn, Syk, Erk, y los receptores de estrógenos, progesterona y andrógenos. En todos los casos se utilizó β-actina como gen constitutivo (control positivo de amplificación) (Morales-Montor et al, 2003). Las secuencias de oligonucleótidos empleadas en los experimentos de PCR se encuentran descritas en la siguiente tabla:

Gen	Sentido	Anti-sentido
c-fos	5'-TGTACGATCA CTGAACTG CA	5'-AGTCAGTTATATCCTGGC
c-jun	5'CTGGAGCATT TACTGCTG	5'-GTG TTGAGATGATGCTTCGAC
Lyn	5'-CCCAAACCTCAGAAGCCATG	5'-TGTCGACTACGGCTGCTGCT
Syk	5'-GAAGCCCTGCCCATGGACACA	5'-AAGCCTCAGTTCCACAGCTGT
Erk	5'-ACAAAGTTCGAGTTGCTATCA	5'-ATTGATGCCAATGATGTTCTC
ER-α	5'-AGACTGTCCAGCAGTAACGAG	5'-TCGTAACACTTGCGCAGCCG
ER-β	5'-CATCTGGGTATCATTACGGTC	5'-GGCACTTCTCTGTCTTCGTAC
PR-A	5'-CAGTGGTGGATTTCATCCATG	5'-CTTCCAGAGGGTAGGTGCAG
PR-B	5'-GGAGGCAGAAATTCCAGACC	5'-GACAACAACCCTTTGGTAGC
AR	5'-GAATGTCAGCCTATCTTTCTTA	5'-TGCCTCATCCTCACACACTGGC
VDR	5'-CTGAATTCCATGAAACACCTGT	5'-GACTCTAGAGGGCTCACACTCACCTCC
TNF-α	5'- CAGAGCTGTCTCTAAACCGT	5'- AGGGTGTCTGAAGGAGGGGA
β-act	5'-GGGTCAGAAGGATTCCTATG	5'-GGTCTCAAACATGATCTGGG

Las secuencias correspondientes a Lyn, Syk y Erk fueron diseñadas a partir de las regiones más conservadas de los sitios SH2 y SH3 de cada gen reportado en ratón, rata y humano. Para demostrar que el tejido obtenido de cisticercos no estaba contaminado con DNA o RNA del hospedero, amplificamos secuencias específicas de los genes murinos VDR (exclusivos en el ratón por pertenecer a la región variable de las IgG) y TNF-α. La reacción de PCR se llevó a cabo de la siguiente manera: 10µg de RNA fueron incubados a 37ºC durante 1 hora en presencia de M-MLV Reverse Transcriptase (1U/20µL, Applied Biosystems, USA). En un volumen de reacción de 20µL se contenían 50µM de cada dNTP y 0.05µg de oligonucleótido dT (Gibco, NY). Una vez obtenido el cDNA, 10 µL de este fue usado como templado para amplificar secuencias específicas de los genes antes mencionados. En un volumen de reacción de 50µL se contenían 10µL de cDNA previamente sintetizado, 5µL de buffer 10X para PCR (Perkin-Elmer, USA), 1mM de MgCl2, 0.2mM de cada dNTP, 0.05µM de cada oligonucleótido y 2.5 unidades de TaqDNA Polimerasa (Biotecnologías Universitarias, México). Después de un paso de desnaturalización inicial a 95ºC durante 5 minutos, las condiciones de amplificación para c-fos fueron las siguientes: 95ºC por 30 segundos, 50ºC por

45 segundos y 72ºC por 30 segundos durante 30 ciclos. Un paso final de extensión fue realizado a 72ºC durante 5 minutos. Por otra parte, después de un paso de desnaturalización inicial a 95ºC durante 5 minutos, las condiciones de amplificación para c-jun fueron las siguientes: 95ºC por 30 segundos, 57ºC por 30 segundos y 72ºC por 30 segundos durante 35 ciclos. Un paso final de extensión fue realizado a 72ºC durante 5 minutos. Los 50µL de producto de PCR fueron corridos en un gel de agarosa al 2% en presencia de PBR321-Ladder como marcador de peso molecular (Biotecnologías Universitarias, México). Los productos de PCR obtenidos fueron revelados por tinción del gel con bromuro de etidio y exposición a radiación ultravioleta. En todos los casos, las condiciones de PCR fueron ajustadas hasta obtener una banda simple correspondiente al peso molecular esperado. Los productos de PCR fueron secuenciados recortando directamente del gel de agarosa la banda deseada y mediante el empleo de un secuenciador automático (Modelo LIC-4200, Aloka Co., Japan). Las secuencias fueron analizadas usando el DNASIS Software (Hitachi Software Engineering, Tokyo, Japan).

Extracción de proteína total

La proteína total de los cisticercos de la *Taenia crassiceps* expuestos *in vitro* a E2, P4, T4 y DHT, fue obtenida por el método convencional de Tris-HCl. Brevemente, cisticercos tratados y controles sin tratamiento hormonal fueron disgregados y homogeneizados en Tris-HCl 100mM (1mL/0.1g de tejido), proteinasa K (100U/mL) y cocktail inhibidor de proteínas. Después de 15 minutos de centrifugación a 14,000rpm, el sobrenadante fue recuperado y la pastilla desechada. La proteína obtenida fue cuantificada por absorbancia a una longitud de onda de 595nm, usando el método de Bradford-Lowry. 30µg de proteína fueron colocadas en Buffer de Laemmli y separadas en un gel de acrilamida al 10% (SDS-PAGE) para los experimentos de electroforesis de proteínas.

Detección de receptores de hormonas esteroides por Western Blot

Las proteínas separadas por SDS-PAGE fueron electro-transferidas a membranas de nitrocelulosa. Las membranas fueron bloqueadas toda la noche en buffer TBTS (Tris–HCl 10mM pH 7.4, NaCl 100mM y 0.5% de Tween-20) conteniendo 5% de leche en polvo. Para la inmunodetección de LYN, SYK, ERK y los receptores de hormonas esteroides en el cisticerco de la *Taenia*

crassiceps, las membranas fueron incubadas a temperatura ambiente con 1μg/mL del anticuerpo monoclonal requerido (ratón α-LYN, ratón α-SYK, ratón α-ERK, cabra α-ERα, cabra α-ERβ, burro α-PR, ratón α-AR. Santa Cruz Biotech) por dos horas. Después de este tiempo, el anticuerpo primario fue retirado y las membranas fueron lavadas cinco veces durante tres minutos con TBS-Tween 20 al 3%. Enseguida, las membranas fueron incubadas en el anticuerpo secundario correspondiente (acoplado a peroxidasa, dilución 1:1000) por una hora a temperatura ambiente. Finalmente, las membranas fueron lavadas cinco veces con TBS-Tween 20 al 3% y las bandas fueron visualizadas usando el sistema ECL de acuerdo a las instrucciones del fabricante (Super signal ECL, Pierce).

Análisis Densitométrico

Los niveles relativos de expresión de cada gen, tomando como base la expresión constitutiva de β-actina como control positivo, fueron determinados por análisis densitométrico de las fotografías correspondientes a los geles de agarosa.

Análisis Estadístico

Todos los ensayos fueron realizados por triplicado y en un total de diez experimentos independientes. Los resultados fueron analizados por ANOVA de una vía (análisis de varianza) seguido de una prueba t-student para determinar diferencias entre medias y una T-Tukey para las diferencias individuales entre grupos. Las significancias estadísticas fueron consideradas cuando $P<0.05$.

RESULTADOS

Cuando los cisticercos fueron tratados con E2 o P4 *in vitro*, la capacidad reproductiva de éstos se vio considerablemente aumentada. En el grupo expuesto a E2, el número de gemas se vio incrementado en 400% sobre el grupo control, mientras que el número de gemas en el grupo tratado con P4 aumentó en un 20% (Figura 6A). La viabilidad, por otra parte, no se vio alterada en estos parásitos, y la motilidad se mantuvo constante durante todo el tiempo del tratamiento.

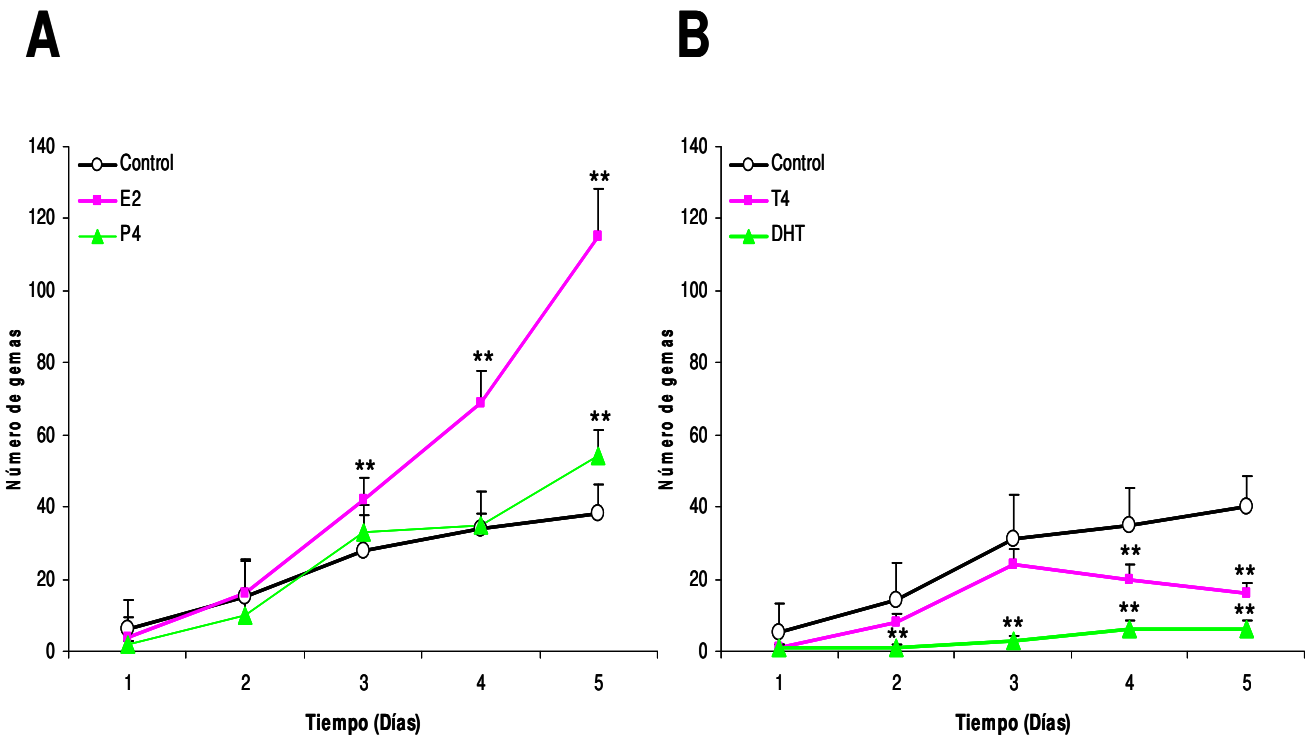

Figura 6. (A) E2 estimula en 400% la reproducción del cisticerco de la *Taenia crassiceps* a partir del tercer día de cultivo *in vitro*, mientras que P4 lo hace solamente al quinto día. (B) Por el contrario, T4 y DHT inhiben la reproducción del cisticerco de la *Taenia crassiceps in vitro*. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

Por el contrario, cuando los cisticercos fueron expuestos a T4 o DHT, la capacidad reproductiva de éstos se vio disminuida en 70% y 85%, respectivamente (Figura 6B), así como su viabilidad, desarrollando áreas opacas y pérdida progresiva de la motilidad. Estos resultados explicarían, al menos parcialmente, porque el cisticerco de la *Taenia crassiceps* crece y se reproduce mejor en hembras que en machos, sentando las bases fisiológicas

para una mejor comprensión del dimorfismo sexual que se presenta durante esta y otras infecciones parasitarias.

En el mismo sentido y con la finalidad de determinar las concentraciones óptimas de los esteroides sexuales en el cultivo *in vitro*, se probaron distintas concentraciones de E2, P4, T4 y DHT, encontrando efectos dependientes de la concentración en cada caso. E2 y P4 no afectan la viabilidad en ninguna concentración probada; sin embargo, concentraciones crecientes de estas hormonas incrementaron claramente el número de gemas en el parásito (Fig. 7). Por otro lado, concentraciones nanomolares de T4 y DHT mostraron un potente efecto cisticida al disminuir dramáticamente el número de gemas en el cisticerco de la *Taenia crassiceps* (Fig. 7).

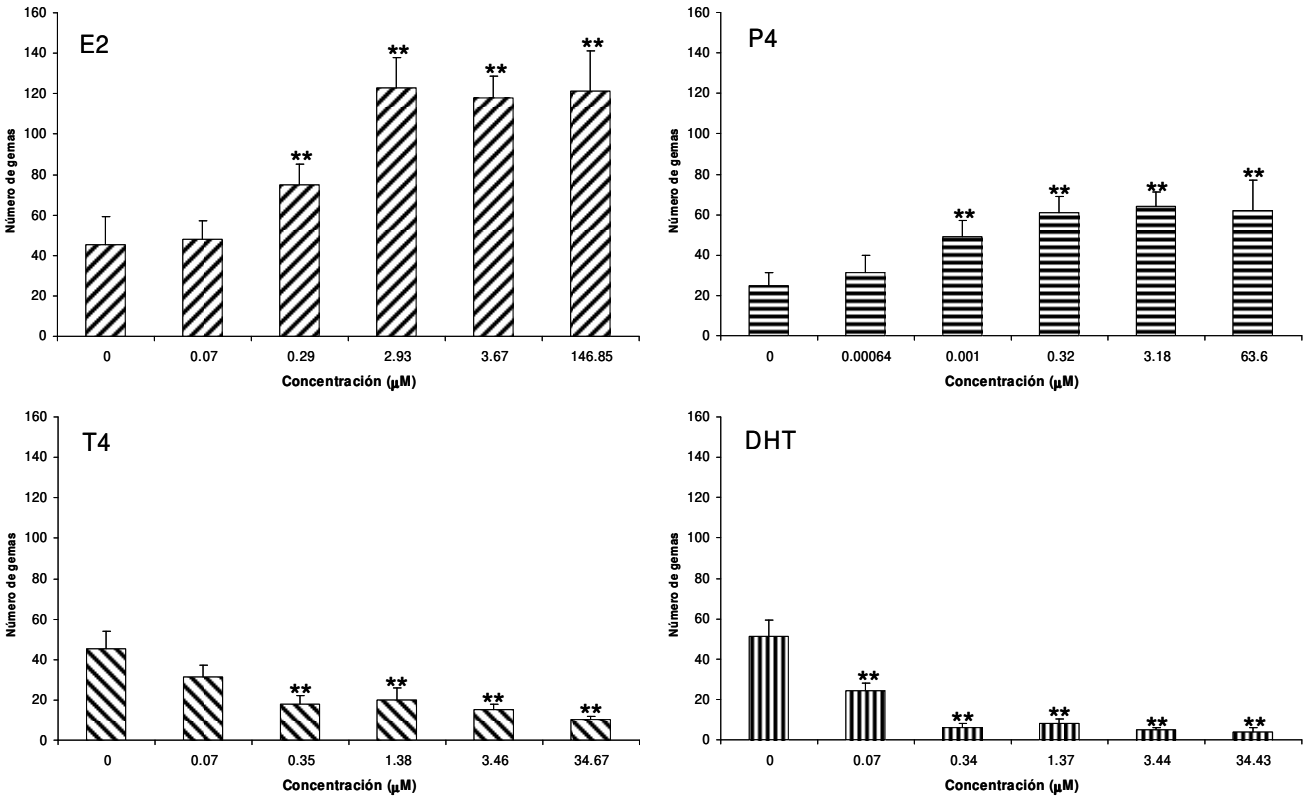

Figura 7. E2 y P4 promueven la reproducción del cisticerco de la *Taenia crassiceps* en forma dependiente de la concentración. Por otra parte, T4 y DHT inhiben la reproducción del parásito independientemente de la concentración usada. ** Diferencias significativas entre grupos tratados y controles (concentración hormonal igual a cero). P<0.05.

Las micrografías de todos los cisticercos tratados con los esteroides sexuales se muestran en la Figura 8. En los tratamientos con E2 y P4 se puede observar que los cisticercos exhibieron un gran número de gemas y aparecieron

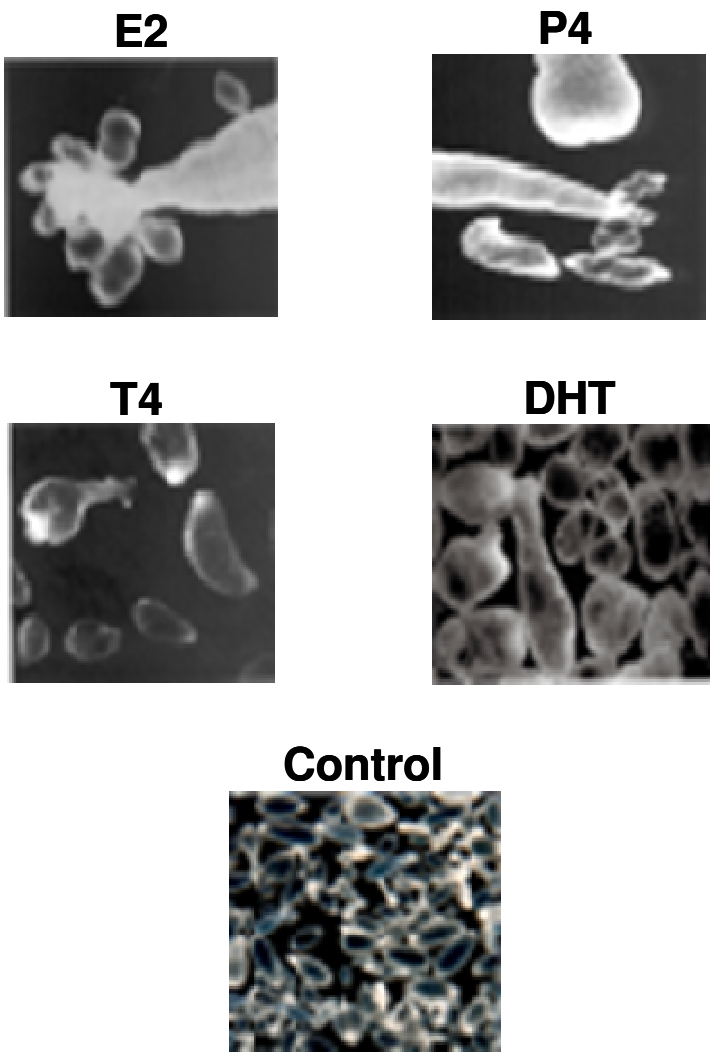

Figura 8. Micrografías de cisticercos de la *Taenia crassiceps* tratados *in vitro* con E2, P4, T4, DHT y controles. Los parásitos tratados con E2 exhibieron una gran cantidad de gemas comparados con el grupo control. P4 mostró un efecto proliferativo sobre el cisticerco pero no de la misma magnitud que E2. Por el contrario, los tratamientos con T4 y DHT inhibieron dramáticamente la formación de gemas del cisticerco de la *Taenia acrassiceps in vitro*. Aumentos en 4X y 6X.

sumamente motiles (Figura 8). Ocasionalmente se encontraron cisticercos multilobulados. Por otra parte, el diámetro aproximado de estos parásitos se mantuvo en 1524 +/- 94.9 micrones. Por el contrario, el tamaño de los cisticercos tratados con T4 y DHT (Fig. 8) fue relativamente pequeño (218 +/- 7.4 micrones), lo cual muestra que el tratamiento con estos andrógenos disminuye no solo la reproducción del cisticerco sino además su crecimiento y tamaño (Fig. 8). Estos parásitos mostraron una progresiva desorganización interna, desarrollo de áreas opacas en el líquido vesicular y progresiva pérdida de la motilidad.

La figura 9 muestra la proliferación de los cisticercos en respuesta al tratamiento hormonal después de ser incubados por 56 horas en presencia de timidina-3H. T4 y DHT aumentaron la incorporación de timidina-3H en el parásito en 50% y 40% respectivamente. En el mismo sentido, P4 aumentó cuatro veces la incorporación total de este nucleótido mientras que el tratamiento con E2 lo hizo seis veces más con respecto al grupo control (Fig. 9A).

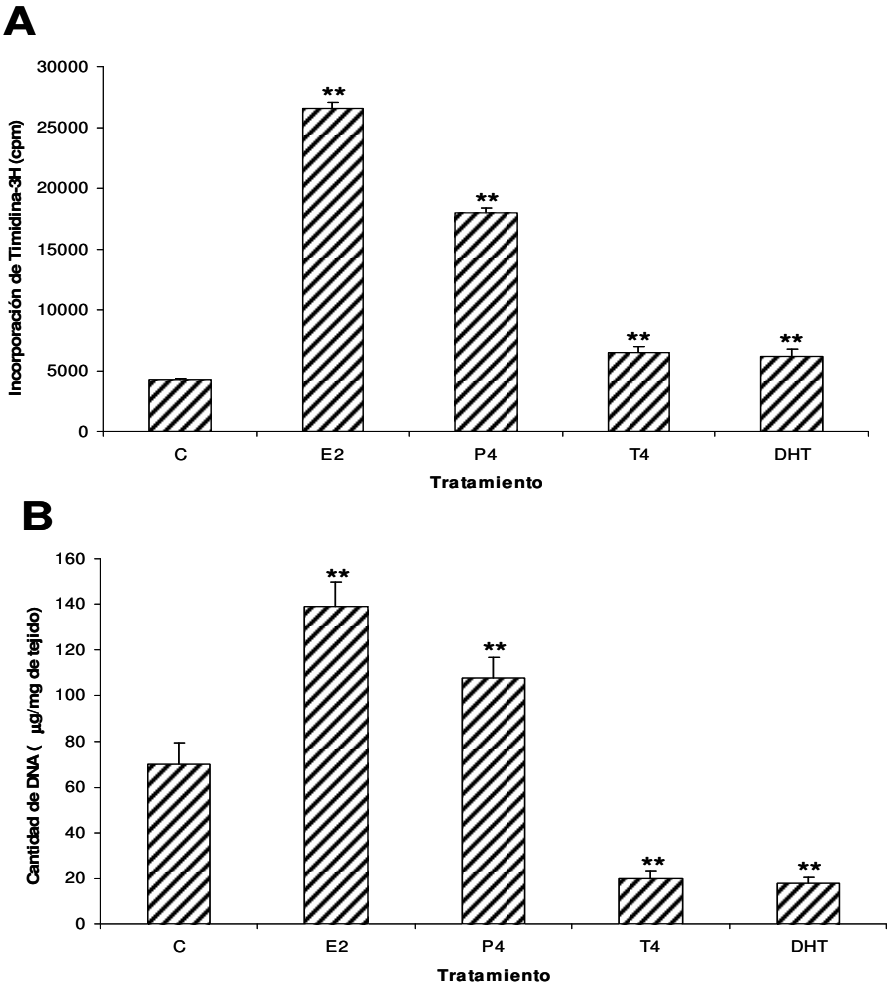

Figura 9. (A) Los tratamientos con E2, P4, T4 y DHT incrementan la incorporación de timidina-3H en el cisticerco de la *Taenia crassiceps*. (B) En una correlación inversa, sólo los tratamientos con hormonas sexuales femeninas promueven la síntesis de DNA mientras que T4 y DHT la disminuyen. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

De manera contrastante, los tratamientos con T4 y DHT disminuyeron en 30 y 25%, respectivamente, la síntesis de DNA en los parásitos tratados con estas hormonas (Fig. 9B). Aunado a esto y a la correlación inversa que existe entre mayor incorporación de timidina-3H y menor síntesis de DNA, estos resultados sugieren que los andrógenos podrían afectar al cisticerco a través

de la activación de un mecanismo de degradación específica de DNA parasitario, lo cual explicaría el contraste entre ambos resultados.

Por otro lado, los tratamientos con esteroides sexuales femeninos mostraron una correlación directa entre la incorporación de timidina-3H y la síntesis de DNA, pues esta última fue aumentada por E2 dos veces más, mientras que P4 la incrementó 1.5 veces, comparada con lo encontrado en el grupo control.

El número de parásitos recuperados, previa exposición en cultivo a E2, P4, T4 o DHT, después de ser reinoculados en ratones hembras y machos de la cepa BALBc/AnN de seis semanas de edad, se muestra en la Figura 10.

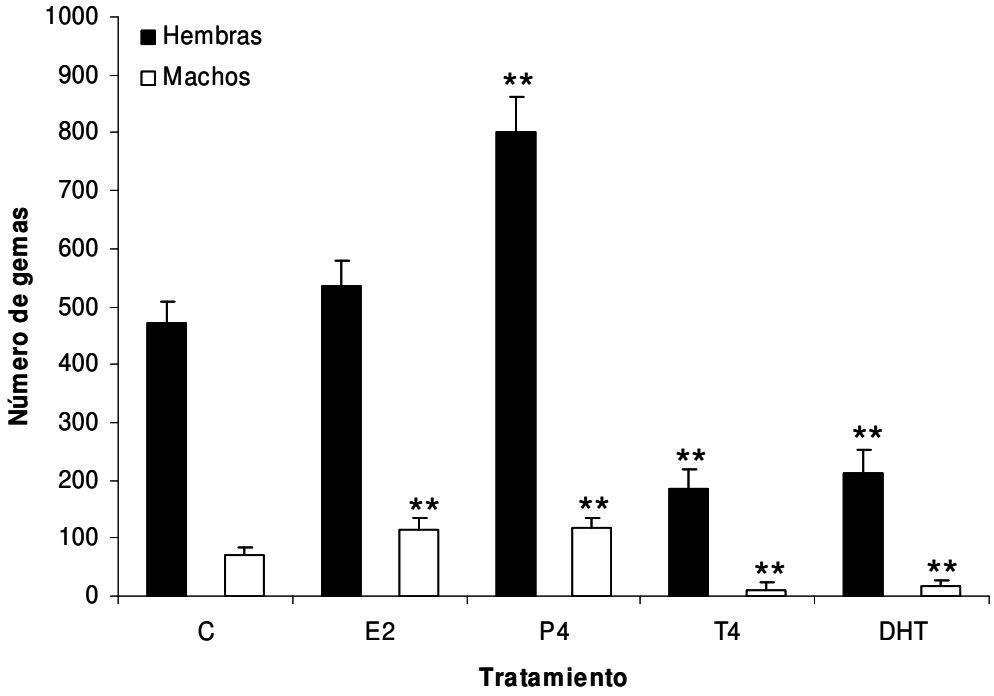

Figura 10. Carga parasitaria en ratones (*Mus musculus*) BALB c/AnN de ambos sexos, inoculados con cisticercos de la *Taenia crassiceps* sometidos previamente a tratamiento hormonal *in vitro*. P4 aumenta la capacidad infectiva de los parásitos mientras que los andrógenos la disminuyen. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

Interesantemente, en hembras y machos el tratamiento con P4 aumentó casi al doble la capacidad infectiva del cisticerco (medida como carga parasitaria), mientras que E2 solo incrementó la infectividad de los cisticercos reinoculados en machos (Figura 10). Por otra parte, los parásitos tratados con T4 y DHT mostraron una disminución en su índice infectivo, en hembras del 50% y en machos del 20%, respectivamente, comparados con el grupo control.

De esta manera se observa que la capacidad infectiva (medida como carga parasitaria) de los cisticercos tratados con E2 aumenta al doble en machos y hembras, mientras que en los parásitos tratados con P4 aumenta al doble sólo en hembras. Por otro lado, T4 y DHT disminuyeron en un 75% la capacidad infectiva de los cisticercos tratados con estos esteroides. Todos estos resultados fueron estadísticamente significativos con $P<0.05$ comparados con los grupos controles.

Por otra parte, con la finalidad de determinar el mecanismo a través del cual las hormonas esteroides afectan la reproducción del cisticerco de la *Taenia crassiceps*, se realizaron cultivos *in vitro* del parásito en presencia de dos tipos de inhibidores: antihormonas que se unen competitivamente al receptor específico del esteroide bloqueando así sus efectos (mecanismo de acción prolongada o genómico) e inhibidores de la fosforilación de segundos mensajeros (mecanismo de acción rápida mediado por cascadas de señalización).

De esta manera, el uso de tamoxifen bloqueó el efecto estimulador de E2 al disminuir en los parásitos tratados el número de gemas al mismo nivel que en los grupos que recibieron el vehículo. Esto sugiere que el 17β-estradiol exógeno estimula la reproducción del parásito, presumiblemente por su unión a una molécula con actividad de receptor de estrógenos (sintetizado por el cisticerco de la *Taenia crassiceps*) ya que, al inhibir farmacológicamente la función de éste, el número de gemas permanece en su nivel basal, véase Figura 11.

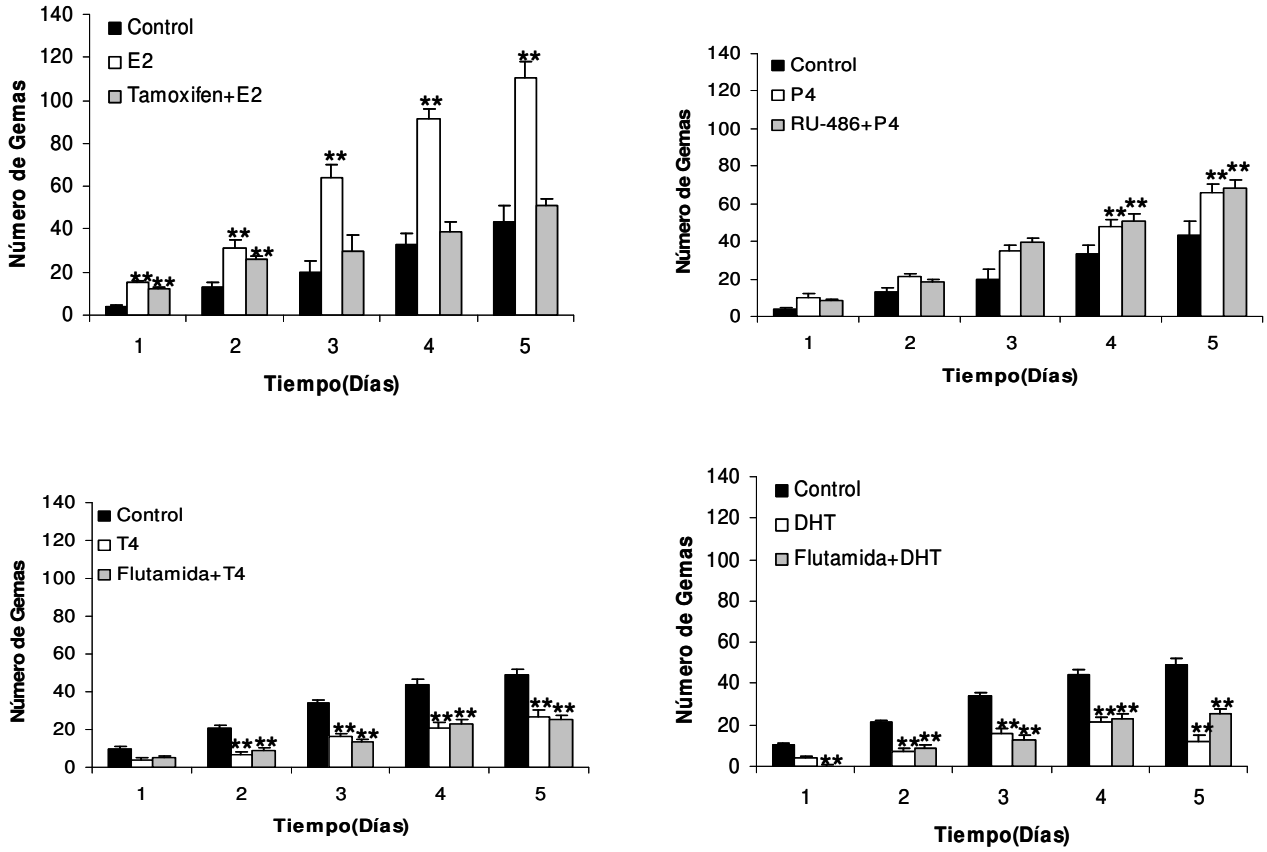

Figura 11. Mecanismo Genómico. Efecto de tamoxifen, RU-486 y flutamida sobre la reproducción del cisticerco de la *Taenia crassiceps in vitro*. Tamoxifen inhibe el efecto de E2 a partir del tercer día de cultivo. Por otra parte, los efectos de P4 y los andrógenos T4 y DHT, no se vieron modificados mediante el uso de RU-486 y flutamida respectivamente. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

De manera contraria, el uso de RU-486 en los cisticercos tratados con P4, y de flutamida en los parásitos expuestos a andrógenos, no revirtió los efectos proliferativos de P4 ni las acciones antiproliferativas de T4 y DHT (Figura 11), lo cual sugiere que los efectos de estas hormonas sobre el parásito, no requieren la presencia de una molécula con actividad de receptor clásico de hormonas esteroides. De esta manera, los efectos de P4 serían mediados por otra vía o simplemente por la conversión de este precursor a E2 (mediante diversas enzimas esteroidogénicas sintetizadas por el parásito), como se ha reportado para cisticercosis en el hospedero murino (Vargas-Villavicencio et al, 2005). Por otra parte, debido a los efectos drásticos de T4 y DHT sobre la reproducción, el crecimiento y la viabilidad del cisticerco de la *Taenia crassiceps* (Figuras 6B y 8), no se descarta la posibilidad de que estos andrógenos posean efectos tóxicos sobre el parásito, lo cual pone en

relevancia el uso alternativo de las hormonas esteroides, en este caso, el de T4 y DHT como potentes agentes cisticidas, lo que explicaría parcialmente porque el macho es más resistente a la infección que la hembra, y porque el cisticerco induce la feminización del macho crónicamente parasitado.

Como se mencionó anteriormente, los mecanismos genómicos no son la única forma en la cual las hormonas esteroides llevan a cabo sus efectos. Alternativamente, se han descrito diversos mecanismos de acción rápida de las hormonas esteroides que poseen múltiples efectos en el organismo, como los de adaptación pronta al estrés y respuesta inmediata a estímulos extracelulares. Por tal razón, se decidió evaluar la participación de este tipo de acciones hormonales sobre el cisticerco de la *Taenia crassiceps*. De esta forma, se estudiaron diversas cascadas de señalización que fueran altamente conservadas en la escala filogenética, que estuvieran involucradas en procesos celulares proliferativos y que pudieran ser activadas por hormonas esteroides sexuales. Así, llegamos al estudio de la vía de señalización activada por un miembro de la familia de las Src-cinasas, LYN, mismo que río abajo posee la capacidad de activar a SYK, proteína que una vez fosforilada inducirá la activación de las cinasas reguladas por señales extracelulares (ERK).

En el cisticerco de la *Taenia crassiceps*, E2 promueve in vitro la reproducción del parásito como se describió anteriormente. Sin embargo, la adición de AG18 (inhibidor de todos los receptores membranales con actividad de cinasa de tirosina) inhibió por completo el efecto proliferativo de E2 y redujo en 50% la formación basal de gemas sin afectar la viabilidad del cisticerco (Fig. 12). Cascada abajo, el uso de genisteína (inhibidor de la activación de LYN) bloqueó los efectos de E2 y, de manera semejante a AG18, disminuyó el número de gemas por debajo del nivel de los grupos controles, aunque esta diferencia no fue estadísticamente significativa (Fig. 12). Otra vez cascada abajo, la exposición a picetanol (inhibidor específico de la activación de SYK), provocó en los cisticercos la disminución del número de gemas formadas al inhibir completamente la acción proliferativa de E2 (Fig. 12). Finalmente, toda esta cascada de señalización celular converge en la activación de ERK, misma que al ser bloqueada por la acción de ERK-Inh, provoca una disminución en la reproducción del parásito, expuesto al mismo tiempo a E2 (Fig. 12).

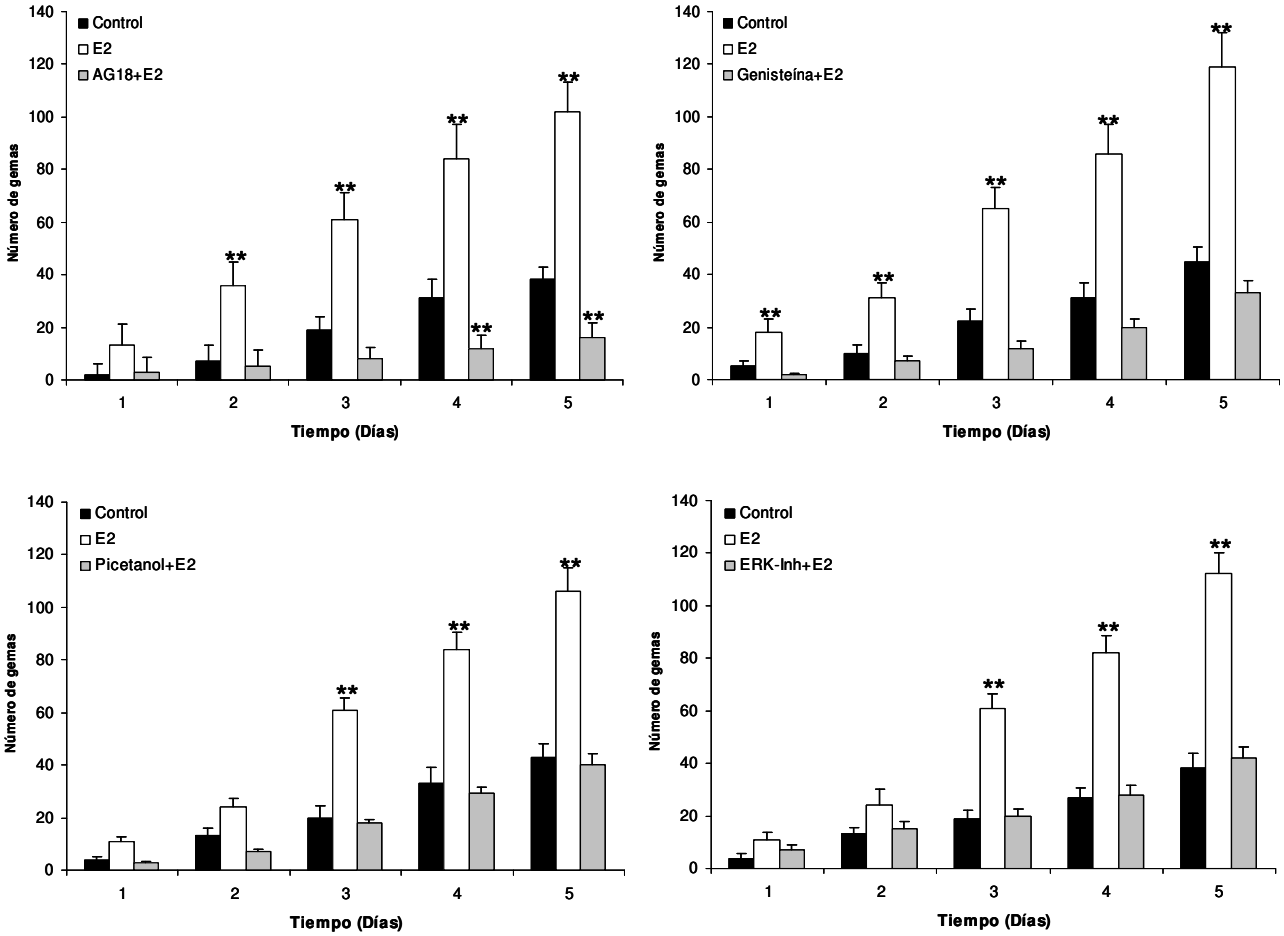

Figura 12. Mecanismo de acción rápida. Efecto de AG18, Genisteína, Picetanol y ERK-Inh sobre la activación de LYN-SYK-ERK y la reproducción del cisticerco de la *Taenia crassiceps* estimulada por E2. El tratamiento con AG18 inhibe el efecto proliferativo de E2 aún por debajo del grupo control, mientras que Genisteína, Picetanol y ERK-Inh inhiben la reproducción parasitaria al mismo nivel que en el grupo control. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

Esto sugiere que E2 promueve la reproducción del parásito también a través de mecanismos no genómicos, induciendo en el cisticerco la fosforilación específica de la cascada LYN-SYK-ERK (cabe resaltar que también se emplearon inhibidores de otras moléculas de señalización como *Fyn* o *Zap-70*, ambas al mismo nivel que *Lyn* y *Syk* en la cascada de activación, respectivamente, y no se encontraron efectos sobre la reproducción del cisticerco estimulada por E2), ya que al inhibir de manera general a todos los receptores con actividad de cinasa de tirosina (AG18) y cascada abajo de forma específica a cada uno de estos segundos mensajeros, el estímulo originalmente inducido por E2 se inhibe completamente (Fig. 12).

Por otro lado, el uso de AG18 en los cisticercos tratados con P4 disminuyó la capacidad reproductiva del cisticerco, aún por debajo de los grupos que sólo recibieron el vehículo (Fig. 13).

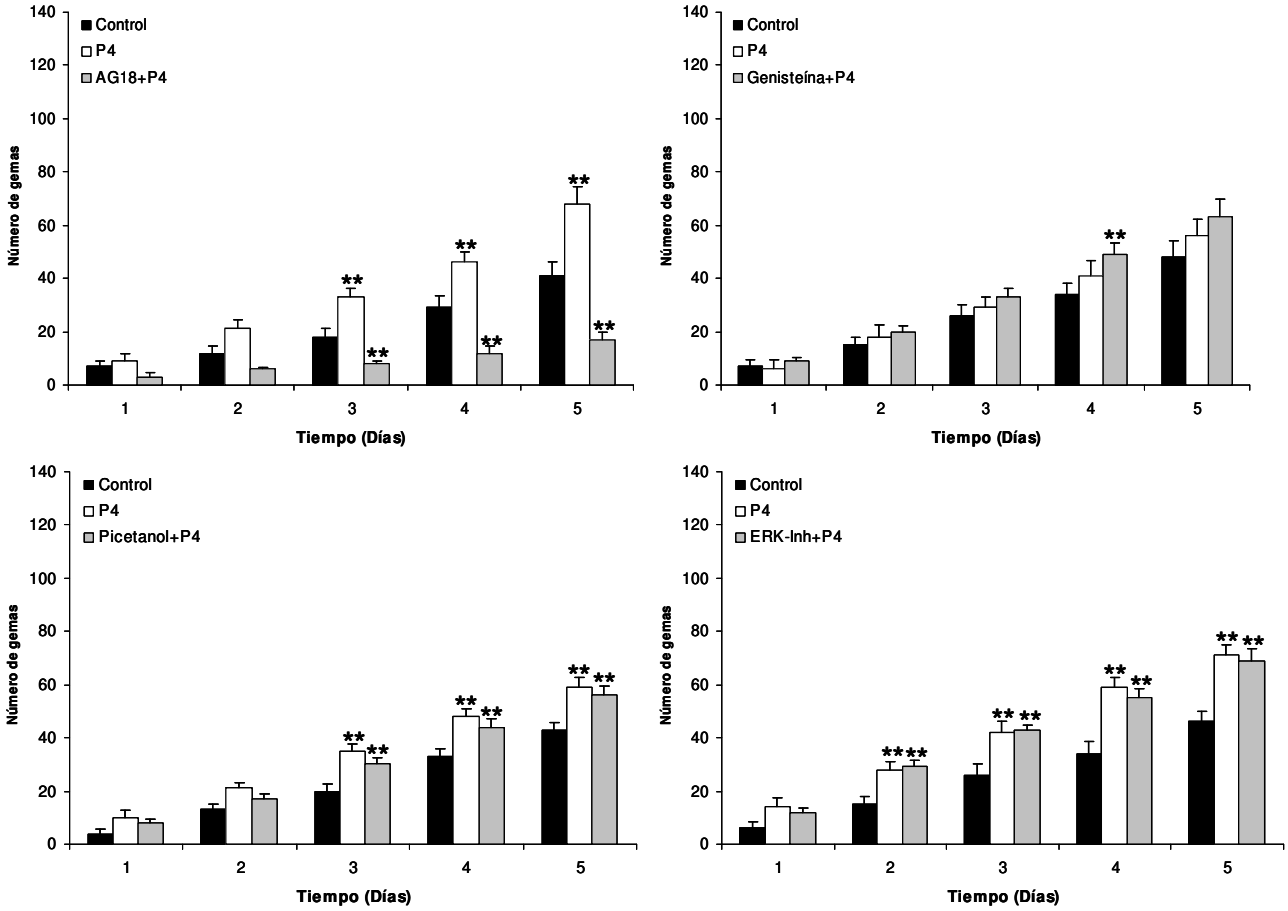

Figura 13. Efecto de AG18, Genisteína, Picetanol y ERK-Inh sobre la activación de LYN-SYK-ERK y la reproducción del cisticerco de la *Taenia crassiceps* estimulada por P4. El tratamiento con AG18 inhibe el efecto proliferativo de P4 sobre el parásito a partir del tercer día en cultivo, mientras que Genisteína, Picetanol y ERK-Inh no mostraron ningún efecto sobre la acción de esta hormona. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

Esto indica que el parásito ha desarrollado diversos mecanismos moleculares y celulares para asegurar su reproducción y sobrevivencia, mismos que no necesariamente dependen de la acción de los esteroides sexuales, aunque su papel dentro de éstos resulta sobremanera relevante. Contrastantemente, Genisteína, Picetanol y ERK-Inh no tuvieron ningún efecto sobre la proliferación inducida por P4 (Fig. 13), lo cual sugiere que los efectos de esta hormona no dependen de un receptor nuclear clásico ni tampoco de la vía de señalización LYN-SYK-ERK, haciendo más fuerte la hipótesis de que la

influencia de P4 sobre el parásito podría deberse a su conversión a E2, u a otra hormona, más que a su actividad misma.

En vista de que los efectos de E2 sobre le cisticerco de la *T. crassiceps* pudieran estar mediados tanto por mecanismos de acción genómica como no genómica, se decidió cultivar al parásito en presencia de ambos tipos de inhibidores, es decir, el cisticerco fue expuesto a tamoxifen y a cada uno de los inhibidores de la cascada LYN-SYK-ERK por separado, AG18, Genisteína, Picetanol y ERK-Inh, respectivamente. Los resultados se muestran en la figura 14.

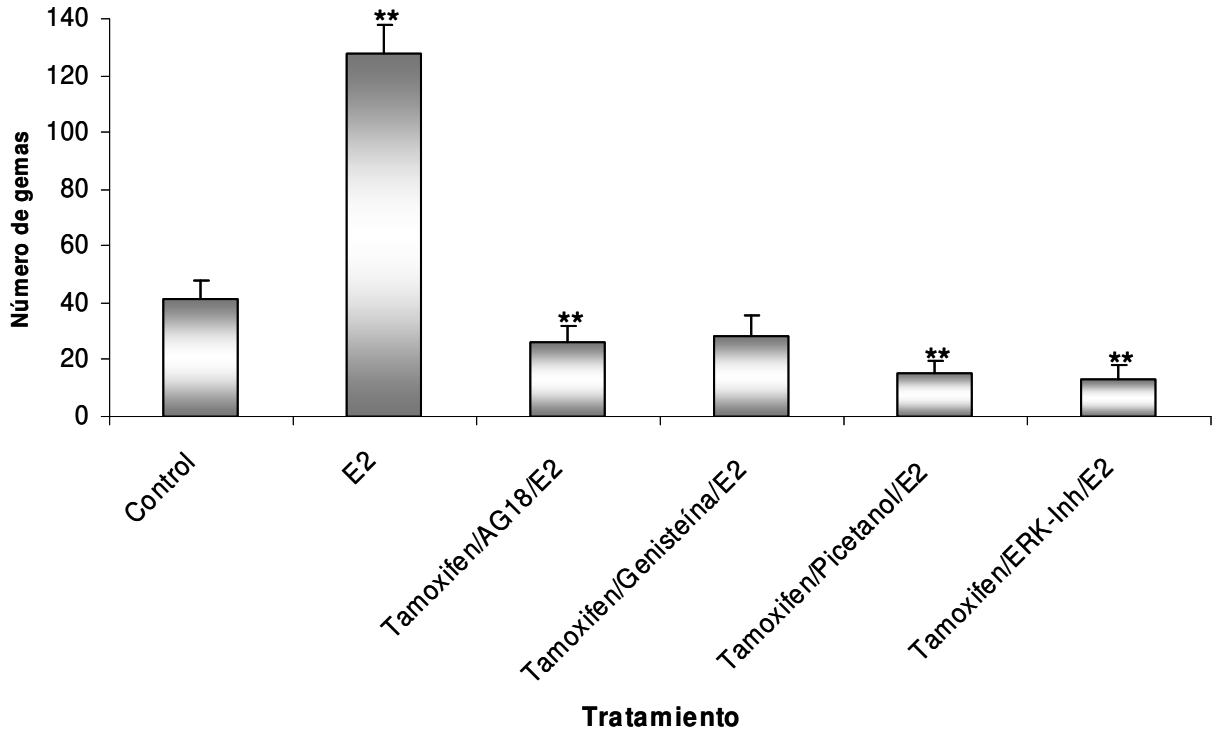

Figura 14. Efecto de antihormonas e inhibidores de proteínas con actividad de cinasa de tirosina sobre la reproducción del cisticerco de la *Taenia crassiceps* estimulada por E2. Los tratamientos con Tamoxifen y AG18, Tamoxifen y Picetanol, y Tamoxifen y ERK-Inh inhibieron la reproducción del parásito aún por debajo de los valores referidos en el grupo control. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

Interesantemente, los efectos acoplados de los inhibidores de vías genómicas y no genómicas, no redujeron completamente la capacidad reproductiva del parásito como se esperaba. Sin embargo, los cisticercos tratados con esta combinación mostraron pérdida progresiva de la motilidad y un marcado decrecimiento. De manera específica, el tratamiento conjunto de Tamoxifen y AG18 mostró resultados semejantes a los encontrados cuando el

parásito es tratado únicamente con AG18, el número de gemas disminuyó en 50% con respecto al valor mostrado en el grupo control (Fig. 14).

Contrastantemente, el uso combinado de Tamoxifen y Genisteína no tuvo efectos estadísticamente significativos sobre la reproducción basal del cisticerco, sin embargo, las combinatorias entre Tamoxifen y Picetanol, y Tamoxifen y ERK-Inh inhibieron totalmente el efecto estimulante de E2 y disminuyeron al 30% la capacidad reproductiva basal del cisticerco de la *Taenia crassiceps*.

Por otro lado, con el fin de determinar los posibles mecanismos moleculares mediante los cuales E2, P4, T4 y DHT llevan a cabo sus efectos en el parásito, se decidió amplificar por PCR los genes c-fos y c-jun, miembros del complejo AP-1, ya que estos genes, además de ser proliferativos, son regulados por esteroides sexuales como el E2. La figura 15 muestra que la expresión de c-fos en cisticercos tratados con E2 y P4 fue fuertemente inducida, ya que los cisticercos que no recibieron tratamiento hormonal (grupo control) expresan escasamente este gen. De forma opuesta, la expresión de c-fos fue abatida en cisticercos tratados con T4 y DHT. Los valores densitométricos de expresión relativa de c-fos son los siguientes: en cisticercos tratados con E2 la expresión de c-fos aumentó 120.3 veces y en los tratados con P4 este índice se incrementó 90.9 veces. Por otra parte, en cisticercos tratados con T4 y DHT, los valores de expresión de c-fos disminuyeron 20 y 80 veces respectivamente (Fig. 15A). El mismo patrón fue observado para c-jun, una inducción clara de la expresión de este gen en cisticercos tratados con E2 y P4 fue observada. El tratamiento con DHT abatió considerablemente la expresión de este gen mientras que el tratamiento con T4 no tuvo efectos significativos sobre la misma. Los valores densitométricos de expresión relativa para c-jun son los siguientes: E2 incrementó 117.3 veces la expresión de c-jun en los cisticercos tratados con este esteroide, P4, de forma muy similar, incrementó 89.9 veces la expresión de este gen, T4 no tuvo efectos sobre su expresión y DHT disminuyó la expresión de c-jun 34.29 veces. En todos los casos se utilizó β-actina como control constitutivo de expresión (Fig. 15B).

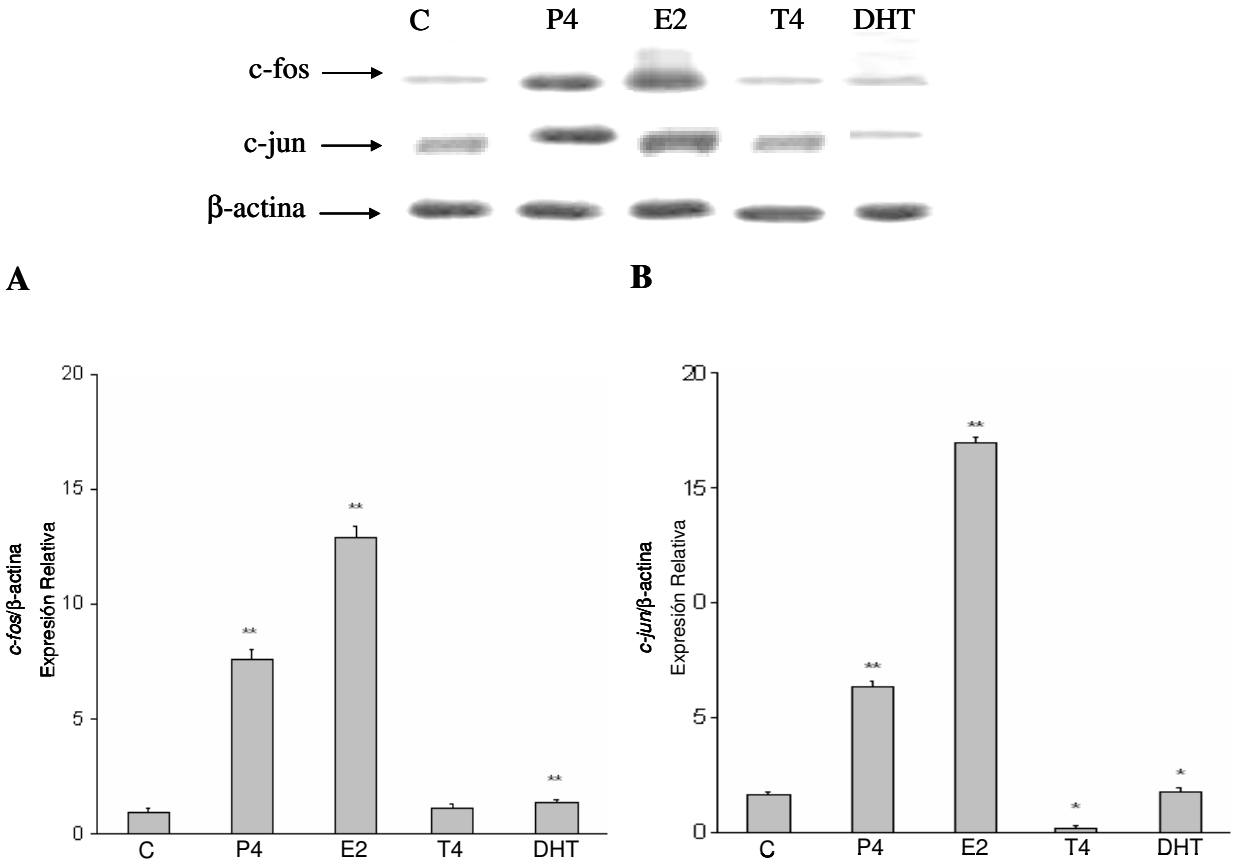

Figura 15. Expresión relativa de c-fos y c-jun en respuesta al tratamiento hormonal en el cisticerco de la *Taenia crassiceps*. E2 y P4 inducen la expresión de c-fos y c-jun mientras que el tratamiento con T4 inhibe casi completamente la expresión de c-jun. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

De esta manera, la determinación de genes parasitarios que pudieran participar en el crecimiento y la reproducción del cisticerco de la *Taenia crassiceps*, y además ser regulados por esteroides sexuales se realizó a dos niveles: el primero de ellos fue estudiando la expresión específica de cada gen en respuesta a la acción hormonal a nivel de RNA mensajero (regulación de la expresión génica por esteroides sexuales). El segundo, fue determinar la presencia de la proteína, sobre todo si los genes estudiados correspondían a receptores hormonales. De esta forma, identificamos y secuenciamos en el cisticerco de la *Taenia crassiceps* (y complementariamente en *T. solium*) los genes completos de *c-fos* y *c-jun*, miembros del complejo de transcripción AP-1, hasta este momento caracterizado solo en mamíferos, además de las secuencias proteicas predichas para cada gen, las cuales mostraron en el caso

de C-Fos, una homología del 96, 93 y 82% con rata, ratón y humano respectivamente, mientras que para el caso de C-Jun la homología con estos tres organismos fue de 98, 92 y 93%, respectivamente (**Ver Apéndice A**).

De forma similar, los genes tipo *Lyn*, *Syk* y *Erk* en el cisticerco de la *Taenia crassiceps* fueron amplificados por RT-PCR y sus secuencias determinadas parcialmente por secuenciación como se muestra en las figuras 16 y 17, respectivamente.

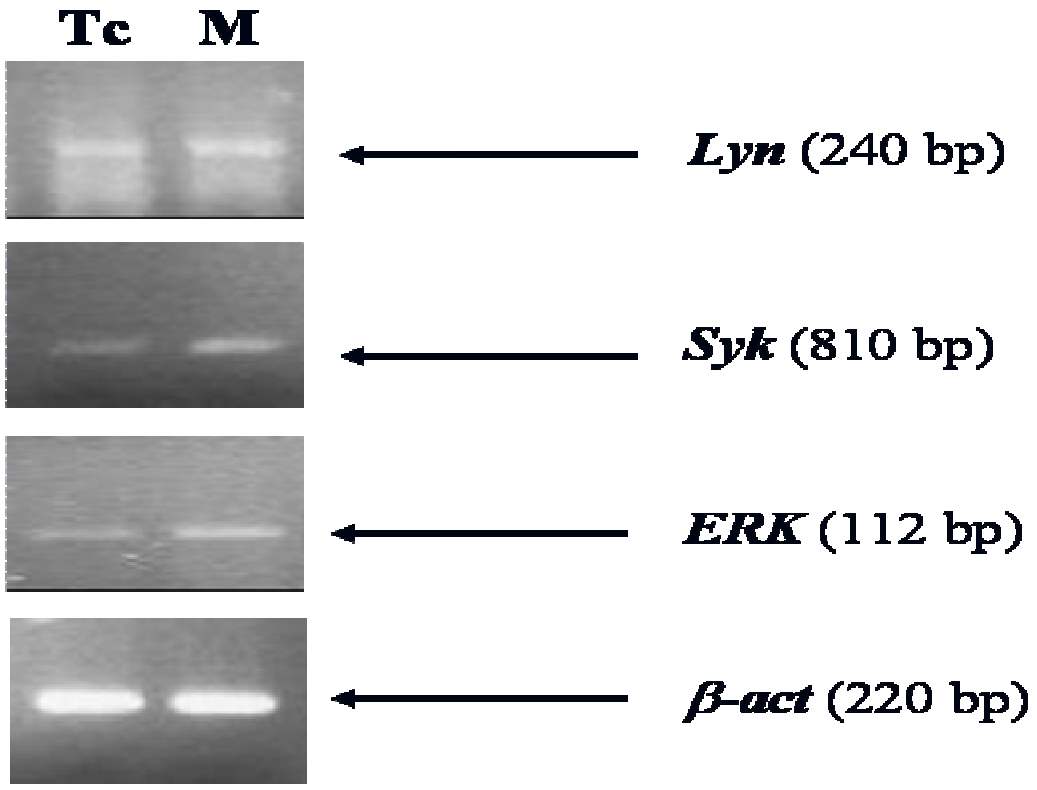

Figura 16. RT-PCR representativo donde se muestran las bandas correspondientes a *Lyn*, *Syk* y *Erk* en el cisticerco de la *Taenia crassiceps* (Tc) y el bazo de ratón hembra (*Mus musculus*) BALBc/AnN (M) como control positivo.

(A) *Lyn*

Taenia crassiceps: 1 AGGCGGGCCCCGCAGGGCTATCCCGTAGCGTGAACAGCTTTTAACCGAAGTCACCGTGGA

Taenia crassiceps: 61 GTTCCGCCGCCCCGAAACTTCCACCACGAGCGAGAAATATGGGATGTATTAAATCAAAAA

Taenia crassiceps: 121 GGAAAGACAATCTCAATGACGATGAAGTAGATTCGAAGACTCAACCAGTTCCTGAATTTC

Taenia crassiceps: 181 ATCTTTTACCAGGACAGAGATTTCAAACAAAAGATCCAGAGGAACAAGGTGACATTGTGG

Taenia crassiceps: 241 TGGCCTTATATCGNNNNNNNNNNN

(B) *Syk*

Taenia crassiceps: 1 CGGTGGGCGGCAGTAGGCAGTGAGCGGAGGAGCCGCGGCAGCGGCATTAAACATTAATTG

Taenia crassiceps: 61 ACTACTTGGAAAGAAAAAAAATCATGGCTTCCAACATGGCCAACCCTGCTAACCATTTGC

Taenia crassiceps: 121 CATACTTCTTTGGCAATATTACACGTGAGGAAGCTGAGGAGTATTTGATGCAAGGAGGAA

Taenia crassiceps: 181 TGAGCGATGGACTATACCTGCTTCGCCAAAGCCGGAATTACCTAGGGGGCTTTGCCTTGT

Taenia crassiceps: 241 CCTTGGCCTATGGAAGGAAGGTTCATCATTACACAATTGAGAGAGAGCTGAGTGGGACAT

Taenia crassiceps: 301 ATGCTATTGCAGGAGGCAAATCACATGCAAGTCCTGCTGAACTCATTAACTATCATTCAG

Taenia crassiceps: 361 AAGAAGCAGATGGCCTTATCTGTCTACTGAGAAAATCTTTCAATCGACCGCCAGGCGTTG

Taenia crassiceps: 421 AGCCCAAAACAGGGCCCTTTGAAGATTTAAAAGAGAACCTCATCAGAGAGTATGTCAAGC

Taenia crassiceps: 481 AAACGTGGAATCTGCAGGGTCATGCTCTTGAACAAGCTATCATTAGCCAAAAACCTCAGC

Taenia crassiceps: 541 TGGAGAAACTGATTGCTACTACAGCCCATGAGAAGATGCCTTGGTTCCATGGGAGGATCT

Taenia crassiceps: 601 CTCGGGAAGAGTCAGAGCACCGTATCCTCATTGGGTCAAGAAACGACGGAAAATTTTTAA

Taenia crassiceps: 661 TACGAGAAAGGGACAGCAATGGTTCCTATGCCCTGTGTTTGCTCAATGATGGAAAAGTAC

Taenia crassiceps: 721 TGCATTACCGTATTGACAGAGATAAGACAGGAAAGCTCTCCATACCAGATGGAAAAAGAT

Taenia crassiceps: 781 TTGACACCCTCTGGCAGTTAGTTGAGCATTAAGTNNNNNNNNNNNNNN

(C) *Erk*

Taenia crassiceps: 1 CCTTAGCCACCGCCGCCGCCATCGCCACCATGGACGAACAGGAGGCATTGAACTCAATCA

Taenia crassiceps: 61 TGAACGATCTGGTGGCCCTCCAGATGAACCGACGTCACCGGATGCCTGGATATNNNNNNN

Figura 17. Secuencia parcial de nucleótidos de genes tipo *Lyn* (A), *Syk* (B) y *Erk* (C) en el cisticerco de la *Taenia crassiceps*.

Por otro lado, la expresión de *Syk* y *Erk* no se vio afectada en el parásito en respuesta a ningún tratamiento hormonal (Figura 18A). Sin embargo, *Lyn* mostró una fuerte regulación positiva por el tratamiento con E2, al aumentar tres veces su expresión con respecto al grupo control, mientras que P4, T4 y DHT no tuvieron ningún efecto sobre la regulación de esta molécula (Figura 18B).

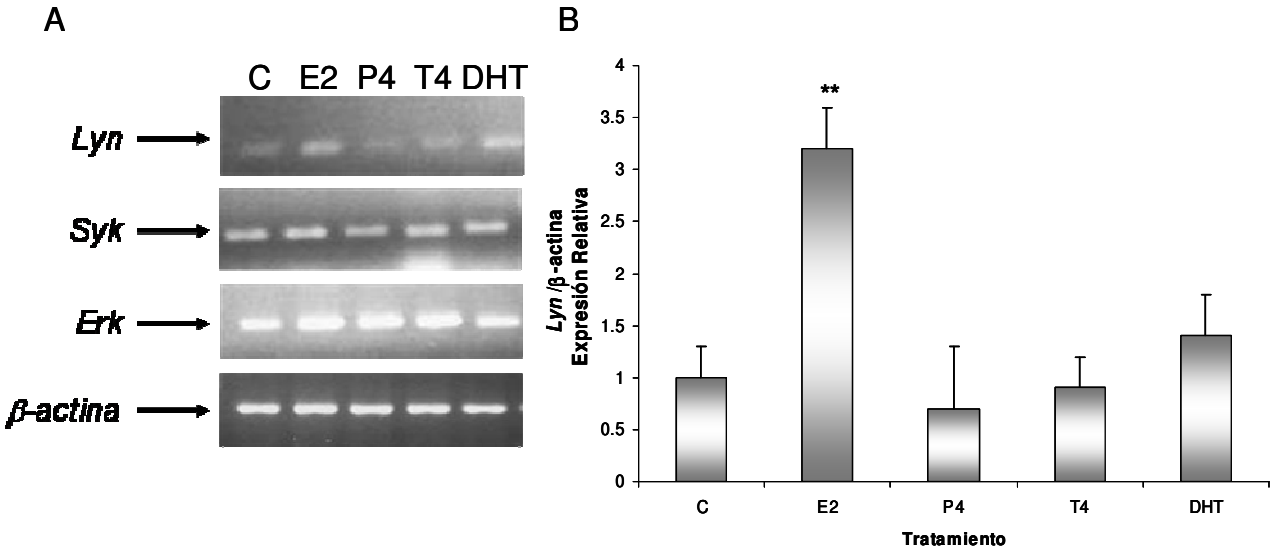

Figura 18. Expresión relativa de *Lyn*, *Syk* y *Erk* en respuesta al tratamiento hormonal en el cisticerco de la *Taenia crassiceps*. (A) RT-PCR representativo. (B) Densitometría óptica de la expresión relativa de *Lyn* (*Lyn*/β-actina). El tratamiento con E2 induce la expresión de *Lyn* en el parásito. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

Estos resultados sugieren fuertemente que moléculas tipo *Lyn*, *Syk* y *Erk*, con actividad de segundos mensajeros, son expresadas por el cisticerco de la *Taenia crassiceps*, y además reguladas diferencialmente en respuesta a E2. Complementariamente, la detección de las proteínas correspondientes a estos genes así como su patrón de fosforilación en respuesta al tratamiento con esteroides fue realizado mediante Western Blot y se muestra en las figuras 19 y 20, respectivamente. En el caso específico de LYN, el anticuerpo monoclonal detectó una sola banda de 55KDa en *T. crassiceps* (Figura 19A), mientras que para SYK el tamaño molecular fue de 80KDa (Figura 19B). Finalmente, un doblete característico de 42 y 44KDa fue detectado en el caso de ERK (Figura 19C). En cada ensayo se utilizó bazo de ratón como control positivo de detección y se ajustaron las condiciones experimentales hasta detectar una sola banda que correspondiera al peso molecular esperado de cada proteína.

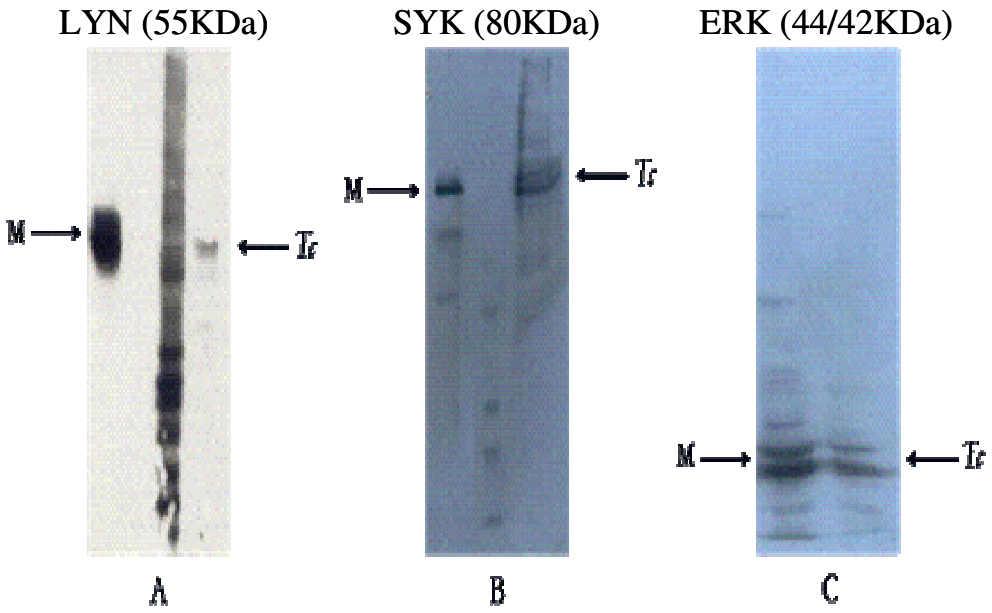

Figura 19. Detección por Western Blot de las proteínas correspondientes en peso molecular a LYN (A), SYK (B) y ERK (C) en el cisticerco de la *Taenia crassiceps* (*Tc*) y el bazo de ratón hembra (*Mus musculus*) BALBc/AnN (M).

Es importante mencionar, que para el caso de este tipo de moléculas no basta con detectar la forma nativa de la proteína, sino además, la forma fosforilada de ésta, misma que lleva a cabo la función de transducción de una señal extracelular al interior de las células. Por esta razón, se realizaron ensayos de Western Blot que nos permitieran estudiar la activación (mediada por la fosforilación proteica) de la vía de señalización LYN-SYK-ERK en el parásito en respuesta al tratamiento con E2, usando por un lado, un anticuerpo que reconoce todos los dominios fosforilados en una muestra de proteína total (Figura 20A y B), y, por el otro, anticuerpos monoclonales diseñados contra la forma nativa de ERK (Figura 20C) y la forma fosforilada de este mismo (Figura 20D), debido a que la activación de este complejo proteínico es el paso limitante en el desencadenamiento de la respuesta proliferativa estimulada por E2 en el parásito.

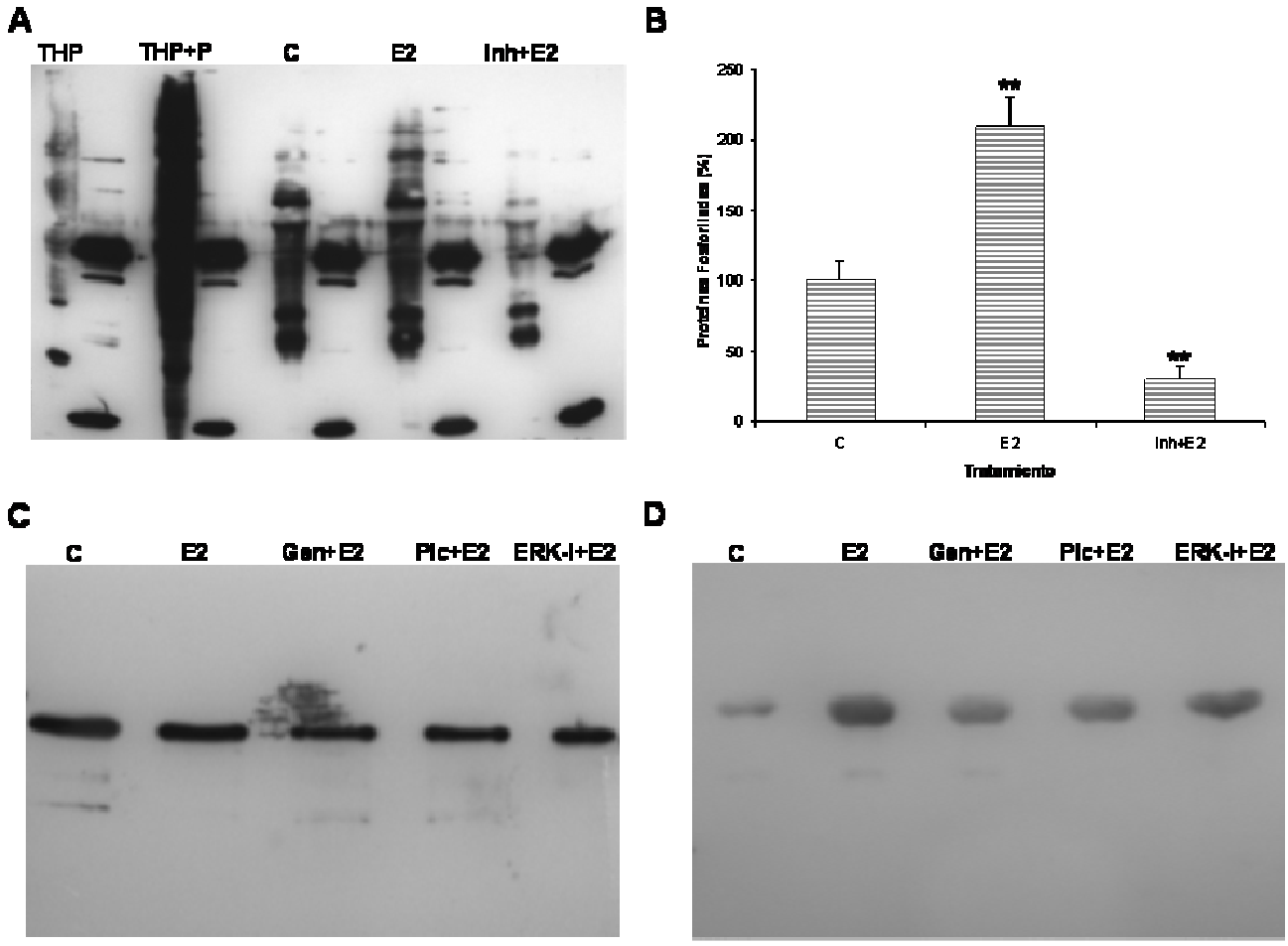

Figura 20. (A) Detección por Western Blot de proteínas fosforiladas totales en respuesta al tratamiento hormonal en el cisticerco de la *Taenia crassiceps*. (B) Cuantificación densitométrica de proteínas fosforiladas totales. Se puede apreciar que el tratamiento con E2 induce la fosforilación de diversas proteínas parasitarias. (C) Western Blot mostrando la forma nativa de ERK. (D) Western blot mostrando la forma fosforilada de ERK. El tratamiento con E2 induce un aumento en la fosforilación de esta cinasa de tirosina. THP=Línea celular derivada de monocitos transformados, P=Pervanadato, Inh=Cocktail inhibidor de cinasas de tirosina, C=Control, Gen=Genisteína, Pic=Picetanol, ERK-I=Inhibidor de ERK. ** Diferencias significativas entre grupos tratados y controles. P<0.05.

De esta manera, las proteínas totales de la línea celular THP en ausencia de estímulo con Pervanadato, mostraron poca fosforilación. Sin embargo, la sola adición de este inhibidor de fosfatasas a estas mismas células incrementó hasta ciento seis veces más la cantidad de proteínas fosforiladas (Fig. 20A). En el caso específico del cisticerco de la *Taenia crassiceps*, el grupo control que no recibió estimulo hormonal presentó una fosforilación basal en el total de sus proteínas. Interesantemente, la sola exposición del parásito a E2

duplicó en éste la cantidad de proteínas fosforiladas, mientras que cuando el cisticerco fue cultivado al mismo tiempo en presencia de Genisteína, Picetanol, ERK-Inh y E2, el porcentaje de proteínas fosforiladas disminuyó dramáticamente hasta un tercio del total de lo referido en el grupo control (Fig. 20A y B). Por otra parte, la exposición del parásito a E2, Genisteína, Picetanol o ERK-Inh, no afectó la síntesis proteica de la forma nativa de ERK (Figura 20C), resultado que correlaciona con lo mostrado a nivel de la expresión del RNA mensajero mostrada en la Figura 18A. Sin embargo, la adición de E2 exógeno indujo potentemente la fosforilación de ERK en el parásito (12:1 con respecto al grupo control) mientras que, contrariamente, los tratamientos con los inhibidores de cinasas de tirosina bloquearon los efectos de este estrógeno, manteniendo la fosforilación de ERK en niveles basales, muy cercanos a los del grupo control (Figura 20D).

Estos resultados apoyan fuertemente que E2 es capaz de estimular directamente la reproducción del cisticerco de la *Taenia crassiceps*, a través de la vía de señalización que comienza con la activación de LYN y SYK, y culmina cascada abajo con la activación de ERK. Adicionalmente, los efectos de E2 sobre el parásito no son a través de la regulación de la expresión génica de estas moléculas de señalización, sino por medio de la fosforilación y activación específica de estas mismas en el cisticerco, hecho que denota el fuerte impacto que el microambiente hormonal del hospedero podría tener sobre la fisiología parasitaria, al regular en éste procesos tan específicos como activación de diversas vías de señalización y expresión génica.

Por último, durante este trabajo se identificaron otros genes que participan directamente en la reproducción y el establecimiento del parásito, y que también pueden ser regulados por hormonas sexuales. Este es el caso de los receptores de hormonas expresados por el parásito, mismos que poseen la capacidad de reconocer esteroides sexuales exógenos (e hipotéticamente hormonas producidas por el hospedero) y de esta manera regular la reproducción, crecimiento y/o metabolismo parasitario en un hospedero inmunocompetente.

De esta forma, identificamos y determinamos en el parásito las secuencias parciales de los receptores de estrógenos subtipos α y β (ER-α y ER-β), así como las correspondientes a los receptores de progesterona isoformas A y B (PR-A y PR-B) y el receptor de andrógenos (Fig. 21).

(A) ER-α

Taenia crassiceps: 1 TTGCTGCACCAGATCCAAGGGAACGAGCTGGAGCCCCTCAACCGCCCGCAGCTCAAGATG

Taenia crassiceps: 61 CCCATGGAGAGGGCCCTGGGCGAGGTATACGTGGACAACAGCAAGCCCACTGTGTTCAAC

Taenia crassiceps: 121 TACCCCGAGGGCGCCGCCTACGAGTTCAACGCCGCCGCCGCCGCCGCCGCCTCG

Taenia crassiceps: 181 GCGCCGGTCTACGGCCAGTCGGGCATCGCCTACGGCCCCGGGTCGGAGGCGGCCGCCTTC

Taenia crassiceps: 241 AGTGCCAACAGCCTGGGGGCTTTCCCCCAGCTCAACAGCGTGTCGCCTAGCCCGCTGATG

Taenia crassiceps: 301 CTGCTGCACCCGCCGCCGCAGCTGTCTCCTTTCCTGCACCCGCACGGCCAGCAGGTGCCC

Taenia crassiceps: 361 TACTACCTGGAGAACGAGCCCAGCGCCTACGCCGTGCGCGACACCGGCCCTCCCGCCTTC

Taenia crassiceps: 421 TACAGGTCTAATTCTGACAATCGACGCCAGAATGGCCGAGAGAGACTGTCCAGCAGTAAC

Taenia crassiceps: 481 GAGAAAGGAAACATGATCATGGAGTCTGCCAAGGAGACTCGCTACTGTGCCGTGTGCAAT

Taenia crassiceps: 541 GNNNTNNNNNTCNNNNNCNN

(B) ER-β

Taenia crassiceps: 1 ATAGGGAGGGATAATACCTCGCATGTGAAGCTGCTCGAATACTTACGTCTCCCCGACAAA

Taenia crassiceps: 61 GCATTAAGTCGTCTACAAACGATTTTGTGCCTCCACACTCATTCCACAATTGGATATCGT

Taenia crassiceps: 121 GTGGGACCCCCACGCAGCACCACAACCATCNGATGCACGCGGTCTTGGTCCACGCTCTGG

Taenia crassiceps: 181 CTTTGCACTACTGTTGTTGCCGCCAGACCACTCGCAGCCGTCACAGCACGCTGGCCGTGT

Taenia crassiceps: 241 ACGAAGACAGAGAAGTGCCACTCTTTCGTAACGTCAAGGGAGGGATCATACCTGGCATGT

Taenia crassiceps: 301 GATAGCTGCTCGAATCTTACGTCTCCCCGACAAAGCATTAAGTCGTCTACAAACGATTTT

Taenia crassiceps: 361 GTGCCTCCACACTCATTCCACAATTGGATATCNGTGTCGACCCCCACGCAGCAACCACAA

Taenia crassiceps: 421 CCATCTGATGCACGCGGTCTTGGTCCACGCTCTGGCTTTGCACTACTGTTGTTGCCGCCA

Taenia crassiceps: 481 GACACTCGCAGCCGTCACAGCACGCTGGCCGTGTACGAAGACAGAGGGAAAGTGCCATTT

Taenia crassiceps: 541 AACGCGCNNNNNNNNNNCNNNGNNNNNNNNNNNN

(C) PR-A

Taenia crassiceps: 1 TCGCCCTATTGCCCCTGTCCCGCACTCCTGGCCGTCGCACCCGGCAGCTGCTGGAGGGGG

Taenia crassiceps: 61 ACAGCTGCCACAGACGTAACACAGGCCATGTTCCTTTATGCCCCACCTGACGGGCTCACT

Taenia crassiceps: 121 CTCCAACGCCATGCCCACCTGCGCCCTGCACGAGACNAACTCACTACTGCATCTACTCTG

Taenia crassiceps: 181 GTCGGAAGGTCAACCTTAACCTTAGACNNNNNNNNNNNNANNNATNNNNN

(D) PR-B

Taenia crassiceps: 1 CGGAAAAGAAGCAGGATTTTAAAAGTGTCTNAGACACGTTGTNGACTCCCTTCGGGACCT

Taenia crassiceps: 61 AACACATTCACGCCAGCCCTTCACCCTGTGAGGGCATTAGTTTCTGGAGTTTTTTTGGGC

Taenia crassiceps: 121 CAAAGCTTACAAAATATCCCCGCAGTGTCCCTGCTCCCAAAGGGTTGGCTGTTAAAGAAC

Taenia crassiceps: 181 ACCGTTNAAACCCTGCAGCGTNNNNCNNNCGANNNNNNNN

(E) AR

Taenia crassiceps: 1 AAGGAGAGGCTTCCAGCACCACCAGCCCCACTGAGGAGACAACCCAGAAGCTGACAGTGT

Taenia crassiceps: 61 CACACATTGAAGGCTATGAATGTCAGCCCATCTTTCTGAATGTCCTGGAAGCCATTGAGC

Taenia crassiceps: 121 CAGGTGTAGTGTGTGCTGGACACGACAACAACCAGCCCGACTCCTTTGCAGCCTTGCTCT

Taenia crassiceps: 181 CTAGCCTCAATGAACTGGGAGAGAGACAGCTTGTACACGTGGTCAAGTGGGCCAAGGCCT

Taenia crassiceps: 241 TGCCTGGCTTCCGCAACTTACACGTGGACGACCAGATGGCTGTCATTCAGTACTCCTGGA

Taenia crassiceps: 301 TGGGGCTCATGGTGTTTGCCATGGGCTNNNNNNNNNNACNNNNNNNNNNNNNNNN

Figura 21. Secuencias parciales correspondientes a los receptores de estrógenos α y β (ER-α y ER-β), receptores de progesterona A y B (PR-A y PR-B) y receptor de andrógenos (AR) en el cisticerco de la *Taenia crassiceps*. ER-β Número de acceso Gene Data Bank: AY596184.

Por otra parte, ER-β fue detectado en mayor cantidad que ER-α, PR-A, PR-B y AR en el cisticerco de la *Taenia crassiceps*, partiendo de la misma cantidad de RNA total (Figura 22A). Adicionalmente, cuando el parásito fue expuesto a P4, ER-β no solo fue sobre-expresado sino también sintetizado en mayor cantidad a nivel de proteína (Figura 22B), mientras que el resto de los receptores de hormonas esteroides no presentaron variación en respuesta al tratamiento con E2 o P4 (Figura 22B). Estos resultados sugieren que el parásito no solo ha desarrollado estructuras proteicas que semejan a los receptores de esteroides sexuales descritos en vertebrados superiores, sino

además que estas moléculas tipo receptor podrían estar reguladas bajo mecanismos endocrinos clásicos, como el que P4 ejerce sobre la expresión de los receptores de estrógenos α y β.

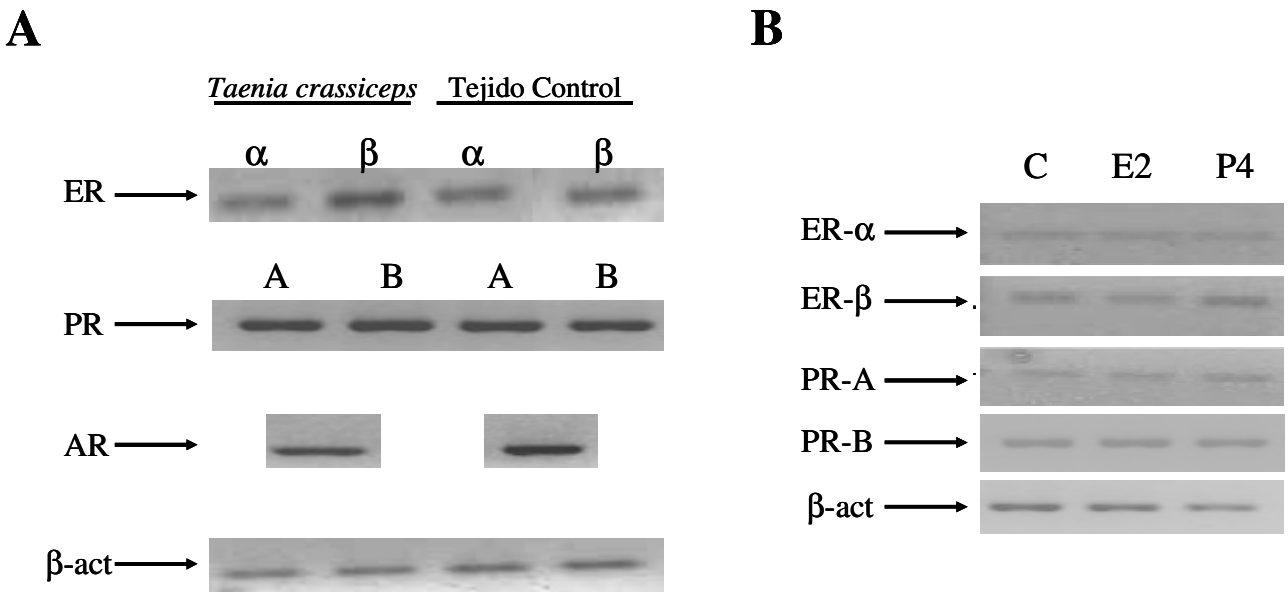

Figura 22. (A) RT-PCR representativo donde se muestra la expresión de ER-α, ER-β, PR-A, PR-B y AR en el cisticerco de la *Taenia crassiceps*. (B) Western Blot mostrando la detección de las proteínas correspondientes a ER-α, ER-β, PR-A y PR-B en el cisticerco de la *Taenia crassiceps* y los efectos del tratamiento con E2 y P4 sobre su síntesis.

DISCUSIÓN

Durante la cisticercosis murina, se conoce bien que una gran cantidad de interacciones entre los sistemas inmune y endocrino, pueden regular diversos aspectos de la infección, tales como el establecimiento parasitario. Sin embargo, el curso final de la infección no solo es decidido por medio de estas complejas interacciones. Nuestros resultados sugieren que el sistema endocrino, a través de sus productos hormonales, también puede ser determinante durante el establecimiento del cisticerco de la *Taenia crassiceps* en el hospedero murino. De manera específica, las hormonas esteroides sexuales (exógenas) poseen efectos directos sobre el parásito, mismos que regulan su reproducción, crecimiento e infectividad *in vitro*.

Es bien sabido que las hormonas esteroides poseen una gama muy amplia de acciones que abarcan desde organismos invertebrados (Ej. *Drosophila melanogaster*) hasta mamíferos como el ratón o el humano mismo. Sin embargo, en el caso de organismos parasitarios aún no se precisan bien los efectos y mecanismos que los esteroides sexuales son capaces de establecer en un hospedero inmunocompetente. Por tal razón, la materia de esta tesis fue determinar si las hormonas esteroides sexuales E2, P4, T4 y DHT poseían efectos directos sobre el cisticerco de la *Taenia crassiceps*, profundizando en sus mecanismos, específicamente en la regulación (a la alta o a la baja) de genes parasitarios como resultado de la influencia hormonal.

De esta manera, se determinó que E2 estimula la reproducción del parásito sin afectar su viabilidad. En este mismo sentido, P4 es capaz de inducir un aumentó en el índice proliferativo del cisticerco, aunque éste no es de la misma magnitud que el observado con E2. Sin embargo, cuando los parásitos son tratados con este progestágeno, su infectividad al ser reinoculados en hembras y machos se potencia fuertemente.

Por otra parte, los andrógenos T4 y DHT poseen el efecto contrario sobre el parásito, pues inhiben casi completamente la reproducción del cisticerco *in vitro*, afectando también su viabilidad.

Estos resultados denotan el carácter multifuncional que los esteroides sexuales poseen sobre una gran diversidad de organismos, como ya se ha mencionado. En el caso específico de esta parasitósis, E2 puede incrementar la reproducción del cisticerco mientras que P4 aumenta su infectividad,

sugiriendo que las hormonas sexuales femeninas poseen en su conjunto un efecto estimulante sobre *Taenia crassiceps*, e individualmente acciones alternativas que podrían beneficiar y favorecer el establecimiento del parásito en las hembras y el macho feminizado.

Otro de los aspectos interesantes de este trabajo, es el mecanismo a través del cual las hormonas esteroides ejercen sus acciones sobre el cisticerco. Por medio del uso de antihormonas, nuestros resultados sugieren que el parásito ha desarrollado estructuras semejantes a los receptores de hormonas esteroides, mismos que poseerían la capacidad de unirse a una hormona exógena (en el caso del hospedero murino a hormonas sintetizadas por éste) y regular de esta manera la expresión de genes implicados en la reproducción, crecimiento e infectividad del cisticerco de la *Taenia crassiceps*, tales como c-fos y c-jun.

Es de llamar la atención, el alto grado de conservación que las hormonas esteroides poseen en cuanto a sus acciones y mecanismos, divergiendo como se conoce desde insectos hasta mamíferos, y ahora también reportado en organismos parasitarios.

Otro aspecto sumamente interesante de estos resultados, es el posible beneficio que el cisticerco obtiene al aprovechar las hormonas exógenas, o bien las sintetizadas por el hospedero durante el proceso de infección. Este beneficio podría resultar en un ahorro metabólico y energético, pues el parásito sólo sintetizaría los receptores de esteroides sexuales y aprovecharía en su completo beneficio el microambiente hormonal del hospedero. Este fenómeno fue denominado por nosotros como trans-regulación, haciendo referencia a la capacidad que un organismo posee al regular la expresión génica de otro, a través de sus productos hormonales y factores de crecimiento (**Ver Apéndice A**). En este caso, el hospedero sería capaz de regular de manera cruzada (trans-regulación) el crecimiento y la reproducción del cisticerco, llevando la relación de parasitismo de este último no solo a niveles fisiológicos sino también a niveles moleculares. Interesantemente, este mecanismo no sólo se podría presentar en este modelo de estudio, sino en otras parasitosis en donde se conoce o se ha reportado dimorfismo sexual en la infección, o simplemente efectos relacionados con el microambiente hormonal del hospedero. Este tipo de estudios son de suma importancia, pues al conocer con exactitud las

estructuras de las cuales depende la reproducción o el crecimiento del parásito, se pueden diseñar drogas o análogos hormonales que afecten exclusivamente al patógeno y bloqueen rutas metabólicas o procesos fisiológicos vitales en éste.

Adicionalmente, la reproducción del cisticerco de la *Taenia crassiceps* no solo se lleva a cabo a través de estructuras con actividad de receptores de hormonas esteroides en éste, sino también por medio de la activación de diversas vías de señalización que pueden ser estimuladas por esteroides sexuales, como es el caso de la vía mediada por los segundos mensajeros LYN-SYK-ERK. Es importante recalcar, que la inhibición de cada una de estas moléculas resultó en la inhibición de los efectos estimulantes de E2 sobre el cisticerco, lo que sugiere que este estrógeno aumenta la fosforilación de estas moléculas, induciendo finalmente la activación de esta vía de señalización, como se mostró anteriormente en la sección de resultados. Estos hallazgos sugieren que el parásito no solo ha desarrollado estructuras semejantes a los receptores de hormonas esteroides, sino también vías de señalización mediadas por cascadas de segundos mensajeros que aseguran todavía más su reproducción y establecimiento en un ambiente hostil como el ofrecido por un hospedero inmuno-competente. Interesantemente, se sabe que este tipo de moléculas poseen dominios proteínicos altamente conservados denominados SH2 y SH3, capaces de interaccionar con receptores de hormonas específicos y así regular la expresión génica de un organismo. Es probable que este sea el punto en el cual ambas vías en el parásito, genómicas y no genómicas, confluyan para potenciar aspectos sumamente importantes de éste como su reproducción, lo cual sugiere que los organismos parasitarios han desarrollado "mecanismos amortiguadores", mismos que le permiten divergir sus estrategias de sobrevivencia y evitar de esta manera el bloqueo de funciones sumamente importantes tales como su diferenciación y los mecanismo que le permiten evadir la respuesta inmunológica.

Por otro lado, queda por esclarecer el mecanismo antiproliferativo que los andrógenos T4 y DHT poseen sobre el cisticerco de la *Taenia crassiceps*. Nuestros resultados indican que el tratamiento con estos andrógenos no afecta la incorporación de timidina-3H en el parásito, sin embargo, la síntesis de DNA se ve disminuida en estos mismos, sugiriendo un mecanismo de daño y

degradación del DNA, probablemente mediado por una vía apoptótica activada por andrógenos.

Adicionalmente, resultados no incluidos en esta tesis demuestran que el tratamiento con andrógenos provoca un desarreglo del citoesqueleto del parásito, debido a la alteración de la estructura de las células flama (células que en su conjunto forman parte del aparato protonefridial del cisticerco), que exhiben una gran desorganización en el halo de actina que las rodea así como pérdida progresiva en la síntesis de tubulina. Es importante mencionar, que la estructura molecular del albendazol (fármaco de elección en el tratamiento de la teniosis/cisticercosis) conserva dominios parecidos al ciclo pentano per-hidro fenantreno, estructura base de las hormonas esteroides, misma que quizá le de a los andrógenos efectos cisticidas parecidos a los que se observan con el uso de este fármaco. No obstante, es necesario profundizar aún más en estos experimentos de microscopia confocal, y en algunas otras estrategias experimentales que nos permitan definir con precisión cuál es el mecanismo antiproliferativo que media las acciones de T4 y DHT sobre el metacéstodo de la *Taenia crassiceps*.

Por último, los genes identificados en el cisticerco de la *Taenia crassiceps* implicados en la reproducción del parásito se muestran en la sección de resultados (c-fos, c-jun, Lyn, Syk, Erk, ER-α, ER-β, PR-A, PR-B y AR). Consideramos que estos no son todos los genes que activamente podrían participar en el crecimiento, la infectividad y la reproducción del parásito, sin embargo, poseen funciones dependientes de hormonas sexuales y desempeñan papeles proliferativos en el cisticerco, lo cual los hace buenos candidatos para ser estudiados en otras parasitosis como la provocada por el metacéstodo de la *Taenia solium*, sabiendo de antemano que su inhibición podría resultar en la interrupción de algún proceso fisiológico vital en el parásito y, por ende, del proceso infeccioso mediado por éste.

Los resultados, hipótesis e ideas vertidas en esta tesis pretenden generar una concepción diferente de la relación hospedero-parásito, abarcando desde el nivel fisiológico hasta el molecular, en el cual el parásito depende en mucho de las hormonas y factores de crecimiento del hospedero.

Además, el que hormonas exógenas (o también las producidas por el hospedero) puedan regular la expresión génica del cisticerco de la *Taenia*

crassiceps, no debe ser exclusivo de esta parasitosis, pues quizás en otras enfermedades provocadas por parásitos extracelulares este mismo fenómeno se repita, a distintos niveles, lo cual abre una interesante ventana en el estudio y diseño de drogas y moléculas que puedan afectar exclusivamente al patógeno y redefinir el curso de la infección parasitaria.

CONCLUSIONES

1) 17β-estradiol, progesterona, testosterona y 5α-dihidrotestosterona afectan directamente la reproducción, crecimiento e infectividad del cisticerco de la *Taenia crassiceps in vitro*.
2) 17β-estradiol aumenta cuatro veces la reproducción del cisticerco de la *Taenia crassiceps*.
3) El tratamiento con progesterona incrementa la capacidad infectiva del cisticerco de la *T. crassiceps* al ser reinoculado en hembras y machos.
4) Testosterona y 5α-dihidrotestosterona inhibieron la reproducción del cisticerco de la *Taenia crassiceps* en 70 y 85%, respectivamente, mostrando un potente efecto cisticida al reducir en 95% la viabilidad del parásito.
5) Los tratamientos con 17β-estradiol y progesterona no afectaron la viabilidad del cisticerco de la *T. crassiceps*.
6) El cisticerco de la *T. crassiceps* sintetiza proteínas con actividad de receptor de hormonas esteroides tipo ER-α, ER-β, PR-A, PR-B y AR.
7) El cisticerco de la *T. crassiceps* expresa segundos mensajeros parecidos en secuencia y función a Lyn, Syk y ERK, descritos previamente en mamíferos.
8) c-fos y c-jun son expresados por el cisticerco de la *T. crassiceps* y parecen estar implicados en el proceso proliferativo mediado por 17β-estradiol.
9) El uso de tamoxifen, AG18, genisteína, picetanol y ERK-Inh inhibe el efecto proliferativo de 17β-estradiol sobre el cisticerco de la *T. crassiceps*, lo cual sugiere que este estrógeno estimula la reproducción del parásito a través de la vía de señalización mediada por LYN-SYK-ERK.
10) El uso de RU-486, AG18, genisteína, picetanol y ERK-Inh no inhibe el efecto proliferativo de progesterona sobre el parásito, lo cual sugiere que sus efectos son mediados por otras vías o simplemente debido a su conversión a otra hormona en el cisticerco.

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APÉNDICE A

PUBLICACIONES EN REVISTAS INDEXADAS

1) Morales-Montor J, Chavarria A, De León MA, Del Castillo LI, **Escobedo G**, Sánchez EN, Vargas JA, Hernández-Flores M, Romo-González T, and Sarralde C. Host gender in parasitic infections of mammals: an evaluation of the female host supremacy paradigm. *Journal of Parasitology* (2004) 90: 531-546.
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APÉNDICE B

ARTICULOS ENVIADOS PARA PUBLICACIÓN

1) **Escobedo G**, Nava K, Soldevila G, Chávez-Ríos JR, Hallal-Calleros C, Ostoa-Saloma P and Morales-Montor J. Detection of LYN-like and ERK-like as Possible Non-genomic Action Mechanism Involved in the Estradiol-Stimulated Reproduction of the Helminth Cestode *Taenia crassiceps*. En preparación.

APÉNDICE C

OTRAS PUBLICACIONES

MEMORIAS IN EXTENSO

1) **Escobedo G**, Larralde C, Ostoa-Saloma P and Morales-Montor J. 17β-estradiol stimulates the reproduction of the metacestode of the helminth parasite *Taenia crassiceps. Proceedings of the IX European Multicongress of Parasitology* (2005). MC29, 45-49.

CAPÍTULOS EN LIBROS

1) Arteaga M, Rodríguez-Dorantes M, **Escobedo G**, Vargas-Villavicencio JA and Morales-Montor J. The host-parasite neuroimmunoendocrine network: Its mechanisms, function and consequences to the host and the parasite. Luis I. Terrazas Editor, en *Advances in the Immunobiology of Parasitic Diseases*, Research Signpost Fort P.O., Trivandrum-695 023, Kerala, India, pp 101-118, 2007, ISBN 81-308-0166-3, First Edition.

2) **Escobedo G**, De León MA and Morales-Montor J. Sexual dimorphism in parasitic diseases: beyond the dogma of female-biased infections. Craig W. Roberts Editor. 2008. *En preparación*.

REVISTAS NO INDEXADAS, DE DIVULGACIÓN Y REGISTRO EN BASE DE DATOS DE GENEBANK

1) **Escobedo G** y Morales-Montor J. Transregularización del Establecimiento, Crecimiento y Reproducción Parasitaria por Hormonas del Hospedero: ¿Un Nuevo Mecanismo de Explotación Utilizado por los Parásitos? *Revista de Educación Bioquímica* (2004) 23 (1): 12-17.

2) **Escobedo G** y Morales-Montor J. La red de interacciones neuroinmunoendocrinas en esquistosomiasis: implicaciones para el hospedero y el parásito. *Gaceta Biomédicas* (2007) 8: 3, 1-3.

3) Genes Secuenciados y Publicados en el GeneDataBank

Gen	Número de Acceso GeneDataBank
c-fos Taenia crassiceps	bankit 46327365 AY436615
c-jun Taenia crassiceps	bankit 46327363 AY436613
c-fos Taenia solium	bankit 46327367 AY436616
c-jun Taeniasolium	bankit 46327363 AY436614
PR-A *Taenia solium*	bankit 46988512 AY596186
PR-B *Taenia solium*	bankit 46988505 AY596185
ER-β *Taenia crassiceps*	bankit 46988499 AY596184

APÉNDICE D

PREMIOS Y DISTINCIONES

1) **Medalla al Mérito Universitario 2002**. Al Estudiante más Destacado de la Licenciatura en Biología Experimental. Universidad Autónoma Metropolitana, Unidad Iztapalapa. Agosto de 2002.

2) **Premio al Mejor Trabajo en al Área de Biología Celular 2004**. XVI Congreso Nacional de Parasitología, CONAPAR. Tlaxcala. México. Octubre de 2004.

3) **Marc Dresden Award 2007**. Otorgado por la American Society for Parasitologist. From Alaska to Chiapas: The First North American Parasitology Congress. Mérida, Yucatán. México. Junio de 2007.

4) **Premio Dr. Ramiro Montemayor 2007**. Al Mejor Trabajo en Ciencia Biomédica Básica. XXIV Congreso Nacional de Investigación Biomédica. Monterrey, Nuevo León. México. Septiembre de 2007.

5) **Global Health Travel Award 2008**. Otorgado por la fundación Melinda and Bill Gates. Keystone Symposia. 8-13 de Abril de 2008. Breckenridge, Colorado, USA.

6) **Premio Canifarma-Veterinaria 2007 Dr. Alfredo Téllez Girón Rode**. Otorgado por la Cámara Nacional de la Industria Farmacéutica. Mayo de 2008.

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MOLECULAR MECHANISMS INVOLVED IN THE DIFFERENTIAL EFFECTS OF SEX STEROIDS ON THE REPRODUCTION AND INFECTIVITY OF *TAENIA CRASSICEPS*

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ABSTRACT: The in vitro exposure of *Taenia crassiceps* cysticerci to 17-β estradiol (E_2) and progesterone (P_4) stimulated their reproduction and infectivity. Testosterone (T_4) and dihydrotestosterone (DHT) inhibited their reproduction and reduced their motility and infectivity. E_2 and P_4 increased, whereas T_4 and DHT reduced, the expression of parasite *c-fos* and *c-jun* and DNA synthesis. In vitro exposure of cysticerci to sex steroids before their inoculation into recipient noninfected mice resulted in large parasite loads when pretreated with E_2 and P_4 and in smaller loads when pretreated with T_4 and DHT. To determine the possible molecular mechanisms by which sex steroids affect *T. crassiceps*, sex steroid receptors were amplified. *Taenia crassiceps* expressed estrogen receptors (both α and β isoforms) and androgen receptors but no P_4 receptors. These results demonstrate that sex steroids act directly on parasite reproduction by binding to a classic and specific sex steroid receptor on the parasite. The differential response of cysticerci to sex steroids may also be involved in their ability to grow faster in the murine female or feminized male host. This is the first report of direct sex steroid effects on the parasite possibly through sex steroid receptors in the cysticerci.

Host and parasite sex-associated biases may be combined to favor their evolution toward a mutually acceptable relationship.

In experimental *Taenia crassiceps* cysticercosis, female hosts of various inbred mouse strains bear larger parasite loads than males in the first 4 wk of infection (Sciutto et al., 1991). Later on during infection, however, the sex-associated differences tend to disappear as male parasite loads approach those of females. Chronically infected males become feminized and develop high serum estradiol (E_2) and low testosterone (T_4) levels (Larralde et al., 1995; Morales et al., 1996; Morales-Montor, Hallal-Calleros et al., 2002) with a negative impact on their sexual and aggressive behavior (Morales et al., 1996; Gourbal et al., 2001). These results strongly suggest that estrogens promote and androgens inhibit parasite reproduction and are well in line with gonadectomy and hormonal reconstitution experiments (Huerta et al., 1992; Larralde et al., 1995; Morales-Montor et al., 1999; Morales-Montor, Baig et al., 2001). Because preliminary experiments failed to detect a direct response of the parasite when exposed in vitro to sex steroids (C. Larralde, pers. comm.), it was assumed that sex steroids exert their actions on parasite reproduction by way of the host's immune system, perhaps by inducing a T-helper type 1/T-helper type 2 (TH1/TH2) imbalance in favor of TH2, in the case of estrogens, and of TH1 in the case of androgens (Terrazas et al., 1994; Bojalil et al., 1993; Morales-Montor, Baig et al., 2002). Notwithstanding the intervention of the host's immune response in dealing with the parasite, the possibility of additional direct effects of sexual steroid hormones on the parasite's physiology should not be hastily discarded. Direct effects of sex steroids have been invoked in explaining the relation between androgen treatment and increased numbers of larval and adult stages of intestinal helminths or of various other parasites in other organs of vertebrate hosts, i.e., *Ancyclostoma caninum* grows better and increases egg production when the host is injected with T_4 (Bhai and Pandey, 1982); T_4 increases viability of *Nematospiroides dubius* larvae in the gut of the rat (Dobson, 1961), and it also does so with *Nippostrongilus brasiliensis* located in the hamster gut (Solomon, 1969). *Leishmania major* systemic infections in mice are strongly affected by T_4 (Mock and Nacy, 1998) as well as by the accelerated larval development of intestinal cestodes such as *Echinoccocus granulosus* (Frayha et al., 1971) and *Mesocestoides corti* (Novak, 1975). However, none of the above observations indicate which are the targets of the sex steroids or if they emanate from hosts or parasites or both. Certainly, the complex interactions existing between the endocrine and the immune systems of mammals (Besedovsky and Del Rey, 2002; Morales-Montor, Chavarria et al., 2004) provide examples of opportunities for sex steroids to exert their parasite-promoting or -restricting actions through the physiological systems of the host. Indeed, early experiments with *T. crassiceps* showed that sex-associated differences in parasite loads tend to disappear after thymectomy (Bojalil et al., 1993; Terrazas et al., 1994). However, the direct effects of sex steroids and other hormones in a variety of other parasites (Maswoswe et al., 1985; Lingnau et al., 1993; Freilich et al., 2000; Morales-Montor, Mohamed et al., 2001) indicate caution in excluding helminths from the general rule of sex steroid capacity to act directly on them. Only a consistent failure of sensitive technology to show the effects of sex steroids on cysticerci outside the host, in vitro, could weaken the possibility of direct action of the hormones on the parasite.

Thus, the present in vitro experiments were designed to thoroughly explore the possibility that sexual steroid hormones have a direct effect on *T. crassiceps* reproduction, viability, and infectivity and to look for preliminary evidence of the possible molecular mechanisms involved.

MATERIALS AND METHODS

Harvesting and preparing cysticerci for experimentation

A new stock of *T. crassiceps* cysticerci (ORF-Kuhn2 strain) (Culbreth et al., 1972; Freeman, 1969) was maintained in our laboratory by serial intraperitoneal passage in BALB/c AnN female mice approximately every 4 mo. Cysticerci for each experimental session were obtained from intraperitoneally infected female mice and placed in tubes containing sterile phosphate-buffered saline (PBS) (1×) supplemented with 100 U/ml of antibiotics-fungizone (Gibco, Grand Island, New York) (Esch and Smyth, 1976). The tubes were centrifuged for 10 min at 1,500 rpm at 4 C and the supernatant was discarded. The packed cysticerci were incubated in AIM-V serum-free medium (Gibco BRL, Rockville, Mary-

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TABLE I. Sequences of the primers used for PCR amplification of total *Taenia crassiceps* RNA reverse transcribed.*

Gene and product size	Sense primer	Antisense primer
c-fos (271 bp)	5′-CCCCTGTCAACACACAGGAC	5′-CCGATGCTCTGCGCTCTGC
c-jun (324 bp)	5′-CAGATCCCGGTGCAGCACCCG	5′-GGTGGCACCCACTGTTAACGTGG
ER-α (239 bp)	5′-AGACTGTCCAGCAGTAACGAG	5′-TCGTAACACTTGCGCAGCCG
ER-β (237 bp)	5′-CATCTGGGTATCATTACGGTC	5′-GGCACTTCTCTGTCTTCGTAC
PR-A (206 bp)	5′-CAGTGGTGGATTTCATCCATG	5′-CTTCCAGAGGGTAGGTGCAG
PR-B (210 bp)	5′-GGAGGCAGAAATTCCAGACC	5′-GACAACAACCCTTTGGTAGC
AR-CO_2H (309 bp)	5′-GAATGTCAGCCTATCTTTCTTA	5′-TGCCTCATCCTCACACACTGGC
AR-NH_2 (301 bp)	5′-GACCTTGGATGGAGAACTACTCCG	5′-GGTTGGTTGTTGTCATGTCCGGC
β-actin (220 bp)	5′-GGGTCAGAAGGATTCCTATG	5′-GGTCTCAAACATGATCTGGG

* Primers were designed based on the most conserved regions of all species sequenced genes or based using the sequence of cloned *T. crassiceps* genes (*c-fos* and *c-jun*). Each PCR product was purified and sequenced as described in Materials and Methods.

land). They were then centrifuged 3 times for 10 min at 1,500 rpm for washing. After the final wash, the numbers of viable (complete, translucent, and motile cystic structures) cysticerci were counted using a binocular microscope. Ten viable nonbudding cysticerci approximately 2 mm in diameter were then selected and dispensed into each well of 24-well culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, New Jersey) in 1-ml AIM-V medium and incubated at 37 C under 5% CO_2. A sufficient number of culture wells were prepared in each experiment to accommodate the complete experimental design for evaluation of in vitro treatment effects of sex steroids on the cysticerci. Cultures were examined daily, and their medium was completely replaced when it turned yellowish (pH 6.5).

Hormone dose–response–time curves

Culture grade E_2, progesterone (P_4), T_4, and dihydrotestosterone (DHT) were obtained from Sigma (St. Louis, Missouri). For in vitro tests, water-soluble E_2, and P_4, were dissolved in AIM-V serum-free culture medium, whereas T_4, and DHT were dissolved in pure ethanol (Sigma) to the desired stock concentration and sterilized by passage through a 0.2-mm Millipore filter. The experiments used the parasite-loaded wells: 4 wells were used as untreated controls, 4 wells were supplemented with the solvent in which hormones were diluted, and 4 wells were treated with different concentrations of E_2, P_4, T_4, and DHT in their respective solvents. Each hormone was prepared in a final volume of 100 μl and added to 1 ml of medium in each well. Final concentrations of ethanol in each well for T_4 and DHT were 0.6 and 0.4%, respectively. The concentrations of hormones used in this study were based on our previous studies of serum levels found in feminized mice levels (Larralde et al., 1995; Morales et al., 1996; Morales-Montor et al., 1999; Morales-Montor, Hallal-Calleros et al., 2001) and were chosen to approximate those levels in our in vitro experimental system. In hormone dose–response–time curves, only the number of buds per cysticercus as a function of days in culture was assessed as the response variable. From the dose response time curves of each hormone, an optimal dose was selected to be used in further experimentation: the dose of each hormone at the shortest time in which the differences with the respective control values were maximal.

Effects of optimal doses of sex steroids on the viability, proliferation indexes, and RNA expression of cultured cysticerci

Visual assessment of reproduction and viability: Parasite reproduction was assessed as the total number of buds that were attached to the 10 cysticerci in each well. Bud count was performed daily under an inverted light microscope. Cysticercus viability was determined daily for the various wells using an inverted microscope (Olympus, MO21, Tokyo, Japan) at ×10 and ×100 magnification. Injury to cysticerci was recognized microscopically by progressive internal disorganization, by development of whitish opaque areas on the parasite's tegument, and by loss of motility. Dead cysticerci were immobile, opaque, and disorganized structures. Photomicrographs of representative treated cysticerci were obtained.

DNA synthesis: Cultured cysticerci were homogenized and digested with proteinase K (100 U/ml) for 18–20 hr at 37 C with continuous shaking. After the protein fraction was extracted with phenol–chloroform, DNA was precipitated with cold ethanol and assessed for high molecular weight integrity by electrophoresis. DNA purity and quantity were evaluated by the ratio of optical density (OD) readings at 260/280 nm.

Proliferation assays: Cultured cysticerci were pulsed with 10 μCi of ^{3}H-thymidine (methyl-^{3}H TDR, spact. 247.9 GBq/mmol, NEN, Boston, Massachusetts), and the parasites were incubated for another 56 hr. Then, the DNA from pulsed cysticerci was purified as described and harvested in an automatic cell harvester (Skatron Instruments, Mod 11028, Oslo, Norway) onto glass paper filters and processed. The filters were then counted on a Betaplate system (Wallac Mod 1205, Turku, Finland).

Total RNA extraction: Total RNA was isolated from *T. crassiceps* hormone treated cysticerci by the method based on guanidine isothiocyanate phenol–chloroform extraction using Trizol reagent (Invitrogen, Carlsbad, California). In brief, cysticerci were disrupted in Trizol reagent (1 ml/0.1 g tissue), and 0.2 ml of chloroform was added per ml of Trizol. The aqueous phase was recovered after 10 min centrifugation at 14,000 *g*. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and redissolved in RNAse-free water. The RNA concentration was determined by absorbance at 260 nm, and its purity was verified after electrophoresis on 1.0% denaturing agarose gel in the presence of 2.2 M formaldehyde.

AP-1 complex, estrogen receptors α and β (ER-α and ER-β), P_4 receptors A and B (PR-A and PR-B), and androgen receptor (AR) gene amplification

Total RNA from hormone-treated cysticerci was reverse transcribed followed by specific polymerase chain reaction (PCR) amplification of *c-fos, c-jun,* ER-α, ER-β, PR-A, PR-B, AR, together with β-actin (control gene) as previously described (Morales-Montor, Rodrigues-Dorantes et al., 2004). Primers were designed based on the most conserved regions of all species of sequenced genes reported in the NIH Gene Data Bank (ER-α and ER-β, PR-A and PR-B, and AR) or using the sequence of cloned *T. crassiceps* genes (*c-fos* and *c-jun*). The sequences of the used primers are given in Table I. The PCR products obtained were observed by staining with ethidium bromide. In all cases, a single band was detected corresponding to the expected molecular weight of the gene. The expression of *c-fos* and *c-jun* are numerically presented as the ratio of the OD of each studied gene relative to the expression in the same preparation of the β-actin gene, a constitutively expressed gene used as an internal control for differences in the amplification procedure between experiments and in the staining of the different gels.

Infectivity assay of pretreated cysticerci

Six-week-old BALB/c AnN mice of both sexes were used in this study as recipients of hormone-pretreated cysticerci. The mice were fed with Purina Diet 5015 and water ad lib. Larvae for intraperitoneal experimental infection were the 10, in vitro–cultured cysticerci after their pretreatment for 5 days with the optimal dose of each of the sex steroids

(E_2, P_4, T_4, or DHT). Each recipient mouse was injected intraperitoneally with the pretreated cysticerci using a 0.8 gauge needle. Recipient mice were killed by cervical dislocation while under pentobarbital anesthesia after 4 wk of infection. The parasite load was estimated by inverted microscope-aided visual counting of the number of free-floating, cysticerci larger than 2 mm, collected from the infected mice after thorough peritoneal washes with PBS.

Experimental design and statistical analysis

Hormone dose–response–time curves were estimated in 10 independent experiments, each performed with cysticerci freshly extracted from different infected donor mice. The response variable used in statistical analyses was the sum of buds in the 6 wells at each hormone dose and time of exposure in each experiment. The hormone used (4 concentrations), the dose of hormone used (5 levels), the time of exposure (10 levels) and experiment (10 levels) were the independent variables. All experiments to evaluate the effect of the optimal dose of hormone on the parasite proliferation indexes (sum of buds, DNA quantity, ^{3}H-thymidine, RNA expression) were performed 5 times. In each experiment, each of the 4 treatments was replicated in 4 wells, with each well containing 10 cysticerci. The data for the 4 replications of each treatment were pooled and expressed as an average. Data were analyzed using analysis of variance (ANOVA) with hormone treatment (5 levels) and the number of experiment (5 levels) as independent variables and the dependent variables were the 4-wells average of the sum of buds, DNA quantity, and ^{3}H-thymidine cpm (for proliferation assays); the average of *c-fos*/β-actin OD ratio and *c-jun*/β-actin OD ratio for RNA expression; and the total number of cysticerci developed in recipient mice of pretreated cysticerci for the infectivity assay. If ANOVA revealed significant differences between treatments, the Student's *t*-test of differences between group means was applied to each experiment using the residual variance estimated by ANOVA to test for significance. Differences were considered significant for $P < 0.05$.

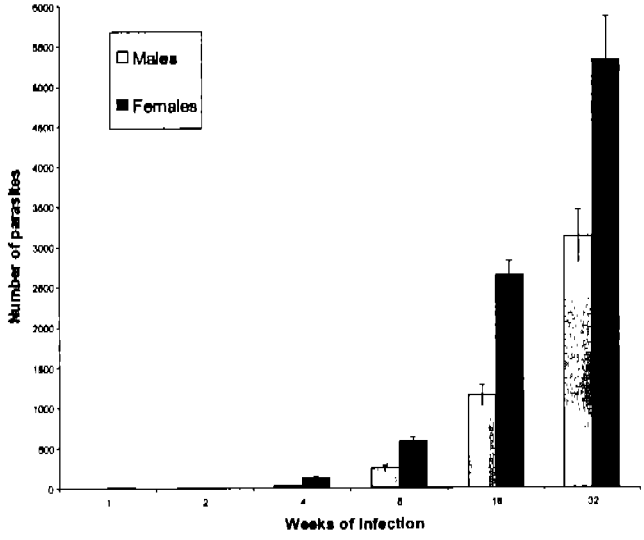

FIGURE 1. Time course infection in male and female mice. Ten cysts were injected into the peritoneal cavity of female and male mice. Initially, *Taenia crassiceps* cysticerci grow faster in females than in males (acute infection), but in chronic infections (more than 4 wk), males are also burdened by huge parasite loads. Data represent mean ± SE of individual parasite loads of a total of 130 females and 125 males, recorded in a retrospective manner in our database, for a number of experiments performed at different times.

RESULTS

Sex-associated susceptibility during *Taenia crassiceps* cysticercosis

A few days after infection, cysticerci begin active asexual reproduction by budding at 1 of the poles. A few months later they number hundreds to thousands, reaching biomasses that may equal that of the host. *Taenia crassiceps* initially grows faster in female than in male mice, but in late infections the numbers in males also may reach thousands (Fig. 1). However, they never become as heavily infected as the female mice. It is important to note that the health of the massively infected mice of both genders is not compromised under laboratory conditions because there is no macroscopic or microscopic sign of serious illness or malnutrition.

Proliferation and viability assays

Time–response curves: When cysticerci were exposed in vitro to E_2 or P_4, it was apparent that both hormones markedly increased parasite bud production (Fig. 2A). In the E_2-treated group the number of buds in relation to control values increased progressively, reaching 4 times their initial number after 5 days in culture, whereas the effect of P_4 was not as powerful (only a 65% increase in the number of buds). After 5 days in culture in the presence of E_2 and P_4, bud production reached a plateau. E_2 and P_4 treatment did not modify the viability of cysticerci during the 10 days of study. When cysticerci were exposed in vitro to T_4 or DHT, the number of buds produced was the same as in the control-cultured cysticerci (Fig. 2B), but the T_4-treated group showed a 10% loss in viability after 4 days, which increased to about 15% after 10 days. DHT had the most profound effect on cysticercus viability, many were found structurally disorganized, scarcely motile, and 20% were dead after 10 days in culture.

Dose–response curves: Different concentrations of hormones in the culture media were studied to establish a dose-dependent response pattern of *T. crassiceps*. The numbers of buds were clearly augmented as the E_2 dose was increased. The 10-μg dose doubled the number of buds (from 6 to 12), reaching the maximum response at 40 μg. As the P_4 dose increased, the effect on bud production was apparent until 20 μg (a 2-fold increment) (Fig. 3A), returning to control levels at higher P_4 doses. E_2 and P_4 did not affect viability at any dose tested, even at concentrations 1.5–4 times higher than those initially used. A dose–response pattern was also revealed when androgens were tested but was opposite to that of estrogens (Fig. 3B). T_4 at a dose of 6 μg apparently reduced the number of buds to half (5 in controls and 2 in T_4-treated cysticerci). DHT showed a reduction in the number of buds to half when the dose of 4 μg was used, returning to control levels at higher doses (6 μg/ml).

Morphological studies: The E_2- and P_4-treated (Fig. 4B and 4C, respectively) cysticerci were transparent, mobile, and surrounded by an increased number of buds, also motile and rather large (the average bud diameter at this time was 1,524 ± 94.9 μm) (Fig. 4B,C); an occasional multilobed giant cysticercus was observed. In contrast, T_4- (Fig. 4D) and DHT-treated cysticerci (Fig. 4E) were significantly smaller (218 ± 7.4 μm) ($P < 0.05$) than E_2 or P_4 or control cysticerci (1,524 ± 94.9 μm) (Fig. 4A), suffering progressive internal disorganization and development of opaque whitish masses in the tegument as well as progressive loss of motility. The optimal hormone doses for

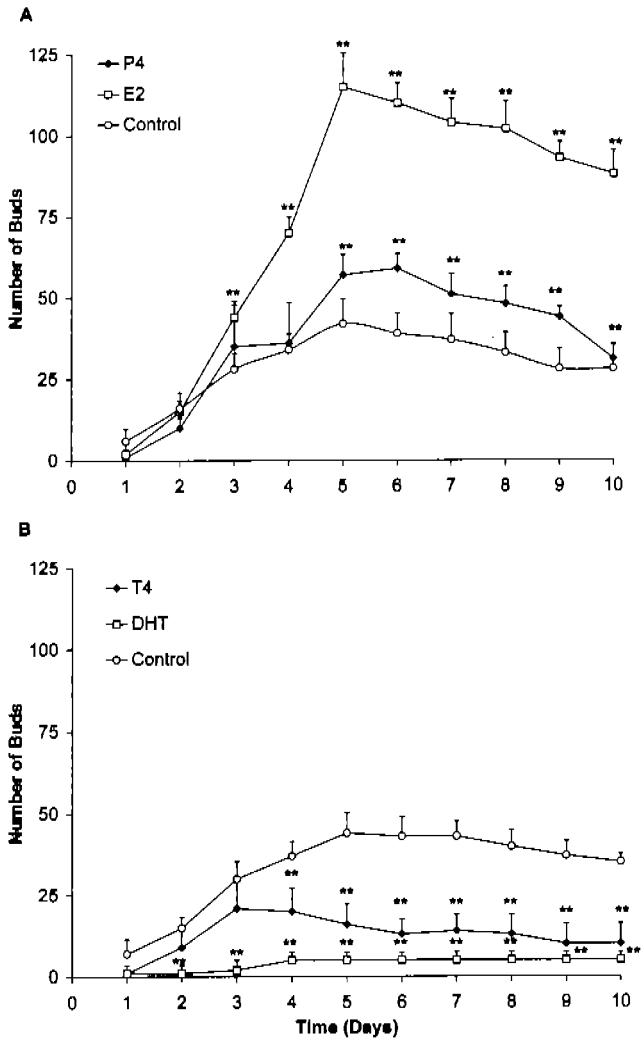

FIGURE 2. Time curves of *Taenia crassiceps* cysticerci reproduction after exposure to estradiol and progesterone (**A**) or testosterone or DHT (**B**). Each point represents the mean (SD) of 5 assays counting the number of buds in each parasite contained in each cultured well. Data were pooled, and the mean was obtained. $*P < 0.05$, $**P < 0.01$ with respect to control and vehicle-treated cysticerci. The concentration of hormones are as follows: E_2, 40 μg/ml, P_4, 20 μg/ml, T_4, 4μg/ml, and DHT, 6 μg/ml.

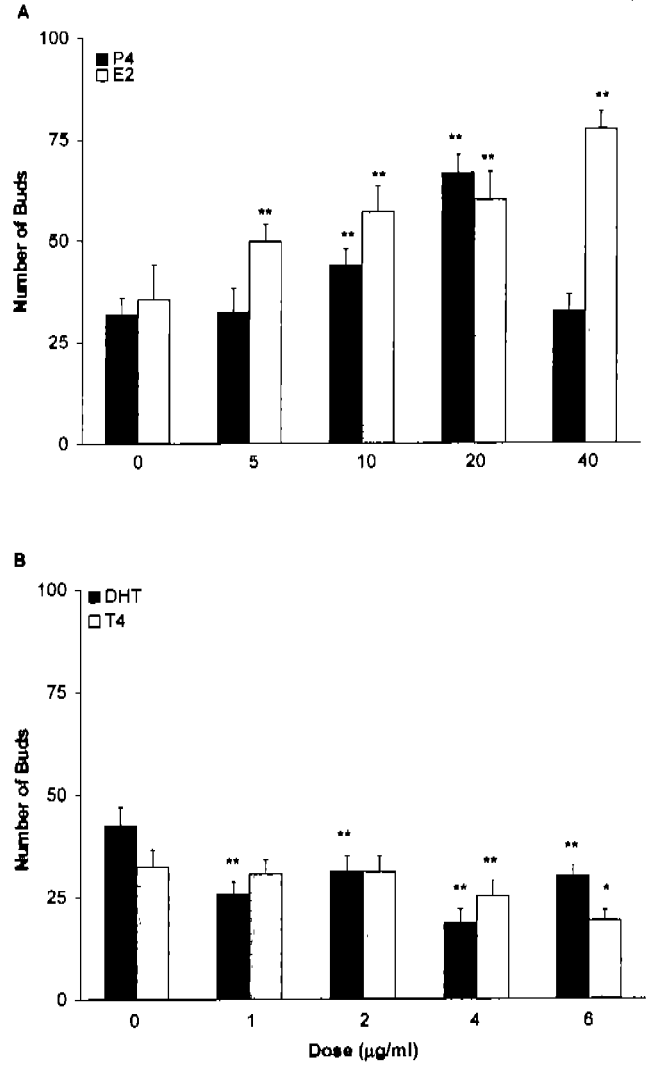

FIGURE 3. Dose-dependent effect of sex steroid hormones on in vitro reproduction of *Taenia crassiceps*. Ten cysticerci were incubated for 5 days under different concentrations of E_2 and P_4 (**A**) or T_4 and DHT (**B**). The bars represent the average of determinations (SD) of the number of buds. $*P < 0.05$, $**P < 0.01$ with respect to control and vehicle groups. Each point represents the mean (SD) of quintuplicate determinations of the number of buds counted and viability of parasites.

further experimentation were 40 μg/ml for E_2, 20 μg/ml for P_4, 6 μg/ml for T_4, and 4 μg/ml for DHT.

Expression of c-fos and c-jun genes in response to hormonal treatment: In view of the known effects of sex steroids on the expression of several gene families, including the AP-1 complex genes (*c-fos* and *c-jun*), which are involved in the control of cell differentiation, reproduction, and apoptosis, these 2 genes were prime candidates for exploration (Hyder et al., 1992, 1995; Jochum et al., 2001). The expression of β-actin as an internal control was also measured to control for differences in amplification procedures and gel staining in each experiment. As shown in Figure 5A, the expression of *c-fos* was markedly enhanced in the E_2- and P_4-treated cysticerci, whereas the T_4- and DHT-treated cysticerci inhibited the expression of this gene (Fig. 5B). All these differences were statistically significant ($P < 0.01$). A similar pattern was also seen for *c-jun* messenger RNA (mRNA) expression, which was clearly augmented in E_2- and P_4-treated cysticerci, but DHT treatment had no effect and T_4 treatment slightly inhibited its expression (Fig. 5B). All these values were significant at $P < 0.01$ in comparison with the expression of both genes in control cysticerci.

DNA quantity and ^{3}H-thymidine incorporation: Figure 6A shows the E_2, P_4, T_4, and DHT stimulation effects on ^{3}H-thymidine uptake in its DNA fraction with respect to controls. E_2 and P_4 treatment augmented ^{3}H-thymidine uptake 5-fold and 3-fold, respectively, compared with the untreated cysts. T_4 and DHT treatment impaired ^{3}H-thymidine uptake by the cysts, with similar incorporation levels to control cysticerci. The same pat-

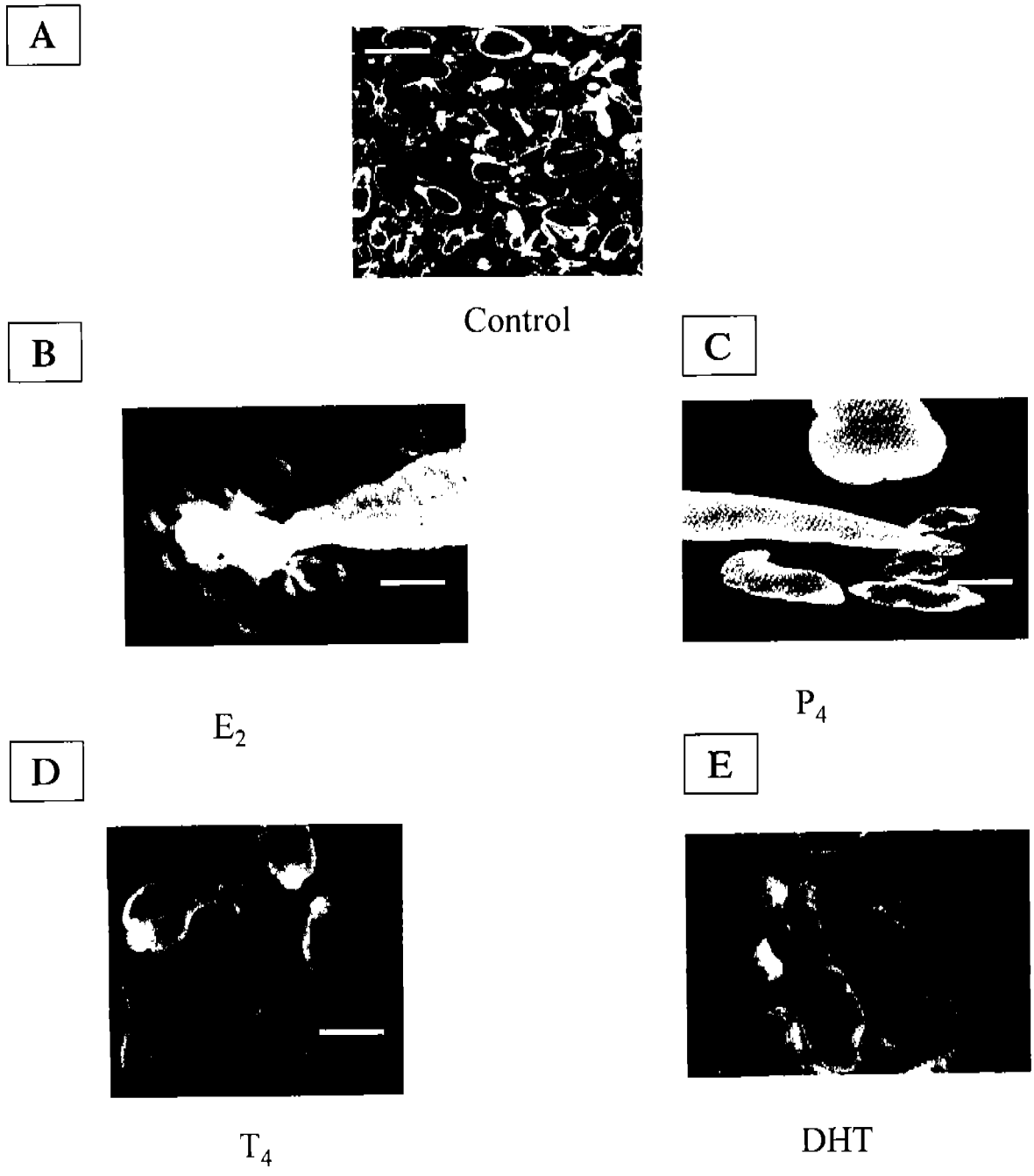

FIGURE 4. Micrographs of *Taenia crassiceps* cysticerci treated in vitro with sexual steroid hormones. The parasites were selected to fairly represent the mean effect on the number of buds for each treatment. White bars in each photograph, 1 mm. (**A**) *T. crassiceps* cysticerci control, (**B**) *T. crassiceps* cysticerci treated with estradiol (40 μg/ml), (**C**) *T. crassiceps* cysticerci treated with progesterone (20 μg/ml), (**D**) *T. crassiceps* cysticerci treated with testosterone (4 μg/ml), and (**E**) *T. crassiceps* cysticerci treated with DHT (6 μg/ml).

tern was observed when DNA was purified and quantified (Fig. 6B). E_2 and P_4 treatment had a stimulatory effect on DNA synthesis when compared with untreated control cysticerci. Thus, E_2 treatment doubled the amount of DNA synthesized, whereas P_4 treatment increased the same 1.5-fold. No effect on DNA synthesis was seen when cysticerci were treated with either T_4 or DHT.

Infectivity assays: Infectivity studies of cysticerci exposed to sex steroids before their inoculation to recipient mice indicated that estrogens promoted and androgens inhibited their infectivity. Figure 7 shows the parasite burdens obtained at 4 wk of infection with cysticerci previously exposed to optimal doses of E_2, P_4, T_4, and DHT 5 days before being inoculated into male and female mice. In male recipients, E_2-treated parasites tripled

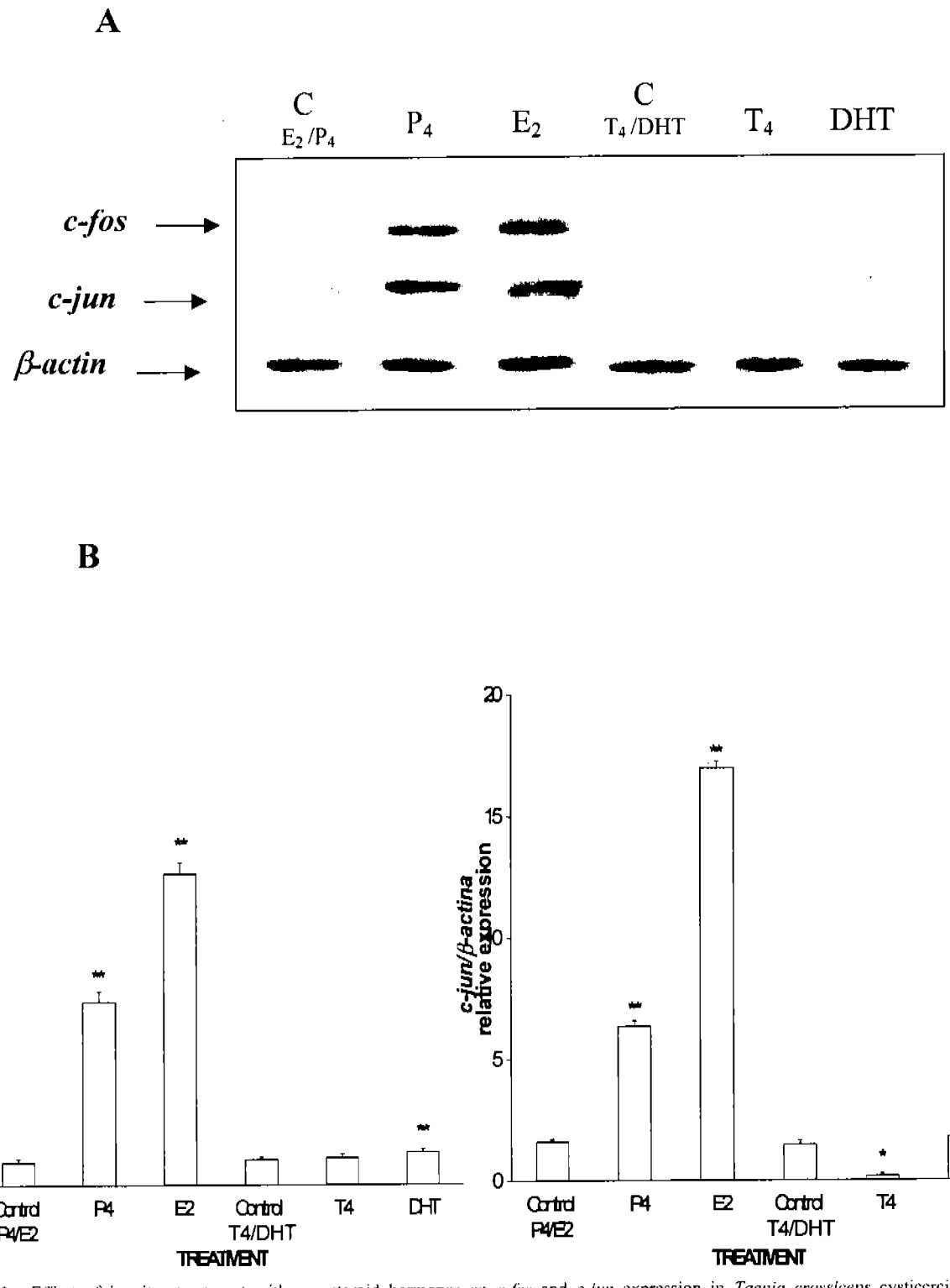

FIGURE 5. Effect of in vitro treatment with sex steroid hormones on *c-fos* and *c-jun* expression in *Taenia crassiceps* cysticerci. (**A**) A representative RT-PCR with total RNA from normal or treated cultured *T. crassiceps* cysticerci showing the detection of *c-fos* and β-actin (used as a control expression gene). (**B**) Results of *c-fos* and *c-jun* expression are reported as densitometric data of the autoradiographic signals. Data represent individual wells by treatment, and each experiment was done in quintuplicate. The relative expression was obtained by correcting the expression of *c-fos* or *c-jun* to that of β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to their respective control group.

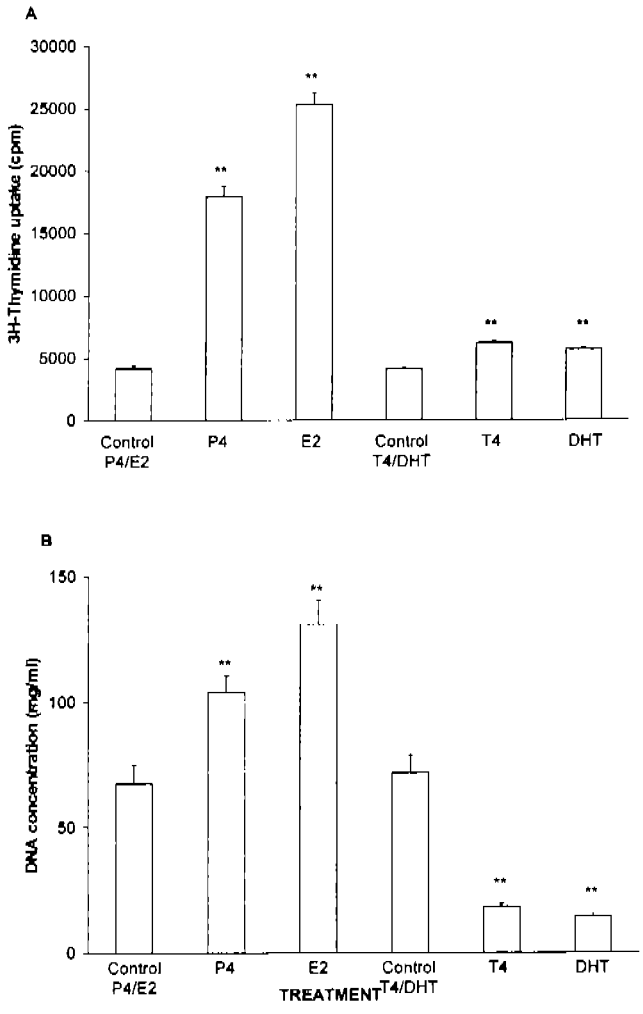

FIGURE 6. Specific proliferation of *Taenia crassiceps* cysticerci measured as ^{3}H-thymidine incorporation (**A**) and total DNA quantities (**B**). Data are represented as the mean SD of 3 different experiments (n = 5). Each cyst culture was done in triplicate. Cysticerci were cultured separately in the presence of each sex steroid and proliferation was measured. DNA was extracted, purified, and quantified from the cysticerci obtained after different treatments. $^*P < 0.05$, $^{**}P < 0.01$ as compared with their respective control group.

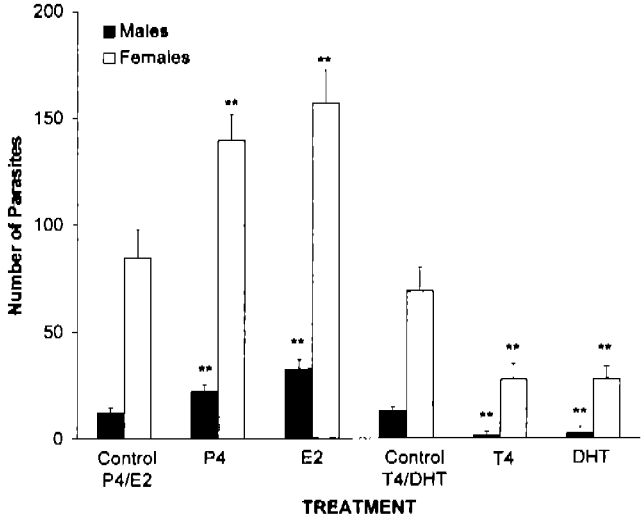

FIGURE 7. Number of *Taenia crassiceps* cysticerci obtained from the peritoneal cavity of BALB/c AnN mice of both sexes with different experimental treatments. Cysticerci were cultured first with a single dose of estradiol, progesterone, testosterone, or DHT, and then reinoculated (10 parasites per mouse) into female and male mice. Bars represent the mean SD of individual parasite loads of a total of 2 experiments (n = 5). $^*P < 0.05$, $^{**}P < 0.01$ as compared with control mice.

in number as compared with controls, and in female recipients they were doubled. P_4 pretreatment also doubled the expected parasite load in both genders. In contrast, prior exposure of parasites to T_4 and DHT significantly decreased the expected parasite load in male and female hosts.

Sex steroid receptors detection in Taenia crassiceps: Because different mRNAs can have varying half-lives and may undergo selective degradation, we determined the quantity and integrity of total RNA extracted from the different sources used in this study. It was clear that the same amount of RNA (1 μg) that was not degraded was used for reverse transcription (RT)-PCR amplification in each studied tissue (data not shown). The amplification by RT-PCR of the ER-α and ER-β, AR, PR-A, and PR-B and β-actin genes for *T. crassiceps* is shown in Figure 8. Specific fragments that correspond in molecular weight to those of ER-α, ER-β, and AR were obtained from both *T. crassiceps* and control tissues, whereas PR-A and PR-B fragments were only detected in the mouse uterus (control tissue) and not in *T. crassiceps*. Sequencing of the *T. crassiceps*–amplified fragments, as well as of control tissues, demonstrated their identity to ER-α and ER-β, AR, and β-actin (data not shown).

DISCUSSION

This work shows that sex steroids act directly on *T. crassiceps* cysticerci proliferation and viability without need of the host's participation, and E_2 and P_4 promote parasite reproduction without affecting their viability. In contrast, T_4 and DHT significantly inhibit parasite proliferation and may lead to their destruction. These effects depend both on the concentration of hormones and the duration of exposure. The effects on reproduction and viability began at 24 hr of culture but were maximally different between experimental and control groups at 5 days of culture and later. DHT was more drastic in its deleterious effects on cysticerci than T_4 ($P < 0.05$) and E_2 more stimulatory than P_4 ($P < 0.05$). The involvement of the AP-1 complex genes (*c-fos* and *c-jun*) of the parasite was shown because the treatment with the 4 sex steroids had an impact on their expression in a way congruent with the proliferation and viability changes. The effects of E_2 and P_4 stimulated the expression of both *c-fos* and *c-jun* and those of T_4 or DHT inhibited their expression.

Previously, we had found that gender and circulating E_2 and T_4 levels in host mice crucially affected the dynamics of parasite loads in mice infected with cysticerci of *T. crassiceps* (Morales-Montor, Baig et al., 2002). The very fact that infection of male mice with *T. crassiceps* leads to striking increases in estrogen levels of the host is consistent with the idea that cysticerci fare better in high estrogen conditions and somehow in-

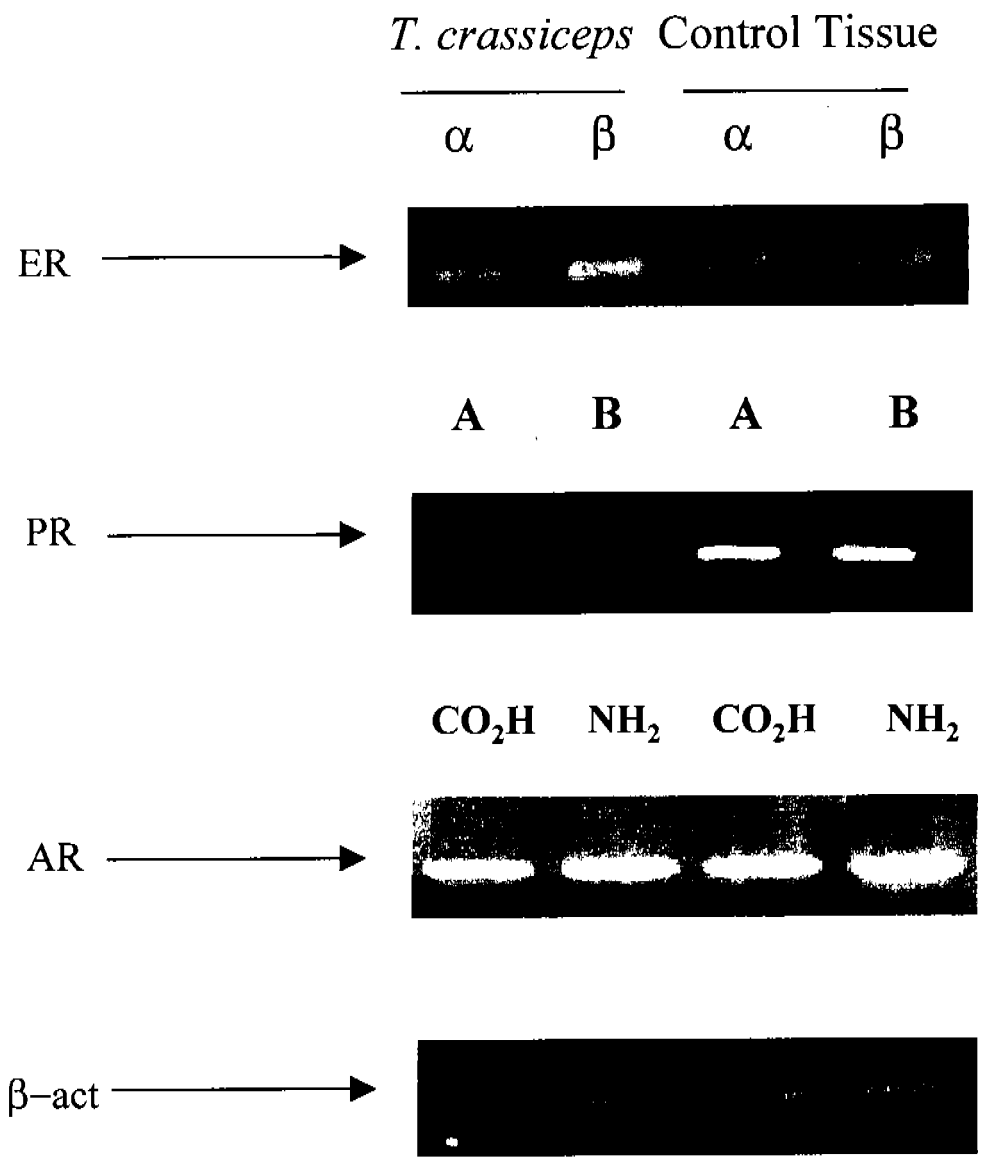

FIGURE 8. Expression of sex steroid receptors in *Taenia crassiceps* cysticerci. In the vertical axis, we show the expression of the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), and β-actin in *T. crassiceps* and control mouse tissue. In the horizontal axis, we show the distinct isoforms of the receptors. The positive expression control tissue for the ER was mouse ovary, for PR it was mouse uterus, and for AR we used mouse testes. For amplification of AR, we used 2 different sets of primers defined in the figure like the –COOH (carboxilic) and $-NH_2$ (amino) terminal domain of the gene. β-actin was the expressed constitutive gene.

duce the host to produce them. However, a more intricate strategy of parasite activity must now be considered. Perhaps low androgen levels are also necessary for the parasite because they are so stunted by them. The basis for the parasite's preference or avoidance of sex steroids is more understandable if overexpression of AP-1 genes favors asexual reproduction, whereas underexpression does not. An inadequate hormonal environment may lead to apoptosis of crucial parasite cells, as has been proposed for other parasite infections, i.e., retinoic acid has been shown to affect female *Litomosoides carinii* and microfilariae of *L. carinii*, *Brugia malayi*, *Brugia pahangi*, and *Acanthocheilonema viteae* (Zahner et al., 1988). Cercariae, schistosomula, and adult worms of *Schistosoma mansoni*, show reduced viability and significantly inhibit in vitro schistosoma oviposition (Morales-Montor, Mohamed et al., 2001) and T_4 does likewise with the mitochondrial function of *S. mansoni* (Fantappie et al., 1999).

Because there is a great deal of conserved sequence homology among most hormone receptors, especially in the ligand and DNA-binding domains (Damian, 1997), we were able to show that cysticerci expressed an androgen receptor, with homology to the androgen receptor that binds both T_4 and DHT.

Such commonalities between host and cysticercus metabolism should come as no surprise, when extensive homologies between species are being documented in other systems as well. The same argument applies for the direct action of estrogens, although their effects are opposite to those of androgens. Interestingly, we showed that both isoforms of the classic estrogen receptor (ER-α and ER-β) are expressed in *T. crassiceps*, but there is no expression of P_4 receptors (neither isoform A nor B). It appears that the effects of estrogens and androgens are caused by the binding of E_2 and T_4 to their respective receptors. The small effects of P_4 could result from nonexpression of its specific receptor or be caused by nongenomic effects or merely reflect its transformation to E_2 as previously shown for androgens (Gomez et al., 2000). Binding of the ER to the classic estrogen-dependent elements could be responsible for the activation of AP-1 complex genes in the normal metabolism of *T. crassiceps*. Previous studies have demonstrated that the genome of *Onchocerca volvulus* encodes at least 3 members of the nuclear receptor family (Unnasch et al., 1999), and this could also be the case for *T. crassiceps*.

With respect to the significance of the differential effects of sex hormones on host and parasite, we found it interesting to speculate on the evolutionary impact of the host gender differential specialization in dealing with the parasite's different stages. Thus, it would appear that gender specialization of the kind described here for *T. crassiceps* (females favoring and males hindering larval asexual reproduction) could be evolving to a more benign host–parasite relationship.

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Parasite regulation by host hormones: an old mechanism of host exploitation?

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Recent experimental evidence suggests that parasites can not only evade immune responses actively but also exploit the hormonal microenvironment within the host to favor their establishment, growth and reproduction. The benefit for parasites of hormonal exploitation is so great that they have evolved structures similar to the steroid and protein hormone receptors expressed in upper vertebrates that can bind to the hormonal metabolites synthesized by the host. This strategy is exemplified by two parasites that respond to adrenal steroids and sexual steroids, respectively: *Schistosoma mansoni* and *Taenia crassiceps*. Understanding how the host endocrine system can, under certain circumstances, favor the establishment of a parasite, and characterizing the parasite hormone receptors that are involved might aid the design of hormonal analogs and drugs that affect the parasite exclusively.

Hormones and parasites

Hormones regulate a variety of cellular and physiological functions of organisms, such as growth, reproduction and differentiation [1,2]. Recently, the ability of hormones to affect the immunological response directed against pathogenic agents has gained attention [3–6]. This is evident during various parasitic diseases such as malaria, schistosomiasis, toxoplasmosis, cysticercosis, trypanosomiasis and leishmaniasis [7–11] in which strong hormonal regulation of the immune response has been described [12–17]. In *Taenia crassiceps* infection, the interaction of the immune and endocrine systems is dynamic and bidirectional (Box 1). However, factors other than the immunoendocrine system affect the course of a parasitic infection.

Parasites have developed diverse mechanisms of survival within the host that facilitate the establishment of infection. These mechanisms can be grouped into two types. The first is to evade the immune response by using strategies such as antigenic variation, molecular mimicry or affecting antigen processing and presentation [18–20]. For example, pathogens such as *Chlamydia trachomatis* and *Coxiella burnetii* have developed molecules that interfere directly with antigen processing and presentation [20]. In the second mechanism, the parasite exploits a host system to its benefit and, thus, obtains an advantage such as establishment, growth or reproduction [21]. For example, *Naegleria fowleri* can internalize antigen–antibody complexes from its surface. This mechanism provides the parasite with a dual benefit: first, obtaining amino acids for metabolism, and second preventing the surface-bound antibody from interfering with parasite–host-cell interactions [22]. A striking example of the exploitation of host molecules is the ability of several parasites to use host-synthesized cytokines as indirect growth factors for themselves [20,21].

Recent experimental evidence [23–32] has led us to propose a mechanism of host exploitation by parasites. In this system of 'transregulation', the parasite benefits directly from hormones or growth factors that are derived from the host to enable rapid establishment, and increased growth and reproduction rates. Transregulation has been described in at least eight parasitic infections that are caused by both protozoan and metazoans (Table 1).

Hormonal transregulation of parasite growth and reproduction

Adrenal hormones

It has been demonstrated that adrenal hormones exert a profound effect on several parasites. *In vitro* cortisol treatment of *Plasmodium falciparum* merozoites was found to increase the number and size of the gametocytes produced [31]. By contrast, when these parasites were treated with the dehydroepiandrosterone (DHEA) analog 16-α-bromoepiandrosterone, growth rates diminished by 25% [27].

Cortisol was found to stimulate *Entamoeba histolytica* proliferation in a dose-dependent manner. By contrast, exposure of trophozoites to DHEA inhibited proliferation, reduced adherence and motility, and caused lysis in a dose-dependent manner. Consistent with this, cortisol increased, whereas DHEA decreased, levels of synthesis of parasite DNA (as determined by ^{3}H-thymidine incorporation). Lysis of trophozoites by DHEA seems to be caused by a necrotic rather than apoptotic process, as determined through patterns of DNA fragmentation and enzymatic *in situ* labeling of apoptosis-induced DNA-strand breaks [detected by Tdt-mediated dUTP–biotin nick-end labeling (TUNEL) assays]. A possible mechanism of action of trophozoite lysis by DHEA was suggested from

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schistosomiasis and, in fact, is as effective as the most effective vaccine antigen candidates [36]. Also, cortisol and DHEA inhibit oviposition by *S. mansoni* both *in vitro* and *in vivo* [37]. *In vitro*, DHEA has strong cercaricidal and schistosomulicidal effects, in addition to decreasing adult worm survival rates by up to 100% [37]. It has also been demonstrated that adrenal function alters with age during primary and secondary *S. mansoni* infection in baboons [38,39] and mice [38], and this change correlates with the intensity and type of infection [38]. Moreover, adrenalectomized infected mice displayed increased mortality rates and numbers of adult worms, and twice as many ova per worm pair in their liver [40]. Thus, the evidence suggests that a lack of adrenal steroids, particularly DHEA, mediates an increment of the adult worm burden and promotes worm fecundity *in vivo* and *in vitro*.

Sex- and pregnancy-associated hormones

Treatment of adult *Schistosoma haematobium* with testosterone diminishes fertility and, thus, the reproductive capacity of this parasite [24]. Treatment of *T. crassiceps* cysticerci with 17-β-estradiol increases their reproductive capacity by increasing the number of buds, whereas treatment with testosterone or dihydrotestosterone (DHT) diminishes this function. In addition, viability, growth and infective capacity of cysticerci are increased to 200% after treatment with estrogen but are inhibited almost completely by androgen treatment [32]. In separate studies, treatment of *P. falciparum* merozoites with estradiol, progesterone or testosterone increased the number of gametocytes that were produced *in vitro* [29].

It has been shown that the *in vitro* exposure of *E. histolytica* trophozoites to several concentrations of sex steroids such as 17-β-estradiol, progesterone, testosterone and DHT has little effect on parasite viability or proliferation [33].

Other host-derived factors

Trypanosoma cruzi amastigotes that are treated *in vitro* with murine epidermal growth factor (EGF) increase their levels of DNA synthesis, growth and metabolic activity considerably [inducing receptors with tyrosine kinase, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) activity] [23]. Treatment of *P. falciparum* merozoites with insulin increases the number of gametocytes produced *in vitro*. Similarly, murine EGF stimulates the *in vitro* development and maturation of *Brugia malayi* microfilariae and other filarial parasites [26]. It has also been demonstrated that *Onchocerca volvulus* and *Onchocerca lienalis* microfilariae increase their metabolic activity when treated *in vitro* with 20-hydroxyecdisone [28]. Granulocyte–macrophage-colony-stimulating factor (GM-CSF) augments the *in vitro* growth of *Leishmania mexicana* promastigotes [30].

Transregulation of parasite signal-transduction pathways

Regulation of gene expression

The mechanisms by which host hormones act on parasites have recently been investigated, and some parasite molecules that are involved in transregulation have been identified and characterized [32,41]. These include receptors, transporters, steroidogenic pathway enzymes and second messengers that are synthesized by parasites and that enable the exploitation of host hormones. Several lines of investigation are in progress to determine whether

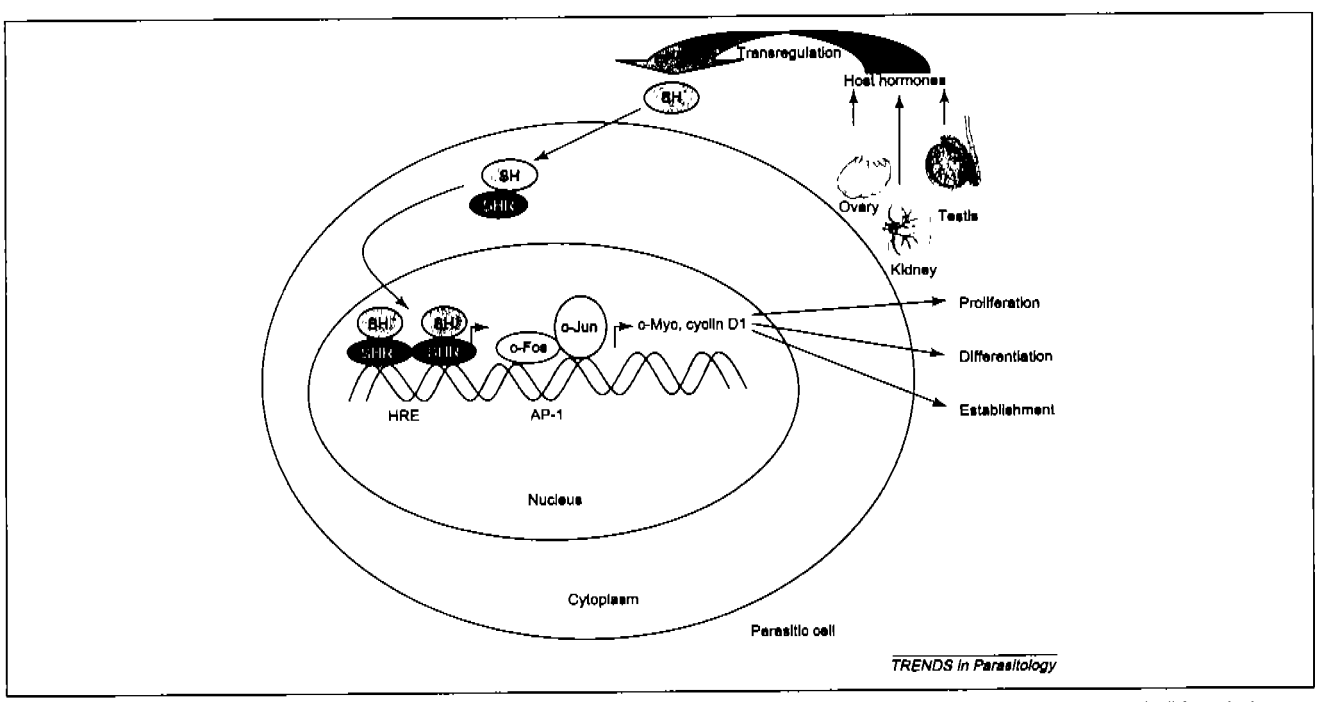

Figure 1. Host steroid hormones can affect several aspects of parasite physiology. Steroids (e.g. estradiol, testosterone, progesterone, DHEA and cortisol) from the host can bind to specific cytoplasmic receptors that are expressed by the parasite. The ligand–receptor complex can regulate, through genomic mechanisms, parasite growth, infectivity, differentiation and reproduction. The ability of the parasite to bind to host molecules and use them to its own benefit is mediated by a mechanism that we have denominated transregulation. Abbreviations: HRE, hormone response element; SH, steroid hormone; SHR, steroid hormone nuclear receptor.

classical nuclear receptors can be found in parasites and, if so, whether they can bind to host hormones to direct downstream transcriptional events (Figure 1).

Sani *et al.* [41] characterized 'specific retinol and retinoic acid-binding proteins' in *O. volvulus*, *Onchocerca gibsoni*, *Dipetalonema viteae*, *Brugia pahangi* and *Dirofilaria immitis*. The role ascribed to these proteins was that of binding to host hormones and mediating the biological effects of retinoids on parasites, such as growth, reproduction and differentiation. The genome of *O. volvulus* codes for at least three classical nuclear receptors [42]; two of these (OvNR-1 and OvNR-2) have been characterized and found to be similar to the retinoid receptors in vertebrates and to the *Drosophila melanogaster* protein EiP78c. Computer modeling suggests that these molecules possess a ligand-binding cavity that is of a size and form capable of binding to a steroid [42,43].

The presence of receptors in *S. mansoni* that can bind to 17-β-estradiol provides a likely mechanism for the protective effect of this hormone in infected mice and hamsters [44]. In the same parasite, classical nuclear receptors for steroids, thyroid hormones and ecdysteroids have been characterized [45]. Homology among these receptors and those described in *Drosophila*, mice and humans ranges from 70% to 95%, hence their enormous capacity to bind to host hormones and affect diverse developmental processes of *Schistosoma* spp. [45]. It has been shown that cysticerci of the helminth parasite *T. crassiceps* express an androgen-receptor-like mRNA and both isoforms of the classic estrogen receptor (α and β), but there is no expression of either isoform (A or B) of progesterone receptors [32]. It seems that the direct *in vitro* effects of estrogens and androgens on *T. crassiceps* reproduction are due to the binding of estradiol and testosterone to their respective receptors. The small effects of progesterone observed in the apparent absence of its specific receptor could be due to non-conventional nuclear receptors or could reflect the transformation of this hormone to estradiol, as shown for androgens [46]. Binding of the estrogen receptor to classic estrogen-dependent elements could be responsible for the activation of activator protein (AP)-1 complex genes in the normal metabolism of *T. crassiceps* [32].

Regulation of protein phosphorylation

Rapid-action or non-genomic mechanisms have been investigated more prolifically than those involving the presence of a classical nuclear receptor (Figure 2). However, this apparent advantage is only relative because the first reports indicating that host hormones might activate cascades of second messengers appeared only at the beginning of this century [23,26]. EGF is a molecule that can activate different signaling pathways in parasites and that has been amply studied [23]. The presence of a complete signaling cascade that corresponds to the Raf kinases has been determined in *B. malayi* [26]. Murine EGF increases transcription levels of Raf kinase and Ran – a nuclear GTPase in *B. malayi* – and has been demonstrated to promote phosphorylation of some microfilarial proteins. Moreover, physical interaction increases between Ran and other proteins (which are yet to be defined) and promotes phosphorylation of some proteins of microfilarial origin. By contrast, *T. cruzi* amastigotes synthesize a receptor that can bind to human EGF to induce the activity of parasite MAPK and PKC cascades in a dose- and time-dependent manner. As proposed by

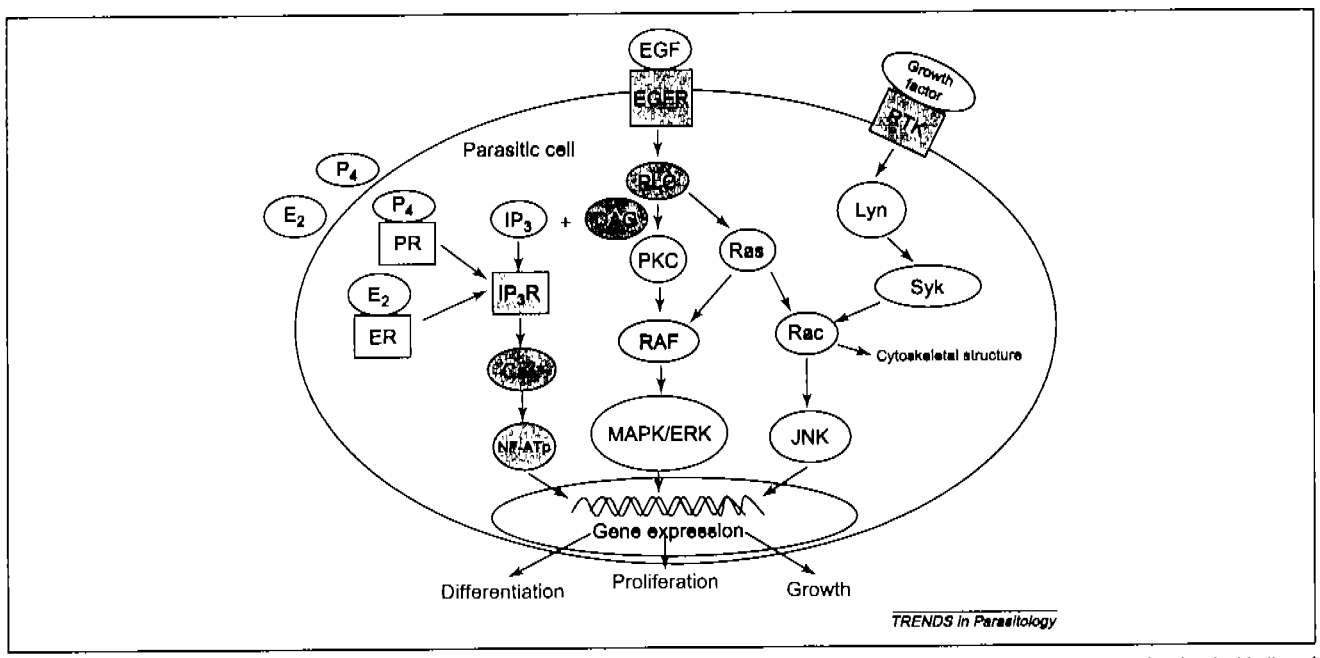

Figure 2. An alternative mechanism of parasite exploitation of the hormonal environment in an immunocompetent host. This mechanism does not involve the binding of hormones to classical nuclear receptors but, instead, the capability of parasites to bind hormones to their cell membrane and activate downstream signal-transduction pathways. The ligand–receptor complex can regulate – through activation of specific signal-transduction cascades such as those involving inositol (1,4,5)-trisphosphate (IP_3), PKC, extracellular-signal-regulated kinases (ERKs) and MAPK – vital processes of parasite physiology, including growth, infectivity, differentiation and reproduction. Abbreviations: DAG, diacylglycerol; EGFR, EGF receptor; IP_3R, IP_3 receptor; JNK, N-terminal c-Jun kinase; NF-ATp, nuclear factor of activated T cells; PLC, phospholipase C; PR, progesterone receptor; RTK, receptor tyrosine kinase.

Ghansah *et al.* [23], these results suggest the existence of a mechanism that regulates growth and parasite proliferation through a signaling pathway that is dependent on human EGF. Similarly, the presence in *S. mansoni* of a receptor (SmRTK-1) with a high degree of structural homology to the insulin receptor catalytic domains has been determined. Preferential localization of SmRTK-1 in sporocysts and oocysts could favor differentiation and growth processes in this parasite [47] because receptors with tyrosine kinase activity typically control aspects of metabolism, growth and development. Recent studies show that *S. haematobium* synthesizes a 28-kDa protein, Sh28GST (*S. haematobium* glutathione-S-transferase of 28 kDa), that can bind to testosterone and facilitate the transport, metabolism and physiological action of this hormone in the parasite [24].

Concluding remarks

In light of this evidence, it is clear that the host endocrine system can not only influence the course of parasitic infection by modulating the immune system but also be exploited directly by parasites. Thus, by means of genomic (Figure 1) and non-genomic (Figure 2) mechanisms, host hormones regulate important parasite processes such as growth, differentiation and reproduction through a mechanism described as transregulation. In some cases, this mechanism enables the parasite to accomplish a more successful infection. In other cases, transregulation might benefit the host by reducing the success of parasite infection.

Comprehension of the concepts of transregulation and host exploitation, in addition to the study of classic nuclear receptors and receptors that regulate the activity of various second-messenger cascades in parasites, provides interesting research perspectives in the complex host–parasite evolutionary relationship. Nuclear receptors in parasites are scarce and have been described in only six parasites to date. However, as more parasite genome projects reach completion, evidence of these receptors in other parasites is likely to accumulate. Recent evidence that cysticerci of *T. crassiceps* possess 17-β-hydroxysteroid dehydrogenase activity that can metabolize androstenedione to testosterone [46] suggests that steroid hormones will also be identified, at least in eukaryotic parasites.

The ability of a parasite to affect a female or male host of the same species differentially (sexual dimorphism of an infection) can be mediated by hormonal regulation of the immune response of the host or by direct hormonal effects on the parasite. Understanding the contribution of each of these effects and the characterization of the parasite molecules involved might facilitate the development of drugs that counteract the effects of hormones on the host immune system or the parasite.

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Differential expression of AP-1 transcription factor genes *c-fos* and *c-jun* in the helminth parasites *Taenia crassiceps* and *Taenia solium*

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SUMMARY

Homologues of *c-fos* and *c-jun* from total DNA of *Taenia crassiceps* and *Taenia solium* were cloned and sequenced. The amino acid alignment analysis revealed that *c-fos* DNAs from *T. crassiceps* and *T. solium* were highly homologous (96%), and both have high homology compared to several mammalian *c-fos* proteins (93% to mouse, 96% to rat and 86% to human). The *c-jun* protein alignment showed higher homology (*T. crassiceps* and *T. solium* have 98%), when compared with mouse, rat and human, being 92%, 98% and 93% respectively. RT–PCR amplification of the parasite's total RNA, showed that *T. crassiceps* expressed both AP-1 complex genes, while *T. solium* only expressed *c-fos*. Southern blot hybridization analysis confirmed the true origin of each amplified gene. AP-1 transcription gene expression is regulated by oestradiol in the same fashion as their mammalian counterparts only in *T. crassiceps*. To study if AP-1 genes are involved in a physiological function of the cyst, reproduction was studied *in vitro*. Oestradiol treatment stimulated reproduction in *T. crassiceps* but not in *T. solium* cysticerci. This is the first report of the detection and functionality of AP-1 transcription factor genes in any species of helminth parasite.

Key words: AP-1 transcription factor, *Taenia crassiceps*, *Taenia solium*, *c-fos* gene, *c-jun* gene.

INTRODUCTION

The metacestode stage of the tapeworm *Taenia solium* causes neurocysticercosis, the Third World major brain disorder that is estimated to affect 50 million people world-wide, and is an emergent disease in the United States (White, 1997). *T. solium* also infects pigs, the intermediate host, leading to major economic loss and transmission to humans (Sciutto *et al.* 2000). Slow data retrieval and high costs in *T. solium* research have led to the use of an experimental disease in mice with another cestode, *Taenia crassiceps*, which exhibits extensive similarities to *T. solium* (Larralde *et al.* 1990; Toledo *et al.* 2001).

Experimental intraperitoneal (IP) cysticercosis of mice is caused by the IP injection of *T. crassiceps* (Culbreth, Esch & Kuhn, 1972) and is characterized by the parasite's rapid rate of asexual reproduction to reach grams of parasite mass per infected host in a few months (Larralde *et al.* 1995). Parasite proliferation is faster in female than in male mice, and is favoured by oestradiol and hindered by androgens (Huerta *et al.* 1992; Bojalil *et al.* 1993; Terrazas *et al.* 1994). The importance of sexual factors for murine cysticercosis is further stressed by a remarkable feminization process that ensues in chronically infected male mice: serum oestradiol levels are increased 200 times their normal values, while those of testosterone are 90% reduced relative to control values (Larralde *et al.* 1995). The infected peritoneum shows a strikingly mild and transient inflammatory process (Padilla *et al.* 2001) and the systemic immune response shows a progressive downregulation of the TH1 cytokine profile and upregulation of TH2 when massive levels of parasite loads are reached (Terrazas *et al.* 1998; Toenjes *et al.* 1999). Sex hormones are proposed to be involved in the regulation of murine *T. crassiceps* cysticercosis by way of thymus-dependent immune mechanisms that obstruct or favour parasite growth (Bojalil *et al.* 1993; Terrazas *et al.* 1994). IL-6 is proposed to favour the expression of P-450 aromatase in infected males, thus shunting testosterone to oestradiol (Morales-Montor *et al.* 2001). Hints that sex hormones may be involved in *T. solium* disease of humans and pigs are progressively being reported (Sotelo & del Bruto, 2000; Morales *et al.* 2002).

In addition to the effects of sex-steroids on the host immune system (Morales-Montor *et al.* 2002), they may also directly affect the parasite if their usual target genes involved in cell proliferation and

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Table 1. Sequences of the primers used for PCR amplification of total *Taenia crassiceps*, *T. solium*, mouse and pig RNA reverse transcribed

(Primers were designed based on the most conserved regions of several species sequenced genes or based using the sequence of cloned *T. crassiceps* genes (*c-fos* and *c-jun*). GenBank accession numbers: *c-fos* (gi55933, gi50399, gi15131232), *c-jun* (gi57819, gi6754401, gi186624), TNF-α (gi395369, gi7305584, gi25952110, gi164694, gi29569762), β-actin (gi38648901, gi6671508, gi5016088, gi27371027, gi156758, gi29603620, gi156758, gi28573581) and VDR (gi184894). Each PCR product was purified and sequenced as described in the Materials and Methods section.)

Gene	Sense primer	Antisense primer
c-fos	5′-CCCCTGTCAACACACAGGAC	5′-CCGATGCTCTGCGCTCTGC
c-jun	5′-CAGATCCCGGTGCAGCACCCG	5′-GGTGGCACCCACTGTTAACGTGG
TNF-α	5′-GGCAGGTCTACTTTGGAGTCATTGC	5′-ACATTCGAGGCTCCAGTGAATTCGG
VDR	5′-TGAATTCCATGAAACACCTGTGGTTCTT	5′-GACTCTAGAGGGCTCACACTCACCTCCCCT
β-actin	5′-CTACAATGAGCTGCGTGTGG	5′-GGTCTCAAACATGATCTGGG

differentiation (such as *c-fos* and *c-jun*) are present in the parasite. These parasite genes could regulate the expression of many other genes in a variety of tissues and cell types (Hyder, Shipley & Stancel, 1995; Hyder *et al.* 1992) and their over-expression in the cysticercotic mice could well influence the outcome of the infection as well as explain its strong dependence on sex-steroids. The specific aim of this study was to investigate whether *T. crassiceps* and *T. solium* cysticerci express sequences of the AP-1 complex proto-oncogenes *c-fos* and *c-jun* (both strongly regulated by oestradiol) and, if so, because oestradiol has been demonstrated to play a major role during cysticercosis, to study if the AP-1 gene complex is involved in regulating reproduction of both *T. crassiceps* and *T. solium* cysticerci *in vitro*.

MATERIALS AND METHODS

Parasites

Total RNA was extracted from cysticerci of the fast growing ORF strain of *Taenia crassiceps* isolated as described by Freeman (1962). The cysticerci used in these experiments were cultivated for 5 days after being collected from the peritoneal cavity of donor female mice. The collected cysticerci were alive and quite clean from debris but were, however, washed several times with NTS to minimize contamination with host cells. *T. solium* live cysticerci were dissected and freed from all surrounding tissues of an infected pig, immediately after being humanely killed in the Facultad de Medicina Veterinaria y Zootecnia, UNAM. The cysticerci were washed several times with saline to minimize contamination with host cells.

Total RNA isolation

Total RNA was isolated from *T. crassiceps* and *T. solium* cysticerci, together with mouse testes and pig muscle (as controls for specific *c-jun* or *c-fos* amplification), using the single-step method based on guanidine isothiocyanate/phenol/chloroform extraction with Trizol reagent (Life Technologies, CA). In brief, parasites, mouse testes and pig muscle were disrupted in Trizol reagent (1 ml/0·1 g of tissue), and 0·2 ml of chloroform was added per ml of Trizol. The aqueous phase was recovered after a 10 min centrifugation at 14000 ***g***. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol and redissolved in RNAse-free water. RNA concentration was determined by absorbance at 260 nm and its purity was verified after electrophoresis on 1·0% denaturing agarose gel in the presence of 2·2 M formaldehyde.

RT–PCR amplification

Total RNA from parasites, mouse testes and pig muscle was reverse transcribed followed by specific PCR amplification of *c-fos* and *c-jun* gene sequences. Nucleotide sequences of the primers used for *c-fos*, *c-jun* and β-actin amplification are shown in Table 1. To demonstrate that cysticerci were not contamined by host cell DNA or RNA, we used primers of pig TNF-α and the mouse VDR gene, which correspond to the mouse variable region of the IgG (Table 1). Briefly, 2 μg of total RNA (either host or parasite) were incubated at 37 °C for 1 h with 400 units of M-MLV reverse transcriptase (Applied Biosystems, Boston, MA) in 20 μl of reaction volume containing 50 mM of each dNTP and 0·05 μg oligo (dt) primer (Gibco, NY). Ten μl of the cDNA reaction was subjected to PCR in order to amplify specific sequences of the specified genes. The 50 μl PCR reaction included 10 μl of previously synthesized cDNA, 25 μl of 10× PCR-buffer (Biotecnologias Universitarias, Mexico) 1 mM $MgCl_2$, 0·2 mM of each dNTP, 0·05 μM of each primer, and 2·5 units of *Taq* DNA polymerase (Biotecnologias Universitarias, Mexico). After an initial denaturation step at 94 °C for 4 min, temperature cycling was initiated as follows: 94 °C for 55 sec, 50 °C for 55 sec and 72 °C for 45 sec during 30 cycles for *c-fos*, while for *c-jun* was: 94 °C for 55 sec, 57 °C for 55 sec and 72 °C for 45 sec during 30 cycles. β-actin was amplified by using same

A

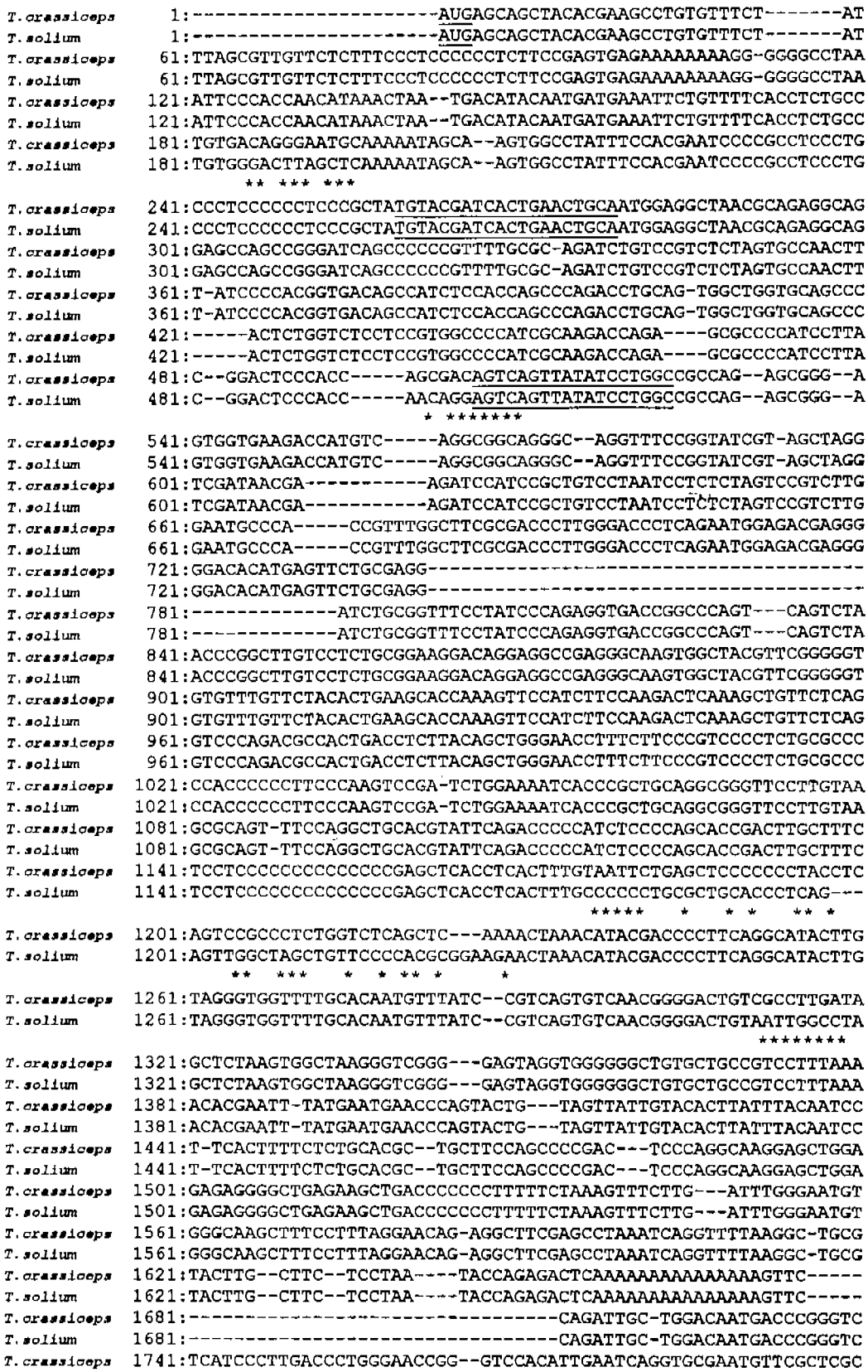
T.crassiceps 1:----------AUGAGCAGCTACACGAAGCCTGTGTTTCT-------AT
T.solium 1:----------AUGAGCAGCTACACGAAGCCTGTGTTTCT-------AT
T.crassiceps 61:TTAGCGTTGTTCTCTTTCCCTCCCCCCTCTTCCGAGTGAGAAAAAAAAAGG-GGGGCCTAA
T.solium 61:TTAGCGTTGTTCTCTTTCCCTCCCCCCTCTTCCGAGTGAGAAAAAAAAAGG-GGGGCCTAA
T.crassiceps 121:ATTCCCACCAACATAAACTAA--TGACATACAATGATGAAATTCTGTTTTCACCTCTGCC
T.solium 121:ATTCCCACCAACATAAACTAA--TGACATACAATGATGAAATTCTGTTTTCACCTCTGCC
T.crassiceps 181:TGTGACAGGGAATGCAAAAATAGCA--AGTGGCCTATTTCCACGAATCCCCGCCTCCCTG
T.solium 181:TGTGGGACTTAGCTCAAAAATAGCA--AGTGGCCTATTTCCACGAATCCCCGCCTCCCTG
** *** ***
T.crassiceps 241:CCCTCCCCCCTCCCGCTATGTACGATCACTGAACTGCAATGGAGGCTAACGCAGAGGCAG
T.solium 241:CCCTCCCCCCTCCCGCTATGTACGATCACTGAACTGCAATGGAGGCTAACGCAGAGGCAG
T.crassiceps 301:GAGCCAGCCGGGATCAGCCCCCCGTTTTGCGC-AGATCTGTCCGTCTCTAGTGCCAACTT
T.solium 301:GAGCCAGCCGGGATCAGCCCCCCGTTTTGCGC-AGATCTGTCCGTCTCTAGTGCCAACTT
T.crassiceps 361:T-ATCCCCACGGTGACAGCCATCTCCACCAGCCCAGACCTGCAG-TGGCTGGTGCAGCCC
T.solium 361:T-ATCCCCACGGTGACAGCCATCTCCACCAGCCCAGACCTGCAG-TGGCTGGTGCAGCCC
T.crassiceps 421:-----ACTCTGGTCTCCTCCGTGGCCCCATCGCAAGACCAGA----GCGCCCCATCCTTA
T.solium 421:-----ACTCTGGTCTCCTCCGTGGCCCCATCGCAAGACCAGA----GCGCCCCATCCTTA
T.crassiceps 481:C--GGACTCCCACC-----AGCGACAGTCAGTTATATCCTGGCCGCCAG--AGCGGG--A
T.solium 481:C--GGACTCCCACC-----AACAGGAGTCAGTTATATCCTGGCCGCCAG--AGCGGG--A
* *******
T.crassiceps 541:GTGGTGAAGACCATGTC-----AGGCGGCAGGGC--AGGTTTCCGGTATCGT-AGCTAGG
T.solium 541:GTGGTGAAGACCATGTC-----AGGCGGCAGGGC--AGGTTTCCGGTATCGT-AGCTAGG
T.crassiceps 601:TCGATAACGA----------AGATCCATCCGCTGTCCTAATCCTCTCTAGTCCGTCTTG
T.solium 601:TCGATAACGA----------AGATCCATCCGCTGTCCTAATCCTCTCTAGTCCGTCTTG
T.crassiceps 661:GAATGCCCA-----CCGTTTGGCTTCGCGACCCTTGGGACCCTCAGAATGGAGACGAGGG
T.solium 661:GAATGCCCA-----CCGTTTGGCTTCGCGACCCTTGGGACCCTCAGAATGGAGACGAGGG
T.crassiceps 721:GGACACATGAGTTCTGCGAGG----------
T.solium 721:GGACACATGAGTTCTGCGAGG----------
T.crassiceps 781:----------ATCTGCGGTTTCCTATCCCAGAGGTGACCGGCCCAGT---CAGTCTA
T.solium 781:----------ATCTGCGGTTTCCTATCCCAGAGGTGACCGGCCCAGT---CAGTCTA
T.crassiceps 841:ACCCGGCTTGTCCTCTGCGGAAGGACAGGAGGCCGAGGGCAAGTGGCTACGTTCGGGGGT
T.solium 841:ACCCGGCTTGTCCTCTGCGGAAGGACAGGAGGCCGAGGGCAAGTGGCTACGTTCGGGGGT
T.crassiceps 901:GTGTTTGTTCTACACTGAAGCACCAAAGTTCCATCTTCCAAGACTCAAAGCTGTTCTCAG
T.solium 901:GTGTTTGTTCTACACTGAAGCACCAAAGTTCCATCTTCCAAGACTCAAAGCTGTTCTCAG
T.crassiceps 961:GTCCCAGACGCCACTGACCTCTTACAGCTGGGAACCTTTCTTCCCGTCCCCTCTGCGCCC
T.solium 961:GTCCCAGACGCCACTGACCTCTTACAGCTGGGAACCTTTCTTCCCGTCCCCTCTGCGCCC
T.crassiceps 1021:CCACCCCCCTTCCCAAGTCCGA-TCTGGAAAATCACCCGCTGCAGGCGGGTTCCTTGTAA
T.solium 1021:CCACCCCCCTTCCCAAGTCCGA-TCTGGAAAATCACCCGCTGCAGGCGGGTTCCTTGTAA
T.crassiceps 1081:GCGCAGT-TTCCAGGCTGCACGTATTCAGACCCCCATCTCCCCAGCACCGACTTGCTTTC
T.solium 1081:GCGCAGT-TTCCAGGCTGCACGTATTCAGACCCCCATCTCCCCAGCACCGACTTGCTTTC
T.crassiceps 1141:TCCTCCCCCCCCCCCCCCCGAGCTCACCTCACTTTGTAATTCTGAGCTCCCCCCCTACCTC
T.solium 1141:TCCTCCCCCCCCCCCCCCCGAGCTCACCTCACTTTGCCCCCCTGCGCTGCACCCTCAG---
***** * * * ** *
T.crassiceps 1201:AGTCCGCCCTCTGGTCTCAGCTC---AAAACTAAACATACGACCCCTTCAGGCATACTTG
T.solium 1201:AGTTGGCTAGCTGTTCCCCACGCGGAAGAACTAAACATACGACCCCTTCAGGCATACTTG
** *** * * ** * *
T.crassiceps 1261:TAGGGTGGTTTTGCACAATGTTTATC--CGTCAGTGTCAACGGGGACTGTCGCCTTGATA
T.solium 1261:TAGGGTGGTTTTGCACAATGTTTATC--CGTCAGTGTCAACGGGGACTGTAATTGGCCTA
****
T.crassiceps 1321:GCTCTAAGTGGCTAAGGGTCGGG---GAGTAGGTGGGGGGCTGTGCTGCCGTCCTTTAAA
T.solium 1321:GCTCTAAGTGGCTAAGGGTCGGG---GAGTAGGTGGGGGGCTGTGCTGCCGTCCTTTAAA
T.crassiceps 1381:ACACGAATT-TATGAATGAACCCAGTACTG---TAGTTATTGTACACTTATTTACAATCC
T.solium 1381:ACACGAATT-TATGAATGAACCCAGTACTG---TAGTTATTGTACACTTATTTACAATCC
T.crassiceps 1441:T-TCACTTTTCTCTGCACGC--TGCTTCCAGCCCCGAC---TCCCAGGCAAGGAGCTGGA
T.solium 1441:T-TCACTTTTCTCTGCACGC--TGCTTCCAGCCCCGAC---TCCCAGGCAAGGAGCTGGA
T.crassiceps 1501:GAGAGGGGCTGAGAAGCTGACCCCCCCTTTTTCTAAAGTTTCTTG---ATTTGGGAATGT
T.solium 1501:GAGAGGGGCTGAGAAGCTGACCCCCCCTTTTTCTAAAGTTTCTTG---ATTTGGGAATGT
T.crassiceps 1561:GGGCAAGCTTTCCTTTAGGAACAG-AGGCTTCGAGCCTAAATCAGGTTTTAAGGC-TGCG
T.solium 1561:GGGCAAGCTTTCCTTTAGGAACAG-AGGCTTCGAGCCTAAATCAGGTTTTAAGGC-TGCG
T.crassiceps 1621:TACTTG--CTTC--TCCTAA----TACCAGAGACTCAAAAAAAAAAAAAAAAGTTC-----
T.solium 1621:TACTTG--CTTC--TCCTAA----TACCAGAGACTCAAAAAAAAAAAAAAAAGTTC-----
T.crassiceps 1681:----------CAGATTGC-TGGACAATGACCCGGGTC
T.solium 1681:----------CAGATTGC-TGGACAATGACCCGGGTC
T.crassiceps 1741:TCATCCCTTGACCCTGGGAACCGG--GTCCACATTGAATCAGGTGCGAATGTTCGCTCGC

Fig. 1. (*cont.*)

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T.solium       1741:TCATCCCTTGACCCTGGGAACCGG--GTCCACATTGAATCAGGTGCGAATGTTCGCTCGC
T.crassiceps   1801:CTTCTCTGCCTTTCCCGGCAGCCGGCGCCTCCCCTCCGCC--CCGGTTCCCCCCCTGCGC
T.solium       1801:CTTCTCTGCCTTTCCCGGCAGCCGGCGCCTCCCCTCCGCC--CCGGTTCTAATTCTGAGC
                                                              *****   *
T.crassiceps   1861:TGCACCCTCAG---AGTTGGCT---AGCTGTTCCCCACGCGGAAGGT-----CAATCCCT
T.solium       1861:TCCCCCCCTACCTCAGTCCGCC---CTCTGGTCTCAGCTC---AAGT-----CAATCCCT
                     * *   ** *       **  *   **    *  * ** *      *
T.crassiceps   1921:CCC----TCCTTTACACAGGATGTCCATATTAGGACATCTGCGTCAGCAGGTTTCCAC--
T.solium       1921:CCC----TCCTTTACACAGGATGTCCATATTAGGACATCTGCGTCAGCAGGTTTCCAC--
T.crassiceps   1981:GGCCGGTCCCTGTTGTTCTGGGGGGGGGACC--ATCTCCGAAATCCTACCCGGCCGCGG-
T.solium       1981:GGCCGGTCCCTGTTGTTCTGGGGGGGGGACC--ATCTCCGAAATCCTACCCGGCCGCGG-
T.crassiceps   2041:TCTAGGAGACCC---CCTAAGATCCCAAATGTGAACACTCATAGGTGAAAGA---TGTAT
T.solium       2041:TCTAGGAGACCC---CCTAAGATCCCAAATGTGAACACTCATAGGTGAAAGA---TGTAT
T.crassiceps   2101:GCCAAGACGGGGGT----------TGAAAGC-------CTGGGGCGTAGAGTTGACGAC-A
T.solium       2101:GCCAAGACGGGGGT----------TGAAAGC-------CTGGGGCGTAGAGTTGACGAC-A
T.crassiceps   2161:GAGCGCCCGCAGAGGGCCTTGGGGCGCGCTTCCCCCCCCTTCCAGTTC--CGCCCAGTGA
T.solium       2161:GAGCGCCCGCAGAGGGCCTTGGGGCGCGCTTCCCCCCCCTTCCAGTTC--CGCCCAGTGA
T.crassiceps   2221:C--GTAGGAAGTCCATCCATTCACAGCGCTTCTATAAAGGCGCCAGCTGAGGCGCC----
T.solium       2221:C--GTAGGAAGTCCATCCATTCACAGCGCTTCTATAAAGGCGCCAGCTGAGGCGCC----
T.crassiceps   2281:TACTACTAAAAAAAAAA----------
T.solium       2281:TACTACTAAAAAAAAAA----------
                    * *   ** ****  *
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B

```
Rattus rattus:  1   MMYQGFAGEYEXXXXXXXXXXXXXXXLTYYPSPADSFSSMGSPVNSQDFCTDLAVSSANF 60
Mus musculus:   1   MMYQGFAGEYEXXXXXXXXXXXXXXXLTYYPSPADSFSSMGSPVNSQDFCTDLAVSSANF 60
Homo sapiens:   1   ************************************************************ 60
Taenia crassiceps: 1   MMFSGFNADYEASSSRCSSASPAGDSLSYYHSPADSFSSMGSPVNTQDFCADLSVSSANF 60
Taenia solium:  1   MMFSGFNADYEASSSRCSSASPAGDSLSYYHSPADSFSSMGSPVNAQDFCTDLAVSSANF 60

Rattus rattus:  61  VPTVTAISTSPDLQWLVQPTLISSVAPSQNRG-HPYGVXXXXXXXXYSRPAVLKAPGGRG 120
Mus musculus:   61  VPTVTAISTSPDLQWLVQPTLISSVAPSQNRG-HPYGVXXXXXXXXYSRPAVLKAPGGRG 120
Homo sapiens:   61  ************************************************************ 120
Taenia crassiceps: 61  IPTVTAISTSPDLQWLVQPTLVSSVAPSQTRAPHPYGLPTPSTGAYARAGVVKTMSGGRA 120
Taenia solium:  61  IPTVTAISTSPDLQWLVQPALVSSVAPSQTRAPHPFGVPAPSAGAYSRAGVVKTMTGGRA 120

Rattus rattus:  121 QSIGRRGKVEQLSPXXXXXXXXXXXXXNKMAAAKCRNRRRELTDTLQAETDQLEEEKSALQ 180
Mus musculus:   121 QSIGRRGKVEQLSPXXXXXXXXXXXXXNKMAAAKCRNRRRELTDTLQAETDQLEEEKSALQ 180
Homo sapiens:   121 ************************************************ETDQLEEEKSALQ 180
Taenia crassiceps: 121 QSIGRRGKVEQLSPEEEEKRRIRRERNKMAAAKCRNRRRELTDTLQAETDQLEDEKSALQ 180
Taenia solium:  121 QSIGRRGKVEQLSPEEEEKRRIRRERNKMAAAKCRNRRRELTDTLQAETDQLEDEKSALQ 180

Rattus rattus:  181 AEIANXXXXXXXXFILAAHRPACKMPEELRFSEELAAATALDLGAPSPAA----AEETF 240
Mus musculus:   181 AEIANXXXXXXXXFILAAHRPACKMPEELRFSEELAAATALDLGAPSPAA----AEETF 240
Homo sapiens:   181 AEIANXXXXXXXXFILAAHRPACKMPEELRFSEELAAATALDLGAPSPAAA----EETF 240
Taenia crassiceps: 181 TEIANLLKEKEKLEFILAAHRPACKIPNDLGFPEEM-SVTSLDLTGGLPEATTPESEEAF 240
Taenia solium:  181 TEIANLLKEKEKLEFILAAHRPACKIPDDLGFPEEMSVASLDLTGGLPEVATPESEEAFT 240

Rattus rattus:  241 ALPXMTEAPPAVPPKEP--SGSGLELKAEPFDELLFSTGPR----EASRSVPDMDLPGAS 300
Mus musculus:   241 ALPXMTEAPPAVPPKEP--SGSGLELKAEPFDELLFSTGPR----EASRSVPDMDLPGAS 300
Homo sapiens:   241 ALPXMTEAPPAVPPKEP--SGSGLELKAEPFDELLFSTGPR----EASRSVPDMDLPGAS 300
Taenia crassiceps: 241 TLPLLNDPEPK-PSLEPVKNISNMELKAEPFDDFLFPASSRPSGSETARSVPDVDLSG-- 300
Taenia solium:  241 LPLLNDPEPKPSVEPVKSISSMELKTEPFDDFLFPASSRPSGSETARSVPDMDLSGSFYA 300

Rattus rattus:  301 SFYASDWEPLGAGSSG------ELEPLXXXXXXXXXXXXXXXXXFVFTYPEADAFPSCAA 360
Mus musculus:   301 SFYASDWEPLGAGSSG------ELEPLXXXXXXXXXXXXXXXXXFVFTYPEADAFPSCAA 360
Homo sapiens:   301 SFYASDWEPLGAGSSG------ELEPLXXXXXXXXXXXXXXXXXFVFTYPEADAFPSCAA 360
Taenia crassiceps: 301 SFYAADWEPLHSSSLGMGPMVTELEPLCTPVVTCTPSCTTYTSSFVFTYPEADSFPSCAA 360
Taenia solium:  301 ADWEPLHSGSLGMGPMATELEPLCTPVVTCTPSCTAYTSSFVFTYPEADSFPSCAAAHRK 360

Rattus rattus:  361 AHRKGXXXXXXXXXXXXXPTLLAL 384 96%
Mus musculus:   361 AHRKGXXXXXXXXXXXXXPTLLAL 384 93%
Homo sapiens:   361 AHRKGXXXXXXXXXXXXXPTLLAL 384 82%
Taenia crassiceps: 361 AHRKGSSSNEPSSDSLSSPTLLAL 384
Taenia solium:  361 GSSSNEPSSDSLSSPTLLAL     380
```

Fig. 1. For legend see opposite page.

conditions as those for *c-jun*. An extra primer extension temperature at 72 °C for 10 min was done for every gene. Then 25 μl of the total PCR reaction products of each sample were electrophoresed on 2% agarose gel. PCR products were visualized by staining with ethidium bromide. A single band was detected in all cases, and the size of each specific amplification product as expected was 274 nt (*c-fos*), 325 nt (*c-jun*) and 327 nt (β-actin).

Probe development

Fifty μl samples of the PCR reaction were electrophoresed on 2% agarose gels. The PCR products obtained were visualized by staining with ethidium bromide, and were detected by using a 100 nt ladder as molecular weight marker (Gibco, BRL, NY). In all cases, the bands correponding to *T. crassiceps c-fos* and *c-jun*, and *T. solium c-fos*, together with VDR and TNF-α from mouse and pig respectively were excised directly from the gel, purified by electroelution, and cloned into the bluescript SK(+/−) (Stratagene, La Jolla, CA) vector. These specific cDNAs were used as probes to perform the *T. crassiceps* and *T. solium* phage library screening and the Southern blot hybridization analysis.

Southern blot analysis

T. crassiceps and *T. solium* cysticerci were homogenized gently and digested by proteinase K (100 units/ml) for 18–20 h at 37 °C, with continuous shaking. After the protein fraction was extracted with phenol/chloroform, the DNA was precipitated with cold ethanol and assessed for high molecular weight integrity by electrophoresis. DNA aliquots (20 μg) were digested with *Eco*RI, separated in 1% agarose gels, denatured with 0·25 M NaOH, and transferred to Hybond nylon membranes. cDNA parasite probes, labelled with [α-^{32}P]CTP, 0·6 kb *c-fos* and *c-jun* were synthesized from linearized cDNA. The membranes were hybridized with the probe overnight at 65 °C and then washed with 0·3 × SSC/0·1% SDS for about 2 h at 65 °C, followed by exposure to X-ray film. The signals were quantitated by densitometric scanning (Molecular Dynamics, Sunnyvale, CA). PCR products of the VDR and TNF-α gels were blotted in the same way as parasite genomic DNA to reveal a very low rate of amplification, and to be sure that no contamination at all from host cells occurred.

DNA sequencing

The DNA sequence was determined by using a Thermo Sequenase cycle sequencing kit (Biorad) and an automatic sequencer (Model LIC-4200, Aloka Co., Japan). The sequence data were analysed using DNASIS Software (Hitachi Software Engineering, Tokyo, Japan). Homology searching on the nucleotide and protein database was carried out with the Blast program at the National Center for Biotechnology Information (Bethesda, Maryland). Pairwase sequence alignment and protein identities were performed using CLUSTLAW 1.6 software.

Treatment of T. crassiceps *and* T. solium *with oestradiol: hormone dose-response and temporal curves*

Culture grade E_2 was obtained from Sigma. For *in vitro* tests, water-soluble E_2, was dissolved in AIM-V free-serum culture medium, and sterilized by passage through a 0·2 mm millipore filter. The experiments employed parasite-loaded wells: 6 wells were used as untreated controls, 6 wells were supplemented with the solvent in which the hormone was diluted and 6 wells were treated with different concentrations of E_2 (5, 10, 20 and 40 μg/ml). Hormone was prepared in a final volume of 100 μl and added to 1 ml of medium in each well. The range of concentrations of oestradiol tested in this study was based on our previous studies of serum levels found in feminized mice (Larralde *et al.* 1995) and were chosen to approximate those levels *in vitro*. In hormone dose-response-time curves, only the number of buds per cysticercus as a function of days in culture was assessed as the response variable (1–10 days in culture). From the dose-response-time curves of oestradiol, an optimal dose was selected to be used in further experimentation: the dose of each hormone at the shortest time in which the differences with the respective control values was maximal. Reproduction was measured as the number of buds that each cyst produced in response to treatment and these were counted directly under an inverted light microscope.

RESULTS

The alignment of the *c-fos* gene sequences and predicted amino acid translation of *T. crassiceps* and *T. solium* with their mammalian counterparts is shown in Fig. 1. *T. crassiceps* and *T. solium* genes

Fig. 1. *Taenia crassiceps* and *T. solium c-fos* gene sequence and predicted amino acid sequence alignment. (A) Nucleotide sequence of *T. crassiceps* and *T. solium c-fos* gene. The positions of the start codon and the oligonucleotides used for PCR amplification are underlined. The nucleotide numbers are by reference to the start of the 5′ UTR when the *T. crassiceps* and *T. solium* sequence is aligned with other known mammalian *c-fos* sequences. Asterisks (*) indicate a change in nucleotide sequence. (B) Alignment of the *c-fos* protein sequences in rat (*Rattus rattus*), mouse (*Mus musculus*), human (*Homo sapiens*), *T. crassiceps*, and *T. solium*. Amino acids are represented by single-letter codes. Dotted lines represent undentified amino acids, and the X represents highly variable regions, meaning that X can be any amino acid.

A

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T.crassiceps     1:AUGTTGGTAACCCTAAGATCCTAAAACAGAGCATGACCTTGAACCTGGCCGACCCGTAGC
T.solium         1:AUGTTGGTAACCCTAAGATCCTAAAACAGAGCATGACCTTGAACCTGGCCGACCCGTAGC
T.crassiceps    61:C-AGAACTCAGCACCGCCGG-AGAGCCGCTGTTGCTGGG-----ACTGGTCTGCGGGCTC
T.solium        61:C-AGAACTCAGCACCGCCGG-AGAGCCGCTGTTGCTGGG-----ACTGGTCTGCGGGCTC
T.crassiceps   121:CAAGGAACCGCTGCTCCCCGAGAGCGCTCCGTGAGTGACCGCGACTTTTCAAAGCTCGGC
T.solium       121:CAAGGAACCGCTGCTCCCCGAGAGCGCTCCGTGAGTGACCGCGACTTTTCAAAGCTCGGC
T.crassiceps   181:ATCGCGCGGGAGCCTACCAACGTGAGTGCTAGCGGAGTCTT----AACCCTGCGCTCCCT
T.solium       181:ATCGCGCGGGAGCCTACCAACGTGAGTGCTAGCGGAGTCTT----AACCCTGCGCTCCCT
T.crassiceps   241:GGAGCGAACTGGGGAGGAGGGCTCAGGGGGAAGCACTGCCGTCTGGAGCGCACGCTCCTA
T.solium       241:GGAGCGAACTGGGGAGGAGGGCTCAGGGGGAAGCACTGCCGTCTGGAGCGCACGCTCCTA
T.crassiceps   301:AACAAACTTT----GTTACAGAAGCGGGGACGCTTCGCCGAGGGCTTCGTGCGCGCCCTG
T.solium       301:AACAAACTTT----GTTACAGAAGCGGGGACGCTTCGCCGAGGGCTTCGTGCGCGCCCTG
T.crassiceps   361:GCTGAACTGCACCGAAACTTCTGCGCACAGCCCAGGCTAACCCCGCGTGAAG-GCCAGCA
T.solium       361:GCTGAACTGCACCGAAACTTCTGCGCACAGCCCAGGCTAACCCCGCGTGAAG-GCCAGCA
T.crassiceps   421:ACTTTCCTGACCACAGACGGACGAAACGACCTTCTACGACGATGCCCTCAACGCCTCGTT
T.solium       421:ACTTTCCTGACCACAGACGGACGAAACGACCTTCTACGACGATGCCCTCAACGCCTCGTT
T.crassiceps   481:CCTCCAGTCCGAGAGCGGTGCCTACGGCTACATCGGAGGCCGGGCGGCAGAGCCGCAGAC
T.solium       481:CCTCCAGTCCGAGAGCGGTGCCTACGGCTACATCGGAGGCCGGGCGGCAGAGCCGCAGAC
T.crassiceps   541:GGTGCCGGAGATG-CCGGGAGAGACGCCGCCCTGTCCCCCATCGACATGGAGTCTCAGGA
T.solium       541:GGTGCCGGAGATG-CCGGGAGAGACGCCGCCCTGTCCCCCATCGACATGGAGTCTCAGGA
T.crassiceps   601:GCGGATCAAGGCGGAGAGGAAGGCGCATGAGAAACCGCATCGCTGCCTCCAAGTGCCGGA
T.solium       601:GCGGATCAAGGCGGAGAGGAAGGCGCATGAGAAACCGCATCGCTGCCTCCAAGTGCCGGA
T.crassiceps   661:AAAGGAAGCTGGAGCGGATCGCCCGGCTAGAGGAAAAAGTGAAAACCTTGAAAGCGCAAA
T.solium       661:AAAGGAAGCTGGAGCGGATCGCCCGGCTAGAGGAAAAAGTGAAAACCTTGAAAGCGCAAA
T.crassiceps   721:CTCCGAGCTGGCGTCCACGGCCAACATGCTCAGGGAACAGGTGG-CACAGCTTAAACAGA
T.solium       721:CTCCGAGCTGGCGTCCACGGCCAACATGCTCAGGGAACAGGTGG-CACAGCTTAAACAGA
T.crassiceps   781:AAGTCATGACCCCGCTTCCCGGCGGAGCAGGAGGTGACGACTGGGCGCGGGTATCCCCCC
T.solium       781:AAGTCATGACCCCGCTTCCCGGCGGAGCAGGAGGTGACGACTGGGCGCGGGTATCCCCCC
T.crassiceps   841:GCTTCCCGGC----------GCGCTGTTGCGGCCGCGGTGGCCTCAGTAGCAGGCGCTGG
T.solium       841:GCTTCCCGGC----------GCGCTGTTGCGGCCGCGGTGGCCTCAGTAGCAGGCGCTGG
T.crassiceps   901:CGGCGGTGGTGGCTACAGCGCCAGCCTGCACAGTGAGCCTCCGGTCTACGCCAACCTCAG
T.solium       901:CGGCGGTGGTGGCTACAGCGCCAGCCTGCACAGTGAGCCTCCGGTCTACGCCAATAT-AC
                                                                  **   *
T.crassiceps   961:CAACTTCAACCCGGGTGCGCTGA-GCAGCGGCGGTGGGCTGGAGCATTTACTGCTGTGTC
T.solium       961:TCATACCAGTTCGCACAGGCGGCTGAAGTTGGGCGGGGCTGGAGCATTTACTGCTGTGTC
                   ** ***  ***  *****  * *  *  ** * **
T.crassiceps  1021:ACCTCCGCGGCACAGCCGGTCAGCGGGCCGGGCTGCAGCCGCCTCCGGTGCAGCACCCGC
T.solium      1021:ACCTCCGCGGCACAGCCGGTCAGCGGGCCGGGCTGCAGCCGCCTCCGGTGCAGCACCCGC
T.crassiceps  1261:GGCTGGCCTTTCCCTCGCAGCCGCAGCAGCAGCAGCAAGCCGTGTTGAGATGATGCTTCG
T.solium      1261:GCCTGGCCTTTCCCTCGCAGCCGCAGCAGCAGCAGCAAGCCGTGTTGAGATGATGCTTCG
T.crassiceps  1321:ACGTGCCGGAGATGCCGGGAGAGACGCCGCCCCTGTCCCCTATCGACATGGAGTCAGCAG
T.solium      1321:ACGTGCCGGAGATGCCGGGAGAGACGCCGCCCCTGTCCCCTATCGACATGGAGTCAGCAG
T.crassiceps  1381:TTGCAAA-CGTTTTGAGAACAGACTGTCAGGGCTGACGCATTGCCGCCTCCAAGTGCCGG
T.solium      1381:TTGCAAA-CGTTTTGAGAACAGACTGTCAGGGCTGACGCATTGCCGCCTCCAAGTGCCGG
T.crassiceps  1441:AAAAGGAAGCTGGAGCGGATCGCTCGGCTAGAGGAAAAAGTGAAAACCTTGAAAGCGCAA
T.solium      1441:AAAAGGAAGCTGGAGCGGATCGCTCGGCTAGAGGAAAAAGTGAAAACCTTGAAAGCGCAA
T.crassiceps  1501:AACTCCGAGCTGGCATCCACGGCCAACATGCTCAGGGAACAGGTGGCACAGCTTAAGCAG
T.solium      1501:AACTCCGAGCTGGCATCCACGGCCAACATGCTCAGGGAACAGGTGGCACAGCTTAAGCAG
T.crassiceps  1561:AAAGTCATGAACCACGTTAACAGTGGGTGCCAACTCATGCTAACGCGGGGCAATGGTCAG
T.solium      1561:AAAGTCATGAACCACGTTAACAGTGGGTGCCAACTCATGCTAACGCGGGGCAATGGTCAG
T.crassiceps  1621:GAGCGGATCAAGG-CAGAGAGGAAGCGCATGAGGAAC-----AAGAAAAAAAATAAC---
T.solium      1621:GAGCGGATCAAGG-CAGAGAGGAAGCGCATGAGGAAC-----AAGAAAAAAAATAAC---
T.crassiceps  1681:-AGAGACAAACTTGAGAACTTGACTGGTTGCGACAAAAAAAAAAAAAAA----------
T.solium      1681:-AGAGACAAACTTGAGAACTTGACTGGTTGCGACAAAAAAAAAAAAAAA----------
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Fig. 2. (*cont.*)

demostrated homology to several known *c-fos* genes sequenced. The *c-fos T. crassiceps* and *T. solium* genes have a high sequence homology between them (96%), while when compared with mouse, rat and human, the scores are lower, but still they are as high as (93%, 96% and 82%) respectively. Comparison of the predicted amino acid sequence of *c-fos* and *c-jun* with the mammalian proteins indicates that *c-fos* and *c-jun* share 92% amino acid identity. Molecular cloning of both genes in both parasites, together with their respective mRNAs, and determination of the full length protein sequence, has confirmed that both belong to the group of proteins of the *c-fos* and *c-jun* family.

B

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Rattus rattus: 1   MTAKMETTFYDDALNASFLQSESGAYGYSNPKILKQSMTLNLADPVGSLKPHLRAKNSDL 60
Mus musculus: 1   MTAKMETTFYDDALNASFLQSESGAYGYSNPKILKQSMTLNLADPVGSLKPHLRAKNSDL 60
Homo sapiens: 1   MTAKMETTFYDDALNASFLQSESGAYGYSNPKILKQSMTLNLADPVGSLKPHLRAKNSDL 60
Taenia crassiceps: 1   MTAKMETTFYDDALNASFLQSESGAYGYSNPKILKQSMTLNLADPVGSLKPHLRAKNSDL 60
Taenia solium: 1   MTAKMETTFYDDALNASFLPSESGPYGYSNPKILKQSMTLNLADPVGSLKPHLRAKNSDL 60

Rattus rattus: 61  LTSPDVGLLKLASPELERLIIQSSNGHITTTPTPTQFLCPKNVTDEQEGFAEGFVRALAE 120
Mus musculus: 61  LTSPDVGLLKLASPELERLIIQSSNGHITTTPTPTQFLCPKNVTDEQEGFAEGFVRALAE 120
Homo sapiens: 61  LTSPDVGLLKLASPELERLIIQSSNGHITTTPTPTQFLCPKNVTDEQEGFAEGFVRALAE 120
Taenia crassiceps: 61  LTSPDVGLLKLASPELERLIIQSSNGHITTTPTPTQFLCPKNVTDEQEGFAEGFVRALAE 120
Taenia solium: 61  LTSPDVGLLKLASPELERLVIQSSNGHITTTPTPTQFLCPKNVTDEQEGFAEGFVRALAE 120

Rattus rattus: 121 LHSQNTLPSVTSAAQPXXXXXXXXXXXXXXXXXXXXXXXXXXXXLHSEPPVYANLSNFNXXX 180
Mus musculus: 121 LHSQNTLPSVTSAAQPXXXXXXXXXXXXXXXXXXXXXXXXXXXXLHSEPPVYANLSNFNXXX 180
Homo sapiens: 121 LHSQNTLPSVTSAAQPXXXXXXXXXXXXXXXXXXXXXXXXXXXXLHSEPPVYANLSNFNXXX 180
Taenia crassiceps: 121 LHSQNTLPSVTSAAQPVSGAGMVAPAVASVAGAGGGGGYSASLHSEPPVYANLSNFNPGA 180
Taenia solium: 121 LHSQNTLPSVTSAAQPVNGAGMVAPAVASVAGGSGSGGFSASLHSEPPVYANLSNFNPGA 180

Rattus rattus: 181 XXXXXXXXXXXXXXXXFXXXXXXXXXXXXXXXXXXXXXXXXXXXRLQALKEEPQTVPEMPG 240
Mus musculus: 181 XXXXXXXXXXXXXXXXFXXXXXXXXXXXXXXXXXXXXXXXXXXXRLQALKEEPQTVPEMPG 240
Homo sapiens: 181 XXXXXXXXXXXXXXXXAXXXXXXXXXXXXXXXXXXXXXXXXXXXRLQALKEEPQTVPEMPG 240
Taenia crassiceps: 181 LSCGGGAPSYGAAGLAFPSQPQQQQQPPQPPHHLPQQIPVQHPRLQALKEEPQTVPEMPG 240
Taenia solium: 181 LSSGGGAPSYGAAGLAFPAQPQQQQQPPHHLPQQMPVQHPRLQALKEEPQTVPEMPGETP 240

Rattus rattus: 241 ETPPLSPIDMESQERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELAST 300
Mus musculus: 241 ETPPLSPIDMESQERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELAST 300
Homo sapiens: 241 ETPPLSPIDMESQERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELAST 300
Taenia crassiceps: 241 ETPPLSPIDMESQERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELAST 300
Taenia solium: 241 PLSPIDMESQERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANM 300

Rattus rattus: 301 ANMLREQVAQLKQKVMNHVNSGCQLMLTQQLQTF 98%                          334
Mus musculus: 301 ANMLREQVAQLKQKVMNHVNSGCQLMLTQQLQTF 92%                          334
Homo sapiens: 301 ANMLREQVAQLKQKVMNHVNSGCQLMLTQQLQTF 93%                          334
Taenia crassiceps: 301 ANMLREQVAQLKQKVMNHVNSGCQLMLTQQLQTF                          334
Taenia solium: 301 LREQVAQLKQKVMNHVNSGCQLMLTQQLQTF                                 331
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Fig. 2. *Taenia crassiceps* and *T. solium c-jun* gene sequence and predicted amino acid sequence alignment. (A) Nucleotide sequence of *T. crassiceps* and *T. solium c-jun* gene. The positions of the start codon and the oligonucleotides used for PCR amplification are underlined. The nucleotide number are by reference to the start of the 5′ UTR when the *T. crassiceps* and *T. solium* sequence is aligned with other known mammalian *c-jun* sequences. Asterisks (*) indicate a change in nucleotide sequence. (B) Alignment of the *c-fos* protein sequences in rat (*Rattus rattus*), mouse (*Mus musculus*), human (*Homo sapiens*), and cestodes *T. crassiceps*, and *T. solium*. Amino acids are represented by single-letter codes. Dotted lines represent unidentified amino acids, and the X represents highly variable regions, meaning that X can be any amino acid.

On the other hand, *T. crassiceps* and *T. solium c-jun* genes (Fig. 2) have a higher sequence homology (98%). Interestingly, the homology is also higher when compared with both helminth gene sequences to their mammalian counterparts (mouse 93%, rat 98%, and human 93%) (Fig. 2). Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the Accession numbers: AY436613, AY436614, AY436615 and AY436616.

In view of the fact that different mRNAs can have varying half-lives and may undergo selective degradation, the quantity and integrity of total RNA extracted from the different sources used in this study was compared. The same amount of RNA (1 μg) that was non-degraded was used for RT–PCR amplification in each tissue studied. The RT–PCR amplification of the *c-fos* sequences for *T. crassiceps* and *T. solium* is shown in Fig. 3A. It is clear that a specific fragment (274 nt) that corresponds in molecular weight to that of *c-fos* was obtained in both control tissues, as well as in *T. crassiceps* and *T. solium*. Note that in both parasites the intensity of the band for *c-fos* amplification is 10 times stronger than the control tissue, using the same total RNA amount. β-actin was constantly expressed in all tissues amplified. In Fig. 3A the amplification by RT–PCR of the *c-jun* gene is also shown in *T. crassiceps* but not in *T. solium*. A specific fragment (325 nt) that corresponds in molecular weight to that of *c-jun* was obtained from mouse testes and pig muscle total RNA. β-actin was constantly expressed in all tissues amplified. By

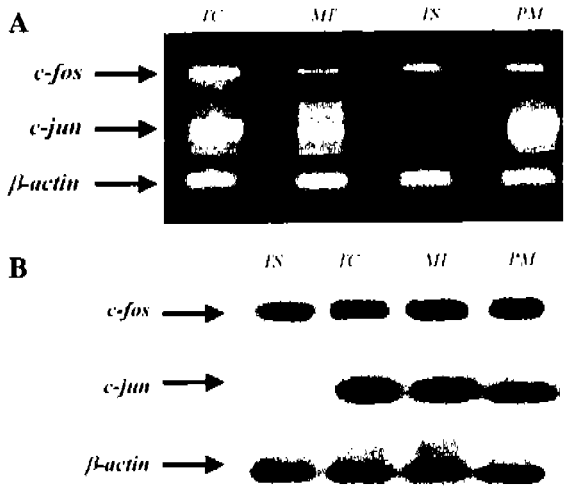

Fig. 3. Analysis of the expression of *c-fos* and *c-jun* gene in *Taenia crassiceps* and *T. solium* cysticerci. (A) A representative RT–PCR of total RNA from *T. crassiceps* (TC), *T. solium* (TS), mouse (MT) testes and pig muscle (PM) showing the detection of *c-fos* and *c-jun*. β-actin was used as the constitutively expressed control gene in all lanes. (B) Representative Northern blot of total RNA from *T. crassiceps* (TC), mouse testes (MT), *T. solium* (TS) and pig muscle (PM), showing the detection of *c-fos* and *c-jun*. β-actin was used as a control of RNA integrity, as well as a contitutively expressed gene to show that *T. solium* does not express the *c-jun* gene.

using degenerated oligonucleotides of the *c-jun* gene as primers, no expression of *c-jun* gene was detected in *T. solium* either (not shown).

As a demonstration that *c-fos* and *c-jun* play a physiological role in both species of cysticerci, and that the lack of expression in *T. solium* found by RT–PCR was not artifactual or due to a trivial reason such as non-specificity of the oligonucleotides used, Northern blot analyses were performed. In Fig. 3B it is shown that both *T. crassiceps* and *T. solium* expressed the *c-fos* gene at similar levels. However, when *c-jun* gene expression was assessed, it was found that only *T. crassiceps* expressed this gene (Fig. 3B). It is important to mention that β-actin was expressed in all RNA samples analysed, supporting the notion that *T. solium* cysticerci do not express the *c-jun* gene.

The fact that *c-fos* and *c-jun* genes are regulated in the same way as their mammalian counterparts in both species of cysticerci, was demostrated by way of RT–PCR amplification of cultured *T. crassiceps* and *T. solium* cysticerci in the presence of oestradiol, a steroid hormone that is known to induce *c-fos* and *c-jun* expression in mammalian cells. In Fig. 4A the pattern of expression for *c-fos*, is shown and it is clear that E_2 treatment strongly augmented *c-fos* expression in *T. crassiceps*, but not in *T. solium*. In Fig. 4A, a representative Northern blot is shown for *c-jun*, in which it is clear that E_2 stimulates *c-jun* expression in *T. crassiceps*, but has no effect whatsoever in *T. solium*.

In Fig. 4B a Southern blot analysis is depicted of *c-jun* and *c-fos* in *T. solium* and *T. crassiceps* cysticerci. Southern blot and hybridization probing of *Eco*RI-digested *T. crassiceps* and *T. solium* genomic DNA identified DNA fragments in each sample that hybridized with the *c-fos* and *c-jun* cDNA probes. *T. crassiceps* and *T. solium* genomic DNA produced restriction fragments of 2·37 kb when hybridized with the *c-fos* probe, while when hybridized with *c-jun* both produced a 4·5 fragment (not shown).

When treated with E_2, *T. crassiceps* cysticerci exhibited amplified copy number of the *c-fos* gene, while *T. solium* did not. The level of *c-fos* amplification in *T. crassiceps* ranged from 2·4 to 3·6. The same pattern was seen when the *c-jun* gene was similarly examined in the same samples in both parasites: an increased (1·9 to 2·3) copy number in *T. crassiceps c-jun* gene as the oestradiol dose increased, while there was no change in the level of *c-jun* gene in *T. solium*. The β-actin gene remained constant.

In Table 2, the effect of oestradiol on *in vitro* reproduction of *T. crassiceps* and *T. solium* is shown. The number of buds was augmented in *T. crassiceps* cysticerci by 120% when they were stimulated with 40 μg of E_2 compared with untreated cysticerci. On the other hand, when *T. solium* cysticerci were treated with E_2, no effect on reproduction was recorded, as compared with untreated cysts. E_2 did not affect viability in any species of cysticerci tested, and reproduction was correlated with AP-1 complex gene expression in *T. crassiceps*.

To further demostrate that *c-fos* and *c-jun* genes were of parasite origin and not obtained artifactually by host cell contamination, we eliminate all possible host cell contamination after parasites being extracted directly from host tissues by culturing them for 1 week in a free-serum medium, which maintains parasites, but not mammalian cells. Parasite cultures were then checked for possible DNA and RNA host cell traces, amplifing a highly specific gene product, which has evolved only in mammals, such as the fragment VDRHC (Variable Region of the Heavy Chain of the immunoglobulins), which showed no amplification in DNA or RNA from the parasite, but gave positive in the host tissues (not shown). The same result was obtained when another highly evolved gene (as TNF-α) was used as a template: the mouse and pig tissues amplified it, whereas *T. solium* and *T. crassiceps* did not (not shown). β-actin was expressed to a similar level as in mouse and pig tissues, demostrating DNA and RNA integrity.

DISCUSSION

This study reports the expression of *c-fos* in *T. crassiceps* and *T. solium* cysticerci and of *c-jun* only in *T. crassiceps*. The presence of both *c-fos* and *c-jun*

Table 2. *In vitro* reproduction and AP-1 relative expression of *Taenia crassiceps* and *T. solium* cysticerci in the absence (C) or presence of a dose of 40 μg of oestradiol (E_2) during five days in culture

Species of cysticerci	Number of buds (av. per cultured well)		Relative expression *c-fos*/β-actin		Relative expression *c-jun*/β-actin	
	C	E_2	C	E_2	C	E_2
T. crassiceps	0	25*	1·2	120·8*	1·6	90·3*
T. solium	0	0	1·5	1·8	0	0

* $P<0{\cdot}01$.

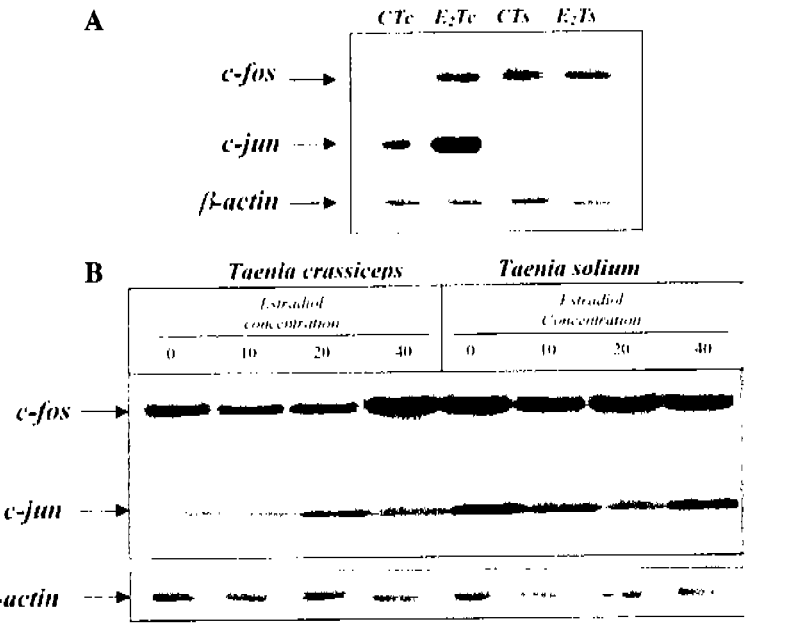

Fig. 4. Northern and Southern blot analyses of *c-fos* and *c-jun* gene expression in cultured and estradiol-treated cysticerci. (A) Representative Northern blot of *c-fos* and *c-jun* expression in response to 40 μg of oestradiol (E_2) from Tc (*Taenia crassiceps*) and *T. solium* compared against unstimulated *T. crassiceps* (CTc) and unstimulated *T. solium* (CTs) cysticerci. (B) Southern blot analysis of genomic DNA for *c-fos* and *c-jun* in *T. crasiceps* and *T. solium* cysticerci. β-actin was used as a control of DNA integrity, and to show that regulation of AP-1 complex genes by estradiol is specific, as well as a contitutively expressed gene to show that *T. solium* possesses the *c-jun* gene, but does not express it. In both blots, β-actin was used as a control, constitutively expressed gene and remained unaltered.

could help to explain the high asexual proliferation rate of *T. crassiceps* in female and feminized male mice, as well as the strong effects of sex-steroids leading to increased parasite loads (Bojalil *et al.* 1993; Larralde *et al.* 1995; Morales-Montor *et al.* 2001, 2002). The sequenced parasite *c-fos* and *c-jun* gene fragments code for the proteins' DNA binding domains, that are the most highly conserved among species, as shown also by our finding that they are identical in *T. solium* and *T. crassiceps*. Most certainly, these parasite genes must be important in the physiology, development and asexual reproduction of the parasite, as they are for many other organisms. Possibly these parasite genes are also important for the host, should the proteins coded reach the host cells and influence their activities in a variety of processes such as inflammation, immunity and cell proliferation (Barnes & Adcock, 1998).

To show the integrity, size and expression level of these genes in parasite RNA, we performed Northern blot analysis. Our results reinforced the notion that *c-fos* is expressed in both species of tapeworm, but *c-jun* is only expressed in *T. crassiceps*, and not in *T. solium*, which is very intriguing. Lack of *c-jun* expression in *T. solium* could result either from host or parasite regulation factors, since Southern blot analysis showed a transcript for *c-jun* in *T. solium*, which demostrates that it does have the gene, but it is not being expressed.

It is well known that *c-fos/c-jun* heterodimers are the most functional at the transcription level (Chinenov & Kerppola, 2001). Thus, *T. solium* cysticerci expressing only *c-fos* would limit the effective transcription of the genes involved in asexual reproduction that characterizes *T. crassiceps* cysticerci. Indeed, the lack of *c-jun* expression in *T. solium* cysticerci is congruent with the fact that this parasite is incapable of asexual reproduction. Although *c-jun* can form a functional homodimer, so far it has not been demonstrated that *c-fos* functions in this way. In this regard, it is very intriguing to understand how *c-fos* alone functions in *T. solium*. It would be important to ascertain which of both possibilities is operating, since *T. solium* cysticerci frequently degenerate and calcify spontaneously and harmessly in its human host's central nervous system for unknown reasons. If these reasons involve regulation of the expression of parasite transcription factors, the possibility of therapy to shut down these genes is opened.

The connection between sex steroids and parasitism is progressively being recognized as decisive in the outcome of several parasite infections (Alexander & Stimson, 1988; Klein, 2000). For instance, in mammals, sex steroids participate in a large number of physiological processes such as sexual behaviour (Maggi & Pérez, 1985), reproduction, and development (Knobil *et al.* 1988). Oestradiol regulates these processes by inducing DNA replication and cellular proliferation through changes in the expression of several proto-oncogenes such as *c-fos* (Kushner *et al.* 2000) and a variety of genes from the *jun* family (Ransone & Verma, 1990; Nephew *et al.* 1993).

Likewise, the influence of *c-fos* and *c-jun* genes on reproductive behaviour affect the evolution of the host and parasite species (McLennan & Brooks, 1991). The inclusion of gene complexes, such as AP-1 in the parasite, with such potent and wide effects on various cellular processes involved in host–parasite relationships may help in understanding a number of major questions in parasitology, like host–parasite specificity, sexual dimorphism, the possible relation with oncogenesis, antigenic parasite variation, and the type of effective immune responses, to name a few.

Comparison of the amino acid sequence encoded by the conserved regions of the taeniid genes further indicates that both *c-fos* and *c-jun* proteins are highly conserved in relation to other parasite genes. Members of the *c-fos* and *c-jun* gene family have two exons encoding two DNA binding domains that are alternatively spliced, producing transcripts that encode proteins having either one or two binding protein domains, which is further indicative that these are transcription factors proteins. Elucidation of features common to the genes encoding these proteins will lead to a better understanding of the role that they play in the parasites' biology.

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HOST GENDER IN PARASITIC INFECTIONS OF MAMMALS: AN EVALUATION OF THE FEMALE HOST SUPREMACY PARADIGM

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ABSTRACT: A review of current literature on mammalian hosts' sexual dimorphism (SD) in parasitic infections revealed that (1) it is a scarcely and superficially studied biological phenomenon of considerable significance for individual health, behavior, and lifestyles and for the evolution of species; (2) there are many notable exceptions to the rule of a favorable female bias in susceptibility to infection; (3) a complex network of molecular and cellular reactions connecting the host's immuno-neuro-endocrine systems with those of the parasite is responsible for the host–parasite relationship rather than just an adaptive immune response and sex hormones; (4) a lack of gender-specific immune profiles in response to different infections; (5) the direct effects of the host hormones on parasite physiology may significantly contribute to SD in parasitism; and (6) the need to enrich the reductionist approach to complex biological issues, like SD, with more penetrating approaches to the study of cause–effect relationships, i.e., network theory. The review concludes by advising against generalization regarding SD and parasitism and by pointing to some of the most promising lines of research.

THE FEMALE HOST SUPREMACY PARADIGM IN PARASITIC INFECTIONS

It is widely held that female mammals are more resistant to parasitic infections than males because of gender-associated differences in exposure and of testosterone's immunosuppressive properties. The paradigm implies that sexual dimorphism (SD) to parasitism is mediated only, or principally, by the host's immune system and usually disregards the parasites' direct response to the distinct sex-steroid profiles of their female and male hosts.

The female supremacy paradigm in parasitic infections has existed for a long time (Addis, 1946), and despite thoughtful recommendations against simplification (Zuk and McKean, 1996), it is rarely questioned. In several prestigious journals, however, it has recently been a matter of debate, particularly in association with mortality trends in humans (Moore and Wilson, 2002; Owens, 2002) and several infectious diseases (Zuk and McKean, 1996; Doprado et al., 1998; Watanabe et al., 1999; Klein, 2000; Ganley and Rajan, 2001; Hughes and Randolph, 2001; Roberts et al., 2001; Verthelyi, 2001). It has also been associated with a number of broader subjects, i.e., evolution of sexual reproduction (Zuk, 1994), decision making of the host, social hierarchy (Barnard et al., 1998; Gourbal et al., 2002), mating behavior (Kavaliers and Colwell, 1993; Morales et al., 1996; Willis and Poulin, 2000), and energy costs of infection and the immune response (Hansen et al., 2003). We decided to reexamine the paradigm in the light of the current understanding of the immune and endocrine systems of potential hosts because we found that it conflicted with our observations regarding experimental murine cysticercosis caused by *Taenia crassiceps*.

EXPERIMENTAL MURINE *TAENIA CRASSICEPS* CYSTICERCOSIS CONFLICTS WITH THE PARADIGM

Taenia crassiceps is an intestinal cestode of canines (definitive host) and of various extraintestinal tissues of rodents (intermediate host) in its larval (cysticercus) stage (Freeman, 1962). Experimental cysticercosis caused by *T. crassiceps* in mice simply requires the intraperitoneal injection of live cysticerci (Culbreth et al., 1972). Intraperitoneal cysticerci reproduce asexually by exogenous budding, developing massive parasite loads in a few months (Smith, Esch et al., 1972; Smith, Parrish et al., 1972) that may even approximate the host's body weight, without causing it apparent discomfort (Larralde et al., 1995). The cysticerci also survive and reproduce in vitro under usual culture conditions in media free of fetal calf serum. These features of experimental murine cysticercosis have made it a convenient model in studying the immunological, genetic, and sexual factors involved in susceptibility to infection and parasite proliferation (Sciutto et al., 2002). Sexual differences to infection in mice are still a matter of research. Thus, experimental findings have shown that in different congenic and syngenic strains of mice, females become infected more often than males and carry more cysticerci than males, with significant between-strain variations (Sciutto et al., 1991; Huerta et al., 1992; Larralde et al., 1995; Terrazas et al., 1998; Morales-Montor, Baig et al., 2001; Morales-Montor, Baig, Hallal-Calleros et al., 2002; Morales-Montor, Baig, Kabbani et al., 2002; Morales-Montor, Hallal-Calleros et al., 2002). Estrogens favor parasite reproduction, whereas androgens appear to inhibit it (Bojalil et al., 1993; Terrazas et al., 1994; Morales-Montor, Baig, Hallal-Calleros et al., 2002). Gonadectomy and thymectomy equalize parasite loads between sexes by greatly increasing those in males and slightly decreasing those in females (Huerta et al., 1992; Terrazas et al., 1994; Morales-Montor, Baig, Hallel-Calleros et al., 2002). Male mice are better protected by vaccination than females (Cruz-Revilla et al., 2000). Externally administered 17β-estradiol and dihydrotestosterone (DHT) are able to restore parasite loads to their normal levels in castrated animals. T cells, but not antibodies, also restore the effects of thymectomy (Bojalil et al., 1993). The TH1 response hinders parasite growth early in infection (Terrazas et al., 1999; Toenjes et al., 1999; Spolski et al., 2000; Rodriguez-Sosa et al., 2002), whereas the TH2 response prevails at later times of infection but is incapable of slowing parasite growth (Terrazas et al., 1998; Toenjes et al., 1999). In chronic infections, the male mouse is feminized (estrogenized and deandrogenized) to a degree that

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inhibits male sexual behavior (Morales et al., 1996). Feminization is apparently caused by overexpression of P-450 aromatase (Morales-Montor, Hallal-Calleros et al., 2002), triggered by the high levels of interleukin-6 (IL-6) in late infections (Morales-Montor, Baig et al., 2001; Morales-Montor, Mohamed et al., 2001). Cytokine profiles of infected male and female mice do not show major differences, except for the levels of IL-4, which are higher in males during early infection only (Terrazas et al., 1998).

These findings led to the initial proposal of a sex steroid, immunoendocrine interaction that controls the reproduction of cysticerci, one in which androgens were postulated to favor a TH1 response that limits parasite growth and in which estrogen favors a TH2 response that permits parasite reproduction (Huerta et al., 1992; Bojalil et al., 1993; Terrazas et al., 1994; Morales-Montor, Baig et al., 2001; Morales-Montor, Baig, Hallal-Calleros et al., 2002; Morales-Montor, Baig, Kabbani et al., 2002). Other studies also support the purported correlation between androgens and TH1 response and between estrogen and TH2 response (Morales-Montor, Baig et al., 2001; Morales-Montor, Baig, Hallal-Calleros et al., 2002; Morales-Montor, Baig, Kabbani et al., 2002; Morales-Montor, Hallal-Calleros et al., 2002).

Signs of SD in cysticercosis were recently reported for other host and taeniid species. For example, sex steroids have been implicated in porcine cysticercosis caused by *Taenia solium* because both castration and pregnancy nearly double the prevalence of naturally acquired cysticercosis in rural pigs (Morales et al., 2002). In humans, women are more frequently afflicted than men by severe neurocysticercosis (Del Brutto et al., 1988) and show higher inflammatory profiles (Fleury et al., 2003). Because sex steroids affect experimental *T. crassiceps* infections in laboratory mice and extend to natural infections of *T. solium* in humans and pigs, as well as *Trichinella spiralis* in rats (Klein et al., 1999), our initial suspicions regarding the general validity of the female supremacy paradigm were strengthened.

Other ongoing research has shown that in *T. crassiceps* murine cysticercosis several physiological systems of the host respond to the host's sex hormones, and so does the parasite either by limiting or by prompting its reproduction. For example, the central nervous system of infected and feminized male mice responds to intraperitoneal infections by overexpresssion of the *c-fos* gene in the hypothalamus, hippocampus, and preoptic area (Morales-Montor, Arrieta et al., 2003). It would appear that hormonal changes induced in the host act to promote the overexpression of the *c-fos* gene involved in cellular differentiation and proliferation of both parasite and host cells (Escobedo et al., 2004), as they do in other stress and immune challenges (Pacheco-Lopez et al., 2002). Thus, in murine cysticercosis, parasite proliferation is responsive not only to the host immune system and testosterone but also to a complex network that integrates the nervous, immune, and endocrine systems of the host and the parasite's physiological systems. The conflict between male biases in *T. crassiceps* cysticercosis with the host female supremacy paradigm expectations is undeniable and requires explanation.

TESTING THE FEMALE HOST SUPREMACY PARADIGM IN A WIDER REPERTOIRE OF PARASITIC INFECTIONS

The paradigm of female host supremacy in parasitic infections of mammals, as well as the robustness of the corresponding endocrinological and immunological factors postulated as its mechanisms, was evaluated using current literature (Medline, n = 110: 1995–2002), as well as several frequently cited classic articles and a few recent ones published in 2003. After examining this literature, however, the general validity of the female supremacy paradigm was seriously weakened by too many exceptions. What emerged was a complex host immuno-neuro-endocrine network that was related to the parasite physiologically and that seems more likely to control the complexities involved in certain host–parasite interactions than testosterone alone.

When infections that documented sex bias in infection parameters (Infection-SD) or immune profiles (Immune-SD) were found, the possibility of a cause–effect relationship was examined further. Each infection was classified as either sexually dimorphic, noting the sex favored by the bias (females > males or females < males), or undefined (females = males). Infection-SD was evaluated in terms of prevalence, intensity, severity, morbidity, mortality, hormonal profiles, or behavioral changes in infected animals. The biological meaning of these parameters differs substantially. Thus, some relate to the probability of infection, i.e., prevalence, and others to the outcome of infection, i.e., mortality, but they were assumed to be equivalent indications of sex bias. Immune-SD parameters include antibody production, lymphoid cell responses to mitogens or antigens, cytokine production, hypersensitivity reactions, and protective effects of vaccination. Immune-SD parameters also have important functional differences in their nature and context of expression, i.e., populations, individuals, cells, and molecules, in their role as effectors or mediators of immune responses, in their operation under in vitro or in vivo conditions, and in their ability to protect from infection. These immune parameters were scored as "greater than" or "smaller than" with respect to the opposite gender. The Immune-SD and Infection-SD data collected were used to examine how the 2 are connected.

FIRST GENERAL SIGNS OF PARADIGM WEAKNESS

The total number of references examined is relatively small, i.e., only 110. The search identified just 46 different parasite species occurring in 10 species of mammalian hosts, a minute sample considering the many thousands of parasite (Hoberg, 1997) and mammalian species (Anderson et al., 1984). Forty-three references reported SD (Table I), and the rest (67) were concerned with molecular interactions between the endocrine and immune systems or with hormonal, behavioral, and immune effects on the host (Fig. 1).

The human medicine bias in SD research is obvious because 56% of all the articles were oriented to the study of infections affecting humans or experimental animal infections (usually in rodents) having a human counterpart, i.e., malaria, schistosomiasis, trypanosomiasis, toxoplasmosis, and cysticercosis. Most references in the list do not directly explore SD but rather describe the in vitro effects of sexual hormones or cytokines on the immediate response of some immunological or endocrinological component derived from hosts of either sex in rather

TABLE I. Infection-SD or Immune-SD in different host–parasite relationships documented to date (some parasites infect more than 1 host species).

Parasite	Host	Dimorphism	Prevalence	Intensity	Severity	Mortality	Mechanisms	Other observations	Reference
Brachylaima cribbi	Mice	Yes	♀ < ♂	♀ < ♂				Expulsion of worms in C57 BL/6J mice is mediated by an immune response	Butcher et al. (2002)
Brugia malayi	Human	Yes	♀ < ♂	♀ < ♂					Ganley and Rajan (2001)
Brugia pahangi	Rat	Yes	♀ < ♂	♀ < ♂					Bell et al. (1999)
Dipetalonema vitae	Hamster	Yes	♀ < ♂	♀ < ♂			17β-estradiol and progesterone are associated with protection in females		Reynouard et al. (1984)
Eimeria vermiformis	Mice							Females distinguish between infected and noninfected males	Kavaliers and Colwell (1993), Kavaliers (1995)
Heligmosomoides polygrus	Mice						Peripheral immune response is reduced in infected males and it is associated to higher levels of corticosterone	High-ranking infected males are less aggressive	Barnard (1998)
Heterakis spumosa	Mice	Yes	♀ < ♂	♀ < ♂			Testosterone favors the development of the parasite and its survival		Harder et al. (1992)
Hymenolepis diminuta	Rat	Yes in response to treatment					Infected males have decreased levels of testosterone in plasma	A deficient diet in vitamin G complex inhibits growth of the parasite in females only. Females distinguish between infected and noninfected males	Addis (1946), Wills and Poulin (2000)
Ixodes ricinus	Voles						Testosterone reduces innate and acquired resistance to tick feeding	Tick feeding favors transmission of other parasites	Hughes and Randolph (2001)
Leishmania donovani	Mice	Yes	♀ < ♂	♀ < ♂				Macrophages treated in vitro with testosterone have an increased number of promastigotes	Zhang et al. (2001)
Leishmania major	Mice	Yes	♀ < ♂	♀ < ♂				Testosterone treatment in females increases parasite number and orchidectomy in males decreases it	Mock and Nacy (1988)

TABLE I. Continued.

Parasite	Host	Dimorphism	Prevalence	Intensity	Severity	Mortality	Mechanisms	Other observations	Reference
Schistosoma mansoni	Hamster	Yes	♀ < ♂	♀ < ♂					Barrabes et al. (1980)
Strongyloides ratti	Rat	Yes	♀ < ♂	♀ < ♂				Testosterone treatment or macrophage blocking increases worm recovery in females	Watanabe et al. (1999)
Strongyloides venezuelensis	Rat	Yes	♀ < ♂	♀ < ♂			Testosterone restitution increases susceptibility and estradiol restitution decreases it	Gonadectomy in male rats decreases parasite loads, whereas it increases the loads in females	Rivero et al. (2002a, 2002b)
Taenia crassiceps	Mice	Yes	♀ > ♂	♀ > ♂			During infection there is a TH1–TH2 shift; in the acute infection IL-12 promotes CD4+-specific proliferation; in the chronic infection, IL-6 predominates and stimulates the aromatase activity, which increases serum estradiol in males	Chronically infected males lose their sexual behavior. Infection changes the response to the predator, facilitating the parasite cycle. Vaccination is more effective in males than in females	Sciutto et al. (1990, 1991), Larralde et al. (1995), Morales et al. (1996), Terrazas et al. (1998, 2002), Gourbal et al. (2001), Morales-Montor, Baig et al. (2001)
Taenia solium	Pigs		♀Nonpregnant > ♀pregnant; ♂noncastrated > ♂castrated				Low levels of androgens or high levels of estrogens probably influence susceptibility to infection		Morales et al. (2002)
Taenia solium	Human	Yes			♀ > ♂			Women develop a greater degree of inflammation when cysticerci are found in brain parenchyma and have more CSF* inflammation and increased cellularity in the CSF than men	Del Brutto et al. (1988), Fleury et al. (2003)
Toxoplasma gondii	Mice	Yes	♀ > ♂	♀ > ♂	♀ > ♂	♀ > ♂	Male SCID* more rapidly produce IL-12 and higher levels of IFN-γ. Males produce higher levels of TNF-α and IFN-γ at the onset of the infection, controlling parasite multiplication	Testosterone treatment reduces parasite numbers and mortality in females. Infection produces infertility in females	Stahl (1994), Roberts et al. (1995), Walker et al. (1997), Liesenfeld et al. (2001)

TABLE 1. Continued.

Parasite	Host	Dimorphism	Prevalence	Intensity	Severity	Mortality	Mechanisms	Other observations	Reference
Trichinella spiralis	Voles	Yes	♀ < ♂	♀ < ♂				Polygamous males have higher testosterone levels than monogamous males concomitant to infection	Klein et al. (1999)
Trypanosoma cruzi	Mice	Yes	♀ < ♂	♀ < ♂			Intact males have higher levels of lytic antibodies	Dominant males have higher levels of testosterone and are less parasitized. Ovariectomy increases infection; estrogen replacement reduces the parasitemia. Orchiectomized males have fewer parasites than controls; testosterone replacement increases parasitemia	Deprado et al. (1998, 1999), Schuster and Schaub (2001)

* CSF, cerebrospinal fluid; SCID, severe combined immunodeficiency disease.

unrealistic in vitro conditions, i.e., cell culture media containing (contaminated with) fetal calf serum rich in growth factors and antibiotics. The biochemical results were then mistakenly assumed to operate in a similar manner in the more complicated context of an in vivo infection.

The analysis developed by the literature search casts doubt on the validity of the general female supremacy paradigm. The most notable shortcomings relate to (1) the poor representation of host–parasite systems among cited references, (2) the heavy human medical bias of the more thoroughly explored infections, (3) the unequal meanings of infection and immune parameters measuring SD in host susceptibility to infection, (4) the questionable protective function for many of the immune parameters, and (5) the excessive use of the reductionist approach in explaining events occurring at higher levels of complexity by way of the direct extrapolation of events occurring in vitro.

THE PREVALENCE AND MECHANISMS OF SD IN SPECIFIC PARASITE INFECTIONS

Table I summarizes the 43 references describing 32 infections in 8 host–parasite systems (some parasite species infect more than 1 host species, and 1 host species is infected by more than 1 parasite species) that provided information on the subject of Infection-SD or Immune-SD. In this data set, in 22 of 32 instances (68%) of the 8 systems, females fared better than males in prevalence, intensity, or consequence of infection (severity), varying from insignificant to pronounced. In 5 of 32 instances (16%), males scored better than females, and in 5 of 32 cases (16%), results could not be defined one way or another. In effect, 32% were exceptions to the paradigm. Furthermore, it is of interest to note (Table I) that severity of infection and mortality indicators were not studied as extensively as prevalence and intensity of infection. Severity of infection was reported in only 28% of the cases cited, and in 60% of these situations, female hosts fared better than males. Only in human schistosomiasis, by *Schistosoma haematobium*, was severity the same for both sexes. Mortality, in contrast, was only reported in 19% of the infections, half of which favored female hosts and the other half males. It is clear, therefore, that SD in severity and mortality have been insufficiently explored to make general and categoric statements.

Sex-associated immunological differences (Immune-SD) were reported in only 10 of 32 infections, and multifeatured immune profiles are described in only 5. Table II focuses on the 5 host–parasite systems in which several infection and immune parameters were evaluated at the time of infection. No uniform pattern or sex bias is discernable. In malaria, toxoplasmosis, and cysticercosis, infections are more prevalent and intense in female than in male mice. However, in leishmaniasis and schistosomiasis, it is the male host that is more frequently and intensely parasitized. The immune parameters studied also vary in each infection, without clear association to infection parameters. Comparison of immune profiles in all 5 infections is possible only for interferon-γ (IFN-γ), which was found to be more elevated in males than in females in all but 1 infection (murine leishmaniasis). Levels of tumor necrosis factor-α (TNF-α) were greater in females than in males with malaria; however, in leishmaniasis, schistosomiasis, and toxoplasmosis, males exhibited higher levels of TNF-α than females. In leishmaniasis

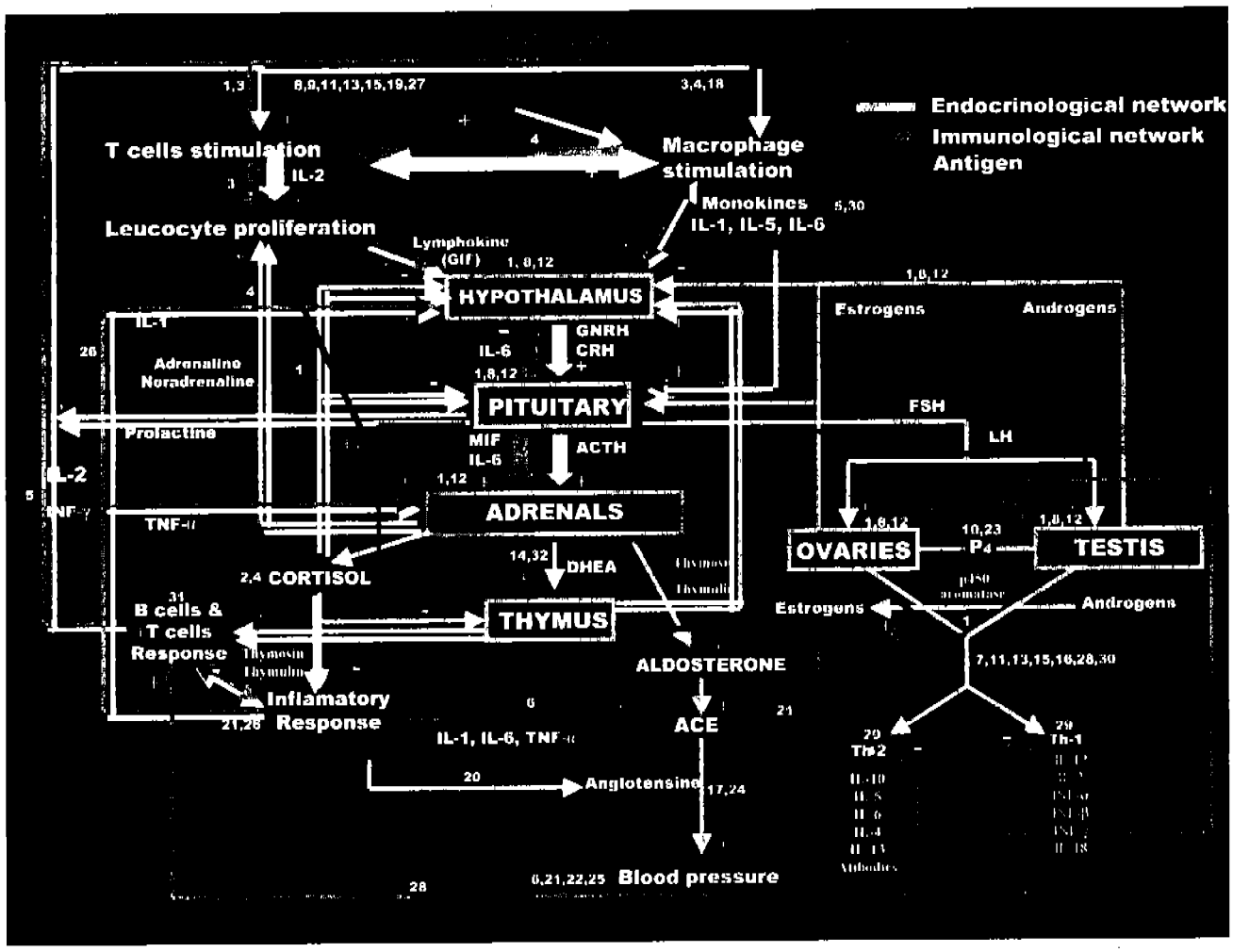

FIGURE 1. The HINEP network circuitry. The magnitude and complexity of the network includes practically all the host's sexual hormones and many of the immunological components described connecting among themselves and with the parasites systems. Arrows (←, ↑, →, ↓) denote connections between nodes; each points to the direction of the signal. ± Signs refer to stimulatory or inhibiting effects. The numbers near each connection code denote the references cited in the figure: (1) Spinedi et al. (2002), (2) Esch (2002), (3) Medzhitov and Janeway (2002), (4) Murtaugh and Foss (2002), (5) Henri et al. (2002), (6) Pramparo (2002), (7) Hughes and Randolph (2001), (8) Verthelyi (2001), (9) Roberts et al. (2001), (10) Thaker et al. (2001), (11) Liesenfeld et al. (2001), (12) Morales-Montor, Baig et al. (2001), (13) Taylor-Robinson (2001), (14) Kurtis et al. (2001), (15) Remoue et al. (2001), (16) Ganley et al. (2001), (17) Salzet and Verger-Bocquet (2001), (18) Zhang et al. (2000), (19) Soliman et al. (2001), (20) Feterowski et al. (2001), (21) Peeters et al. (2001), (22) Chae et al. (2001), (23) Barnea (2001), (24) Franco et al. (2001), (25) Gavras (2001), (26) Weinstock and Elliott (2000), (27) Grossman (1989), (28) Zhang et al. (2000), (29) Balemba et al. (1998), (30) Benedetto et al. (2000), (31) Hunter and Reiner (2000), and (32) Freilich et al. (2000).

and schistosomiasis of mice and humans, respectively, Immune-SD is observed in IFN-γ and TNF-α. In contrast, infection by *Leishmania mexicana* shows no dimorphism in IL-4, IL-10, and IL-12, whereas in infections with other species of *Leishmania*, there is a clear increase in IL-4 and IL-10 in males measured at the site of the lesions. In schistosomiasis, IL-10 production is clearly dimorphic (higher levels favoring females). Murine leishmaniasis exhibited the least dimorphic TH2 cytokine profile of the 5 infections, which contrasts with its very significant favorable female bias toward infection. In male-biased murine cysticercosis, INF-γ was higher in males; IL-2, IL-6, and proliferative responses were equal in both sexes, and IL-10 was higher in females. In addition, in murine cysticercosis, there was a reversal in the sexual bias toward IL-4 with time of infection. Males have higher amounts of IL-4 than females in early infection, but this is reversed in chronic infection. No change in profiles with time of infection was reported for the other 4 parasitic infections. These observations support the suspicion that the relationship between SD to infection, and the immune system's mediating effects are not simple and clearly involve many of the immune effectors. The host's immune response does not seem to be gender specific because no clear sex-related strategy can be detected. One would expect that hosts would have evolved immunological responses that are complementary to parasite strategies at different times of infection, number of parasites, location in the host's tissues, and offensive and defensive mechanisms. For example, extracellular stages of the parasite would be vulnerable to antibodies and

TABLE II. Host–parasite relationships in which several infection and some immune parameters were measured at some point during infection. The immune parameters collected varied in each infection.

Parasite	Host	Dimorphism	Prevalence	Intensity	INF-γ	TNF	IL-2	IL-4	IL-6	IL-10	IL-12	IgA	Specific proliferation	Reference
Leishmania mexicana	Mice	Yes	♀ < ♂	♀ < ♂	♀ > ♂	♀ < ♂		♀ = ♂		♀ = ♂	♀ = ♂			Satoskar and Alexander (1995)
Leishmania spp.	Hamster	Yes	♀ < ♂	♀ < ♂				♀ < ♂		♀ < ♂				Travi, Arteaga et al. (2002)
Schistosoma haematobia	Human	Yes	♀ < ♂	♀ < ♂	♀ < ♂	♀ < ♂				♀ > ♂		♀ > ♂	♀ < ♂	Remoue et al. (2001)
Taenia crassiceps	Mice	Yes	♀ > ♂	♀ > ♂	♀ < ♂*		♀ = ♂	♀ < ♂*	♀ = ♂	♀ > ♂*			♀ = ♂	Terrazas et al. (1998)
								♀ > ♂†						Li (1999)
Plasmodium chabaudi	Mice IL-10$^{-/-}$	Yes	♀ > ♂	♀ > ♂	♀ < ♂	♀ > ♂		♀ = ♂			♀ = ♂			
Toxoplasma gondii	Mice	Yes	♀ > ♂	♀ > ♂	♀ < ♂	♀ < ♂					♀ < ♂		♀ < ♂	Roberts et al. (1995), Walker et al. (1997)

* In acute infection.
† In chronic infection.

complement because of their direct effect on the parasite's external surface (Philipp et al., 1980). Such antibody-mediated damage has been documented to affect some parasite life cycle stages but certainly not all, i.e., tachyzoites in toxoplasmosis (Johnson and Sayles, 2002), early larvae in cysticercosis (Restrepo et al., 2001), merozoites in malaria (Daly and Long, 1995), trophozoites in amoebiasis (Ghosh et al., 1998), and promastigotes in leishmaniasis (Rafati et al., 2001). However, some of the extracellular stages of parasites manage to escape from the circulation and become sequestered inside their target cells, apparently unscathed by antibodies or other harmful immune effectors. An immune response against intracellular parasites would largely depend on the expression of parasite antigens on the infected cell's membrane (Kyes et al., 2001) and the triggering of innate immune effectors. Attraction of effector leukocytes (polymorphonuclear leukocytes, eosinophils, and basophils), cytotoxic T lymphocytes, and natural killer (NK) cells to the site of parasite infection should aid in parasite destruction or at least in containment of their growth or dispersal. Thus, an inclination toward TH2 profiles, with high levels of antibody production, would be most appropriate against extracellular stages of parasites. A TH1 response, which kills infected cells, would be most effective against intracellular parasites (Sher et al., 1992). If female hosts truly favor TH2 responses, they should be more resistant to extracellular parasites and to intracellular infections in their initial stages when the parasites are migrating toward their protected intracellular locations. In contrast, if androgens favor TH1 responses, then males should handle intracellular parasites more effectively, especially during late stages of infection. Despite the attractive congruence of these speculations on immunity and SD to infections, Table II shows no clear sign of such patterns in the strategies of hosts or parasites. Lack of congruity between expected and observed results most likely come from unsound expectations regarding the role of the immune system in sexually dimorphic parasitic infections.

A detailed understanding of the mechanisms leading to the destruction of host or parasite or to a mutually tolerable stalemate requires further research and the application of a more potent and discerning technology. It might also be useful to use other conceptual approaches in exploring host–parasite systems, i.e., SD and host–parasite specificity, among others. Most research on these topics uses a reductionist approach, attempting to link microscopic events and effectors. However, the network or systems approach, where some functions are distributive to the entire network and not localized in some of its parts, is rarely considered (Oltvai and Barabasi, 2002; Strohman, 2003).

THE HOST–IMMUNO-NEURO-ENDOCRINE–PARASITE NETWORK IN CHARGE OF INFECTION AND SD

The usual experimental strategy for examining the mechanisms of immunoendocrine interactions is to add a sexual hormone or a cytokine to an isolated component of the immune, neurological, or endocrine system and then to measure its response. With results obtained by this approach and also with some of the in vivo data in Tables I and II, we constructed a flowchart of the immune and neuroendocrine systems that included all interactions across species, sexes, cells, and types of responses. The result was an all-encompassing host–immuno-

neuro-endocrine–parasite (HINEP) network connecting components within and between the systems of the host and the parasite (Fig. 1). The magnitude and complexity of the HINEP network includes practically all sexual hormones and many of the known immunological components. Simple inspection suggests the networks possible relevance to many other biological scenarios other than infection. Some of the events in the immunoendocrine network involve cellular differentiation, reproduction or death and de novo synthesis of receptors. The prominent and varied connections of the HINEP network with other hormones, well known to be related to stress and the inflammatory process (Besedovsky and del Rey, 2002; Dantzer et al., 2002), suggest that the network can also influence innate immune mechanisms (Yokoyama and Scalzo, 2002). The HINEP network of the host is related directly with the parasite (Morales-Montor, Baig et al., 2001; Morales-Montor, Mohamed et al., 2001), affecting its reproductive capacity through sexual and adrenal steroids that favor the expression of genes related to cellular differentiation and proliferation (Escobedo et al., 2004). The HINEP network contains circuits with forward and backward regulation, producing a great range of effects on the parasite or the host using several venues. Because some of the events in the HINEP network involve cellular reproduction and de novo synthesis of receptors, the network would seem capable of adapting and evolving.

There are other immunoendocrine networks described in the literature, which focus on the connections of the immune system with adrenals and nervous system, but secondarily or not at all with the gonads (Besedovsky and del Rey, 2002). The HINEP network presented in this study adds to the existing networks by incorporating the gonads and sex steroids as intrahost connections and the parasites' physiological systems as interhost connections.

Sex steroids act on a variety of immunocompetent cells affecting clonal expansion, phagocytosis, apoptosis, antigen presentation, and physiological responses to cytokines and chemokines. Thus, there is no question regarding the capacity of sex hormones to modulate the immune response. The significant question is, rather, what is their end effect on the host–parasite relationship and at which point does it act in each gender of host? A node in the network likely to be present under a strong sex-steroid modulation of acquired immunity includes the TH1–TH2 immune responses (Rook et al., 1994; Martin, 2000). Conflicting effects of androgens and estrogens on TH1–TH2 may possibly adjust the relationship of each host sex with the parasite and achieve either "pacifist" coexistence or "belligerent" confrontation. Antigen presentation, clonal expansion, cell activation, or apoptosis and effector macrophage functions, inflammation, and chemotactic responses are also likely candidates for significant hormonal control. The exploration of direct sex-steroid effects is as yet incomplete, and their end effects on the whole immune system, especially when acting in unison, are seldom studied. As can be gathered from the summary of a single hormone's actions on some of the immune parameters cited in Table III, estradiol seems to stimulate TH2, but there is no proof that it shuts down TH1 other than in experiments using mitogens instead of antigens. Similarly, testosterone decreases some B-cell-associated effector functions by reducing the levels of some TH2 cytokines (IL-1, IL-6, TNF-α) but has not been shown to interfere with TH1 functions. Dehydroepiandrostenedione (DHEA) stimulates TH1 immune parameters without apparent effect on TH2. DHT has effects similar to testosterone. Progesterone downregulates effector mechanisms (NK cytotoxic activity and macrophage cytokine and nitric oxide production), and prolactin also acts on the TH1–TH2 modulation node. Based on these observations and considering they probably are not independent effectors, however, it would be adventurous to predict a single hormone's end effect on a host's immunological protection or vulnerability to infection. This is even more likely when many of the sex hormone levels are not independent effectors and some hormones are probably operating simultaneously on the host's immune system when confronted with a parasite. Furthermore, more complexity and less predictability are to be expected from the likelihood of immune cytokines acting directly on the parasite, as do the sex steroids.

Notwithstanding the problem in understanding how real physiological networks actually work, their nodes might differ in terms of the number of connections. Herein lies the only hope for understanding relatively simple cause–effect relationships in parasitism. Firing of the most connected nodes may extend widely and rapidly throughout the network, inducing a significant change of phase in its equilibrium state and prompting the emergence of new properties (Oltvai and Barabasi, 2002; Strohman, 2003). Identification of the most connected nodes would be a way to begin their study as principal participants in SD to infection. To clarify the relationship existing between a host's SD and immunity with susceptibility to infection, we must look for these hierarchic nodes in the HINEP network. Some of them may be apparent at sexual maturity, but others might be more difficult to identify, having operated in the early ontology of the female or male immune and endocrine systems and then disappearing by the time of sexual maturation. One could hypothesize that important neuroendocrine system connections with the immune system are established during embryonic development, when gonadal differentiation occurs (Klein et al., 2002; Sinisi et al., 2003) and principal criteria for immunological self- and danger signal recognition also appear to be set (Matzinger, 2002; Medzhitov and Janeway, 2002).

To illustrate the HINEP network's explanatory and predictive properties of parasite infections, circuits that are turned on in experimental cysticercosis and schistosomiasis are illustrated with different colors in Figure 1. The circuits are not identical, but in both, IL-6 is a prominent feature, and the consequences of its overexpression, i.e., feminization, a TH2-leaning immune response, would be expected in other infections with all the rest being equal. In the network, there are also circuits capable of masculinizing the infected female if P-450 aromatase is directly inhibited, or by inhibition of GNRH in the hypothalamus, mediated or not by IL-6, or by enhancing the expression of 5α-reductase type II by means of an IL yet to be identified. In the opposite direction, the DHEA upregulation of TH1, for instance, endows the endocrine system with an ability to participate in immunological defense, a prediction that has yet to be verified in TH1-sensitive infections (Baszler et al., 1999; Suzuki, 1999; Rogers et al., 2002). In the network's schistosomiasis example, the parasite actively induces an immune response, which progressively leans toward TH2. Then, the increase in IL-β expression in the hypothalamus stimulates CRH production, which, in turn, stimulates pituitary adrenocorticotropin hormone (ACTH) (Morales-Montor, Newhouse et al.,

TABLE III. Effects and mechanisms of action by hormones on immunocompetent cells.*

Hormone	Effect on immune system cells	References
Estradiol	Polyclonal B cell activator; promotes B cells into plasma cells; ↓ bone marrow and thymus mass; ↑ IL-10 and IL-6 secreting cells; ↓ IFN-γ and IL-2 production; downregulates NK activity; upregulates phagocytocis by macrophages; ↑ serotonin and histamine release	Mandrup-Poulsen et al. (1995), Gaillard and Spinedi (1998), Chen et al. (2001), Roberts et al. (2001), Verthelyi (2001), Spinedi et al. (2002), Kitaya et
DHT	↓ T-cell response to mitogen; ↓ mast cell secretion; ↓ IL-1, IL-6, and TNF-α production; ↑ IL-2, TNF-α, and IFN-γ mRNA	Bijlsma et al. (2002), Morales-Montor, Baig, Hallal-Calleros (2002), Tanriverdi et al. (2003)
Testosterone	↓ B-cell response to mitogen; ↓ mast cell secretion of histamine and serotonin; ↓ IL-1, IL-6, and TNF-α production	al. (2003), Maret et al. (2003), Ou et al. (2003) Zhang et al. (2000), Bijlsma et al. (2002), Morales-Montor, Baig, Hallal-Calleros (2002), Tanriverdi et al. (2003)
Progesterone	↓ NK cytotoxic activity; ↑ TNF-α secretion; ↓ macrophage cytokine secretion; ↓ NO production	Mandrup-Poulsen et al. (1995), Gaillard and Spinedi (1998), Verthelyi (2001), Spinedi et al. (2002)
Cortisol	↓ Prostaglandins and leukotrienes production; modulates T- and B-cell maturation; affects trafficking and activation of proinflammatory cells; ↓ the production of IL-1, IL-2, IL-6, IL-8, IL-10, IL-12, and TNF-α	Derijk and Berkenbosch (1991), Mandrup-Poulsen et al. (1995), Loria et al. (1996), Nussdorfer and Mazzocchi (1998), Feterowski et al. (2001), Besedovsky and del Rey (2002), Esch (2002), Morales-Montor, Mohamed et al. (2003)
DHEA	↑ IL-2 production; ↑ IFN-γ production; ↓ IL-6 secretion; ↓ TNF-α production; protects against neurooxidative damage; ↑ T-cell immunity; ↑ DTH reaction	Derijk and Berkenbosch (1991), Mandrup-Poulsen et al. (1995), Loria et al. (1996), Nussdorfer and Mazzocchi (1998), Feterowski et al. (2001), Besedovsky and del Rey (2002)
CRH	↑ IL-1 and IL-6 production; ↑ chemotaxis and superoxide production; ↑ B-cell proliferation; ↑ expression of T cells IL-2 receptors	Derijk and Berkenbosch (1991), Mandrup-Poulsen et al. (1995), Nussdorfer and Mazzocchi (1998), Besedovsky and del Rey (2002), Esch (2002)
ACTH	↑ Antibody production; cytokine secretion and proliferation	Panerai and Ottaviani (1995), Nussdorfer and Mazzocchi (1998), Ottaviani et al. (1999)
Prolactin	↑ Lymphocyte proliferation in response to antigen and mitogens; ↑ IFN-γ and IL-2 secretion; ↓ cell death mechanisms in immune cells; induces NK cells to their differentiation to prolactin-activated killer cells	Derijk and Berkenbosch (1991), Matera et al. (2001), McMurray (2001), Yu-Lee (2002)
VIP	↓ Production of proinflammatory agents; ↑ production of anti-inflammatory cytokines; both functions in activated macrophages; ↑ Th2 cell differentiation	Delgado et al. (2001), Voice et al. (2002), Ganea and Delgado (2003)
GH	↑ Adhesion of thymocytes to thymic epithelial cells; ↑ release of thymocytes from thymic nurse cells; ↑ intrathymic T-cell traffic	Sternberg (1997), Weinstock and Elliott (2000)
Thyroid hormones	Affects primary B-cell development because of reduced proliferation of immature B-cell precursors	Dorshkind and Horseman (2001)
Vasopresin and oxitonin	↑ Cell proliferation	Dorshkind and Horseman (2001)
Encephalins	Low doses: ↑ activates B and T cells; high doses: immunosuppression	Dorshkind and Horseman (2001)
Endorphins	↓ Antibody production and proliferation	Machelska and Stein (2002)
hCG	↓ Proliferation of T and NK and induction of T suppressors	Pope (1990)
Melatonin	Affects thymocyte maturation and differentiation	Hotchkiss and Nelson (2002)

* Abbreviations and symbols: DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; CRH, corticotrophin; ACTH, adrenocorticotrophic hormone; VIP, vasointestinal peptide; GH, growth hormone; hCG, human chorionic gonadotrophin; DTH, delayed-type hypersensitivity; ↓, decrease activity; ↑, increase activity.

2001). Concomitantly, IL-6 and migration inhibitory factor could be regulating ACTH production directly in the pituitary gland. The ACTH production, in turn, stimulates adrenal glands to produce cortisol and DHEA, together with TNF-α, which can directly inhibit parasite growth. Infection triggers the immune response of the host by mediating the neuroendocrine system at HPA axis level (Morales-Montor, Mohamed et al., 2003). The consequent microenvironment could be more permissive for the establishment and growth of the schistosome. The network also exhibits the possibility of HPA axis hormones to act directly on the parasite, affecting its reproductive capacity through adrenal steroids that favor the expression of genes related to cellular differentiation and proliferation (Morales-Montor, Mohamed et al., 2001). On the other hand, in cysticercosis, the nodes of interaction are by way of the hypothalamus–pituitary–gonadal axis. Infection with the metacestode results in a feminization process and in a TH1–TH2 shift of the host's immune response. The cysticerci actively induce a TH2 immune re-

sponse. Then, the increase in IL-6 production in turn stimulates estradiol production by directly activating the enzyme P-450 aromatase and at the same time increasing pituitary follicle-stimulating hormone levels. Estradiol would then cycle back to favor the TH2 response further. In turn, the decreased testosterone production inhibits the TH1-dependent cellular response. The consequence is the change of the host's hormonal microenviroment from restrictive (male) to permissive (female) for cysticerci growth. The network also illustrates the possibility that the host's sex hormones may act directly on the parasite, affecting its reproductive capacity through sex steroids that favor the expression of genes related to cellular differentiation and proliferation in many animal species (Fig. 1).

The HINEP network's prowess, with its manifold forward and backward regulations in operation, is congruent with the variegated profiles of Infection-SD and Immune-SD in the various host–parasite systems. It does not, however, necessarily imply or deny that there will be differences between sexes in the final results of a given confrontation with a parasite, reached perhaps by different mechanisms in different host sexes. A functional feature of complicated, random, or scale-free networks is its stability before random perturbations, whereas strategic perturbations "break" them to pieces (Oltvai and Barabasi, 2002; Strohman, 2002). This could explain why there are some sex-unbiased parasitic diseases. Perhaps only a few parasites are capable of triggering a profound general change in the state of the network that results in Infection-SD, a property limited to those parasites connecting with the complex hierarchical immunological nodes of the network and the sex steroids.

IS THE HINEP NETWORK INVOLVED IN OTHER MEDICAL AND BIOLOGICAL PUZZLES?

The literature search revealed an extremely complex HINEP network, involving hormones and cytokines that predict potent interactions in events generally attributed to the exclusive operation of single systems in response to simple precepts (reproduction and defense). Therefore, much plasticity and multifunctionality in a network are not without risk. Absence of control could lead to the loss of tolerance and autoimmune problems (Derijk and Berkenbosch, 1991; Lechner et al., 1996) or be involved in the immune compromise of aging (Pancrai and Ottaviani, 1995), in the pathophysiology of some infections in which inflammation is a prominent effector of pathology (Mandrup-Poulsen et al., 1995; Henri et al., 2002), or even in some combination of all the above. Moreover, the HINEP network could connect parasite infections with other diseases that seem alien to the immunological and endocrinological domains, such as arterial hypertension (Peeters et al., 2001), atherosclerosis (Chae et al., 2001), and cancer (Herrera and Ostrosky-Wegman, 2001; Polat et al., 2002).

Many other biological questions emerged from the review of the literature on SD, each pointing to avenues for future research. We shall focus on 2 of the more prominent ones, which, in turn, involve many subsidiary possibilities. First, why is there SD in the immune response? Second, is there evidence to suggest that parasites have influenced the evolution of their hosts' Immune-SD?

The very complexity of the HINEP network hints at reasons for Immune-SD other than the self or foreign concept. Perhaps it has evolved as the best mechanism for individuals of either sex to confront infection successfully, even if by different mechanisms, and also to solve with precision gender-specific challenges, like pregnancy (Grossman, 1989), or perhaps the consequences of their territorial, mating, and social behaviors (Zuk, 1994; Kavaliers et al., 2001).

The selective pressure driving evolution toward Immune-SD, matching in importance the defense of the host against infection, is to permit reproduction in a dioecious species without much immunological compromise (Grossman, 1989; Gaillard and Spinedi, 1998; Agrawal and Lively, 2001; Charles et al., 2002; Moore and Wilson, 2002; Owens, 2002; Potti et al., 2002; Tella et al., 2002). This compromise could be achieved by a transient, immunologically specific allowance of female pregnancy with an offspring that is half-foreign, designed in terms of immunoendocrine signaling that does no damage to an effective response to a pathogen (Martal et al., 1995; Matzinger, 2002; Medzhitov and Janeway, 2002). Pregnancy demands for immunological allowance would originate from the advantage of species diversification gained through gender dichotomy. Its satisfaction would call for occasionally fastidious but transient immunoendocrine regulation by hormones and cytokines so that the fetus is not damaged (Barnea, 2001). Immune-SD may provide males with the specialized ability to better cope with their more stressful and dangerous lives when displaying their sex-specific behaviors (Kavaliers et al., 2001; Spinedi et al., 2002). Thus, the hosts that get the best trade-off between the need to diversify and the need to survive would appear to have the better chances to evolve. Even the parasite could benefit from the host's Immune-SD. For example, the parasite-restrictive males in the case of murine cysticercosis may be regarded as behaviorally enhanced vehicles toward the parasite's final destiny in the gut of carnivores (Willis and Poulin, 2000; Gourbal et al., 2001). In turn, the cysticercus-permissive females, when infected with the eggs of *T. crassiceps*, would act as the optimal hosts for their massive reproduction (Poulin and Thurn, 1996; Zuk and McKean, 1996; Panhuis et al., 2001).

The level of complexity introduced in the decision-making process of immune events by the powerful HINEP network regulatory capacity promises to enlighten persistent immunological puzzles such as tolerance and autoimmunity, the connection with infection of seemingly unrelated physiopathological events such as hypertension and cancer, and the role of Immune-SD in species diversification and individual behavior. The roles of sex steroids in the ontological development of the immune system and in acquired and innate immune responses promise invaluable insights and beg for more research.

CONCLUSIONS

There are many exceptions to the female host supremacy paradigm in parasitic infections of mammals, too many to leave unquestioned. Indeed, testosterone is involved in the immunoendocrine interactions triggered by infection, but so are many other hormones and cytokines that act as a network in which the contributions of its single effectors are unclear. Instead, important properties, like infection and immune sex-associated differences, may emerge from the network as a whole. The role of the host's immune system as the only effector of SD in parasitism is not clear; it is insufficiently explored, and it is not

uniformly implemented, even in the most studied host–parasite systems. Finally, the parasite's direct response to the hormonal environment of each host sex has been overlooked as a significant contributor to host SD in parasitic infections. In fact, it would appear that the conflicting findings in murine *T. crassiceps* cysticercosis with the female supremacy paradigm in parasite infections provoke even greater sensitivity to the host's sex steroids, i.e., parasite driven estrogenization and deandrogenization of infected male mice and permissiveness of the female mice TH2 inclined immune profile toward cysticercus proliferation.

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We now approach the possibility of a holistic understanding of parasitism through genomics (Parasite Genomics) as a fascinating perspective in biology, with implications for human health and economy [2]. Parasite Genomics will aid to address fundamental questions such as the origin and evolution of parasites [3,4]; the molecular basis of host–parasite specificity [5]; the definition and dynamics of the parasites host range [6]; the mechanisms of parasite pathogenesis [7]; the various mechanisms that parasites use to evade the host's immune response [8]; the signaling that controls the regulation of genes in the differentiation stages within the parasite life cycle in concert with those of the hosts physiology [9]; the complete antigen profile of a given parasite for vaccine and diagnostics development [10–12]; the complete and precise connectivity between the host and the parasite metabolic pathways for a strategic drug design [13], among others.

2. Comparative parasite genomics

Some contributions on human parasites come from phylogenetical and evolutionary studies of Comparative Genomics: comparison among parasite genomes help to understand evolutionary events that generated genetic diversity [4]; some gene sequences associated with the parasite's survival in its host display high levels of identity among related parasite species [8]; other genes are clearly associated to symbiotic parties including parasites [3]. Comparative studies among parasites have also identified potentially useful sequences for multiple diagnosis of parasite species [14]. Host–parasite meta-genomes establish a plastic network of interactions, largely through mutual regulation of gene expression, to deal with selective pressures from inside and outside a given host–parasite relationship [15]. The set of genes which code for different proteins in parasites shows remarkable concordance with the requirements imposed by the environmental niche in which the parasite resides within the host, a feature possibly involved in host, cell and tissue specificity of the parasite [16]. Horizontal gene transfer between parasites and hosts, some involving viral–bacterial–parasite–host chains might be of great consequence for the evolution of hosts and parasites [17].

The strategies that parasites use to evade the host defense mechanisms are diverse, some involving modification of their displayed antigens as well as of the host physiology [8]. *Trypanosoma brucei* and *Plasmodium falciparum* provide prime examples of antigen variation within the same host, which results from the successive activation of genes that code for different surface antigens, allowing them to expose hundreds of antigen variants and evade the hosts immune response [8,18]. The evolution of parasitism certainly involves a series of adaptations, including the loss of genes, as indicated by the absence of proteins widely distributed in eukaryotes [19]. Parasitism seems to involve "an increasing employment of the metabolic machinery of the host for most of the parasite's requirements, although not all losses are easily explained" [4]. As a result of the loss of genes and lineage-specific innovation, parasites proteomes are somewhat different from other non-parasitic eukaryotes that have similar number of genes. Parasite's proteome has a great component associated with pathogenesis, evasion of immune response and post-transcriptional regulation [4]. There is a debate concerning the processes by which identical duplicated genes diverge in sequence and function. It is clear that after gene duplication, one copy can be subjected to silencing by degenerative mutation, but also, both copies can be preserved through natural selection, specially if one suffered mutations that slightly alter its function (paralog conversion), resulting in new properties. As an example, it has been suggested that there is a connection between the rates of intron gain/loss and protein evolution in malaria parasites [20].

An intriguing aspect of parasitism is the parasite's restricted host range, or host specificity, which varies from several to a single host species. A number of plausible mechanisms may be involved but hard data qualifying the speculations are still scarce. The availability of complete genomes of both parasite and host may enlighten the issue of specificity, when based on their differences and on their similarities, as well as recognize possible parasite niches within other eukaryotes [21]. Thus, complete genomes of potential hosts, when compared with established hosts, may allow for predictions for the most likely expansion of the parasites' host range based on their homology.

3. *Taenia solium*

Cestodes are Platyhelminthes that originally parasitized a number of predator–prey associations. It is conceivable that *T. solium* eventually colonized humans that scavenged their kills, and their associated parasites were passed intraspecifically to other humans and to the first domesticated pigs [22]. Progressively, the population growth of human species, the intensive rearing of pigs, the facilitation of human migration carrying along the porcine stock and rustic forms of rearing, firmly rooted the parasite and distributed it worldwide [23].

The metacestode stage (cysticercus) of the tapeworm *T. solium* causes neurocysticercosis in humans, a debilitating and not rarely mortal disease of insidious onset, difficult diagnosis and not thoroughly resolved by treatment. It is claimed to be the Third World's major parasite responsible for brain disorders. Nowadays *T. solium* infects ~50 million people worldwide and millions of their porcine livestock, and is now considered an emergent disease in developed countries mainly brought-in by migrant workers from countries where the disease is endemic [24]. Practically neglected for decades in the twentieth century [25], research on *T. solium* in the last decades has recruited scientists from all over the world [26].

Because of the *T. solium* great damaging impact on human health and economy of developing countries, most of the current research focuses on the study of clinical aspects of the human disease and in developing diagnostic, therapeutic and prophylactic technologies. Studies on the biology of the parasite are underway in laboratories of Mexico, where many important questions could find an answer, and provide stronger strategies for control. To this end, we have constituted a consortium of key laboratories at the National Autonomous University of Mexico to carry out a genomic project for *T.*

solium. This project will provide powerful resources for the study of taeniasis/cysticercosis, and, in conjunction with the *E. granulosus* and *E. multilocularis* genome project of EST, will mark the beginning of genomics for cestode parasites. Our project is planned in two consecutive stages. The first stage is being carried out to determine some basic parameters of the *T. solium* genome: size, karyotype, gene density, diversity of repeated sequences, highly abundant transcripts, intron density and average size, etc. Afterwards, we will evaluate the best strategy for the second stage, a full blown genome project.

We have estimated the genome size by two different approaches: cytofluorometry on isolated cyton nuclei from *T. solium* metacestodes, stained with propidium iodide, and by means of probabilistic calculations on the coding density of the genome (based on ~2000 sequenced genomic clones, ~3000 ESTs, and a tentative gene number of 17,000), resulting in size estimates of 270 and 251 Mb, respectively.

In terms of sequencing, our goal for the first stage is to characterize several thousand ESTs (from adult worm and cysticerci cDNA libraries) and genomic clones. Results obtained so far from about 16,000 ESTs reads from an adult worm cDNA library, show that only about 40% of the *T. solium* coding sequences have a previously sequenced homologue in the nr protein database. The hit rate with SwissProt is about 24%, with many of the best hits to mammalian genes, especially humans. However, ~1.5% of the SwissProt hits lack homologues in humans, making these genes immediate candidates for investigation on pharmaco-therapy, diagnostics and vaccination. As expected for a metazoan eukaryote, many *T. solium* ESTs are related to gene regulation and signal transduction. Other important functions are protein synthesis, housekeeping, metabolism, cell division, cytoskeleton, proteases, vacuolar transport, hormone response, and extracellular matrix activities.

Results from the sequencing of genomic clones indicate that coding density for the *T. solium* genome appears relatively high: ~9% of all nucleotides are involved in protein coding. Intron size and number within genes appears unexceptional. Preliminary results also suggest that highly repetitive sequences comprise less than 5% of the genome. Many genes show duplications with highly divergent introns, suggesting that these events are not recent. As the project has been conceived as an assemblage of human and physical resources in parasitology, molecular biology and bioinformatics, all data and materials are immediately available to members of the consortium. Information obtained will be annotated and made public soon. At present, there is no generally agreed method as to how this might be done and several schemes are being considered [27].

Development of the project will result in a series of useful products including: cDNA coding sequences to be used as templates for the study of individual genes; as the project is planned to include extensive sequencing of ESTs from eggs, larval and adult stages, maintaining a plasmid backup for each identified coding sequence. Templates will be available upon request to the consortium for future use. Synthetic oligonucleotides from identified ESTs will be produced for the study of gene expression or transcriptional analysis through microarrays. A database containing updated information of the sequencing progress, include all genomic and ESTs sequences, BAC maps, as well as repositories of other data generated by the consortium.

These tools will make proteomics more accessible for the study of the cysticerci responses to different conditions: drug treatment, stress, human vs. pig, geography (cysticerci from different countries), interaction with human hormones, etc. Other questions such as host-parasite gene transfer, parasitism-specific and host-specificity genes, analysis of promoter genes for the development of transformation vectors to express particular proteins or the possible use of cysticerci as bioreactors.

4. Concluding remark

T. solium disease poses unique biological questions and medical needs as to merit the development of a complete genome project. The consortium for the *T. solium* genome project wishes to use this international symposium on Cestode Parasites to make a worldwide call for collaborative research.

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Sex-steroids induce scolex evagination and growth of the human parasite *Taenia solium*: evolutionary implications to the host-parasite relationship

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Abstract

The metacestode stage of *Taenia solium* causes porcine cysticercosis and human neurocysticercosis, both serious veterinary and emergent health problems in third world countries. One of the most important aspects of this infection is the scolex evagination process through which the cysticercus starts its differentiation until to generate the flatworm that will produce and release to the environment thousands of infective eggs. Here, *Taenia solium* cysticerci were *in vitro* exposed to several concentrations of progesterone (P_4) and 17β–estradiol (E_2) in order to explore their effects on parasite differentiation, which is favored in castrated and gestant pigs. Our results suggest a time and dose dependent effect on scolex evagination and growth. To determine the molecular mechanisms by which P_4 and E_2 affected *Taenia solium, in vitro* cultures using RU-486 and Tamoxifen were performed, as well as parasite sex-steroid receptors amplification by means of RT-PCR and detection by western blot. Both anti-hormones blocked the stimulatory effect of P_4 and E_2 on *Taenia solium* cysticerci, respectively. Concomitantly, cysticerci expressed both α and β subtypes of estrogen receptor and A and B isoforms of progesterone receptor, as well as their proteins. Hormonal treatment did not affect sex-steroid receptors expression, excepting the progesterone receptor A which expression was diminished at fifty percent by progesterone treatment. Present results suggest that sex-steroids act directly upon *Taenia solium* metacestodes differentiation, possibly by binding to a parasite sex-steroid receptor, which does have serious implications on their host-parasite relationship at molecular, physiological and evolutionary levels.

Key Words: Progesterone; estrogen; anti-hormones; *Taenia solium*; scolex evagination; parasite differentiation.

1. Introduction

Cysticercosis, caused by the metacestode stage of the tapeworm of *Taenia solium*, is a serious health problem in many developing countries [1-3]. *Taenia solium* metacestodes cause neurocysticercosis, the Third World major brain disorder that affects ~50 million people worldwide, and is considered as an emergent disease in developed countries [4]. *T. solium* also infects pigs, its intermediate host, leading to major economic loss and transmission to humans [5]. Humans are the sole final host of *T. solium*, developing a tapeworm infection, following the ingestion of raw or undercooked contaminated pork meat. *Taenia* eggs are passed with the stools and contaminate the environment [6]. When *T. solium* eggs are ingested by pigs or humans, larvae may develop throughout the body. In the human, most clinical symptoms are induced when cysticerci become established in the central nervous system [1,7]. It is important to underline, *Taenia solium* cysticercus evagination represents the limitant and crucial step in the parasite differentiation inside of the definitive host intestine, because when the human ingest a cysticercus from contaminated pork meat, evagination process is the first differentiation step into developing of an adult worm [8]. Once started parasite diferentiation from cysticercus to worm, through developing of both rostellum and suckers, the flatworm gets to fix at intestinal wall. This process is necessary to start to form proglottides (proliferative units that in their mature stage develop testicles and ovaries) and, therefore, become an adult tapeworm, the infective stage of major importance for cysticercosis and neurocysticercosis propagation, due to the production of thousands of *T. solium* eggs [8]. Then, *T. solium* scolex evagination results a transcendental physiological process not only as part of the parasite's life cycle but also for transmission of the infection. Nevertheless, even though this is a very important parasite physiological process, if hormonal factors are involved in the *T. solium* differentiation from cysticercus to tapeworm, inside of the definitive host intestine has not been yet studied.

On this sense, reciprocal endocrine interactions between parasite and host are receiving increased attention regarding their role in parasite success [9-13]. Direct effects of sex-steroids have been invoked in explaining the relation between steroid hormone treatment and increased numbers of larval and adult stages of intestinal worms or of various other parasites in other organs of vertebrate hosts [14]. In *Taenia solium* porcine cysticercosis, pregnancy as well as castration of males, increased prevalence in wild living pigs [15]. Thus, hormonal environment at the time of infection may play a significant role in determining both susceptibility and immune responses during pig cysticercosis, and possibly human neurocysticercosis.

Another cestode, *T. crassiceps* (which is philogeneticaly very closely relative of *T. solium* and can asexually reproduce itself by budding) has shown to be strongly affected by sex-steroids treatment *in vivo* and *in vitro*. Specifically, 17-β-estradiol increases *T. crassiceps* cysticerci reproduction (manifested by the increasing in number of buds) while treatment with testosterone or dihydrotestosterone (DHT) diminishes this function [16]. In addition, viability, growth and infective capacity of cysticerci are also increased up to 200% following treatment with estradiol, but are almost totally inhibited by androgen treatment [16]. It appears that the direct *in vitro* effects of estrogens and androgens upon *T. crassiceps* reproduction are due to the binding of estradiol and testosterone to their respective receptors [16].

However, direct effects of sex-steroids and other hormones in a variety of other parasites [17-20], indicate caution in excluding helminths from sex-steroid capacity to act directly upon them. Only a consistent failure of sensitive technology to show effects of sex-steroids upon cysticerci outside the host, *in vitro*, could weaken the possibility of direct action of the hormones upon the parasite.

Because sexual hormones play an important role in murine (*Taenia crassiceps*) cysticercosis and there are data in wild living pigs which suggest that also porcine *T. solium* cysticercosis is affected by sex-steroids [15], we decided to study the role of sex hormones in *T. solium* cysticerci *in vitro*, focusing on a very important aspect of them, their role during scolex evagination. As we have said, this represents a transcendental differentiation step which marks worm developing and eggs production, both of them necessary in the life cycle maintenance and a succesful infection. Finally, because of the potential application of hormone-based control and therapeutic measures, the present experiments were designed to thoroughly explore the possibility that sexual steroid hormones have a direct effect upon *T. solium* and one of the most important physiological events of this parasite, scolex evagination.

To our knowledge, this is the first report of direct effects of sex-steroids upon *T. solium* cysticerci, possibly through specific sex-steroids parasite receptors. These findings may mean a relevant contribution to a better understanding of the host-parasite relationship at molecular and evolutionary levels and specific design of anti-cysticerci drugs, which affect exclusively to the parasite, considerating sexual dimorphism and steroid hormone effects on infections by helminth cestodes.

2. Experimental

2.1. Harvesting and preparing cysticerci for experimentation

Taenia solium cysticerci were dissected from the muscle of male infected pigs in independent experiments. Pigs were humanely sacrificed by using head electroshock and posterior bleeding. The method was previously evaluatedsed by the University Animal Care and Use Committee and governmental agencies to ensure compliance with federal regulations and guidelines. The fibrous capsule that surrounded each parasite was carefully separated under a dissection microscope. Once dissected, cysticerci were placed in tubes containing sterile PBS (1x) supplemented with 100 U/ml of antibiotics-fungizone (Gibco,Grand Island, NY) [21]. The tubes were centrifuged for 10 min., at 800 g at 4 C and the supernatant was discarded. The packed cysticerci were incubated in AIM-V serum-free medium (Gibco, BRL, Rockville, MD). They were then centrifuged 3 times for 10 min at 800 g for washing. After the final wash, the numbers of viable (complete and translucent cystic structures) cysticerci were counted using a binocular microscope. Five viable cysticerci were then selected and dispensed into each well of 6-well culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, New Jersey) in 3-ml AIM-V medium and incubated at 37 C under 5% CO_2. Cultures were examined daily and their medium completely replaced when turned yellowish (pH 6.5).

2.2. Hormone dose–response-time curves

Culture grade E_2, P_4, tamoxifen and RU-486 were obtained from Sigma. For *in vitro* tests, water soluble E_2, and P_4 were dissolved in AIM-V free-serum culture medium, whereas tamoxifen and RU-486 were dissolved in pure ethanol (Sigma) to the desired stock concentration, and sterilized by passage through a 0.2-mm millipore filter. The experiments employed the parasite-loaded wells as follows: 4 wells were used as untreated controls, 4 wells were supplemented with

ethanol at the final concentration of 0.06 percent. Series of 4 wells were separately treated with 0.0002, 0.0004, 0.1, 1 and 40 μg/mL of P_4; 0.02, 0.08, 0.8, 1 and 20 μg/mL of E_2 and a combination of both P_4/E_2, prepared at the same time as one single hormonal solution. Final doses for P_4/E_2 solution were 0.02/0.0002, 0.08/0.0004, 0.8/0.1, 1/1 and 20/40 μg/mL of progesterone and estradiol, respectively. Concentrations of hormones used in this study were physiological and pharmacological, as we have previously reported [9,22,23,20,16], and designed in order to approximate levels in humans, gestant pigs and feminized mice [9,15,16,22]. Tamoxifen and RU-486 were added at the same doses than E_2 and P_4, respectively. Each hormone was prepared in a final volume of 100 μl and added to 1 ml of medium in each well. In hormone dose–response-time curves, only the number of scolex differentiated from cysticerci as a function of days in culture was assessed as the response variable. From dose-response curves of each hormone, we selected an optimal dose for P_4 (80 ng/mL), E_2 (400 pg/mL) and P_4/E_2 (800/100 ng/mL, respectively), in order to be used in time-response curves.

2.3. Sex-steroids effects upon viability, scolex length and RNA expression of cultured cysticerci

Cysticercus viability and scolex lengthh were determined daily for all wells using an inverted microscope (Olympus, MO21, Tokyo, Japan) at 10x and 20x magnification. Injury to cysticerci was recognized microscopically by progressive internal disorganization, development of whitish opaque areas on the parasite's tegument and by loss of motility. Dead cysticerci were immobile, opaque and structurally disorganized. Photographs of representative treated cysticerci were obtained.

Total RNA was isolated from hormone-treated *T. solium* cysticerci by the method based on guanidine isothiocyanate/phenol/chloroform extraction using Trizol reagent (Invitrogen, Carlsbad, California). In brief, cysticerci were disrupted in Trizol reagent (1ml/ 0.1 g tissue) and

0.2 ml of chloroform was added per ml of Trizol. The aqueous phase was recovered after 10 min., of centrifugation at 14,000 g. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol and redissolved in RNAse-free water. RNA concentration was determined by absorbance at 260 nm and its purity was verified after electrophoresis on 1.0% denaturing agarose gel in presence of 2.2 M formaldehyde.

2.4. Estrogen receptors α and β and progesterone receptors A and B gene amplification

Total RNA from hormone-treated cysticerci was reverse-transcribed followed by specific PCR amplification of estrogen receptors α and β (ER-α and ER-β) and progesterone receptors A and B (PR-A and PR-B), together with β-actin (control gene) as previously described [24]. Primers were designed based on the most conserved regions of all species of sequenced genes reported in the NIH Gene Data Bank (ER-α and β and PR-A and B) as well as using the sequence of previously cloned *T. crassiceps* genes [16]. Primer sequences are as follows: ER-α, sense 5'-AGACTGTCCAGCAGTAACGAG and antisense 5'-TCGTAACACTTGCGCAGCCG; ER-β, sense 5'-CATCTGGGTATCATTACGGTC and antisense 5'-GGCACTTCTCTGTCTTCGTAC; PR-A, sense 5'-CAGTGGTGGATTTCATCCATG and antisense 5'-CTTCCAGAGGGTAGGTGCAG; PR-B, sense 5'-GGAGGCAGAAATTCCAGACC and antisense 5'-GACAACAACCCTTTGGTAGC; β-actin, sense 5'-GGGTCAGAAGGATTCCTATG and antisense 5'-GGTCTCAAACATGATCTGGG. PCR products obtained were visualized by staining with ethidium bromide. In all cases, a single band was detected corresponding to the expected molecular weight of the gene. Mouse ovary and uterus were used as expression positive controls for this experiment (these results were performed as internal control, data are not shown) The expression of ER-α, ER-β, PR-A and PR-B in the parasite are numerically presented as the ratio of the optical densitometry (OD) of each studied

gene relative to the expression in the same preparation of the β-actin gene, a constitutively expressed gene used as an internal control for differences in the amplification procedure between experiments and in the staining of the different gels.

It is important to underline that in order to demostrate that cysticerci were not contamined by host cell DNA or RNA, we performed the same RT-PCR using specific primers of pig TNF-α and the mouse VDR gene, which corresponds to the variable region of the IgG and therefore shows high specificity exclusively for mouse sequence. Sequences of primers are as follows: TNF-α sense 5'- CAGAGCTGTCTCTAAACCGT -3', and antisense 5'-AGGGTGTCTGAAGGAGGGGA -3', and VDR sense 5'-CTGAATTCCA TGAAACACCTGTGGTTCTT -3', and antisense 5'-GACTCTAGA GGGCTCACACTCA CCTCCCCT -3'.

2.5. ER (α and β) and PR (A and B) western blotting detection on Taenia solium cysticerci

Total protein was obtained from *T. solium* un-treated cysticerci by Tris-HCl conventional isolation. In brief, untreated cysticerci were disrupted in Tris-HCl (1ml/ 0.1 g tissue), Proteinase K (100 units/ml) and proteases inhibitor cocktail (Calbiochem). Supernant was recovered after 15 minutes of centrifugation at 8000 g. Pellet was discarded. Protein quantity was obtained by absorbance at 320 nm using the Bradford-Lowry method. Total Ag *T. solium* extract (100 μg per well) was boiled in reducing Laemmli sample buffer, separated by SDS-PAGE (10% acrylamide) and electroblotted onto nitrocellulose membranes. Membranes were blocked overnight in TBST buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.5% Tween 20) containing 1% BSA. For protein immunodetection, membranes were subjected for 2 hrs to immunoblotting with 1 μg/ml of anti- ER-α (MC-20, Santa Cruz Biotech. SC-542), anti-ER-β (Y-19, Santa Cruz Biotech. SC-6821), and anti-PR-A/PR-B polyclonal antibodies (C-20, Santa Cruz Biotech. SC-539), all of

them diluted 1:1000, followed by HRP-conjugated antibobies anti-mouse or anti-rabbit IgG (Santa Cruz Biothecnology; diluted 1:10,000) for 1 hr, at room temperature. Next, membranes were washed five times in TBST and the bands were visualized using ECL system according the manufacturer's instructions (Super Signal ECL, Pierce). Chemiluminiscent signals were captured in Kodak Bio-Max film, and the bands were quantitatively analyzed from digitized images captured from the films with the Gel-Doc system (BioRad, Richmond, CA), using the Bio-Rad Quantity One software. Mouse ovary and uterus were used as positive controls for this experiment.

2.6. Experimental design and statistical analysis

Hormone dose-response-time curves were estimated in 4 independent experiments, each performed with 5 cysticerci, freshly extracted from different infected pigs. The response variable used in statistical analyses was the sum of scolexed cysticerci in the 4 wells of each hormonal concentration and time of exposure in each experiment. Hormone used, hormonal concentration, time of exposure and number of experiments, were the independent variables. Per each experiment, 4 treatments was replicated in 4 wells, each well containing 5 cysticerci. The data for the 4 replications of each treatment were pooled and expressed as an average. Data were analyzed using analysis of variance (ANOVA) with hormonal treatment and number of experiments as independent variables and, as dependent variables: the average of scolexed cysticerci per each cysticerci group and their length (mm); the average of ER-α/β/β-actine OD ratio and PR-A/B/β-actine OD ratio for RNA expression. If ANOVA found significant differences between treatments, the Student t-test and t-Tukey were applied to each experiment using the residual variance estimated by ANOVA to test for significance. Differences were considered significant when $P< 0.05$.

3. Results

When cysticerci were exposed *in vitro* to P_4, E_2 or P_4/E_2 combination, it was apparent that progesterone and P_4/E_2 markedly increased parasite scolex evagination meanwhile estradiol effect was not so powerful (Fig. 1). In the P_4-treated group, the percent of scolexed cysticerci in relation to control values increased progressively, reaching a hundred percent their initial average after 25 days in culture, while the effect of E_2 only was on fifty percent of the treated parasite but at the same level that in control groups. The effect of P_4/E_2 also increased at a hundred percent the *T. solium* cysticerci scolex evagination, probably more due to P_4-effect than E_2. No synergistic effects were observed at the lowest doses of P_4/E_2. To the opposite, RU-486 and tamoxifen treatment, even in presence of P_4, E_2 and the combination of both hormones, showed a powerful anti-cysticercus effect, due to they completely inhibited scolex evagination in *Taenia solium* (Fig. 1).

Moreover, P_4-treated cysticerci started to evaginate since sixth day in culture achieving a total length of 3 fold-times (9.5mm) upper than control groups (3.25mm) at fifty day (Fig. 2). In addition to, estradiol treatment increased by 2 fold-times the scolex length (6.25mm) comparing with control cysticerci and accelerated scolex evagination, starting evagination at fourth day in culture, upper controls and P_4-treated parasites. The effect of P_4/E_2 was higher than E_2-effect but no differences were observed with P_4-treated parasites (Fig. 2).

On the other hand, different concentrations of P_4 and E_2 in the culture media were studied to ascertain a dose-dependent response pattern of *T. solium* cysticerci. P_4, at the doses 80 and 800 ng/mL, clearly augmented scolex evagination in a hundred of the treated parasites (Fig. 3). The E_2 dose of 400 pg/mL increased sixty percent the parasite differentiation, accelerating scolex formation before than control and P_4-treated cysticerci (Fig. 3). In addition to, combinated P_4 and E_2 did have their most powerful effect at 800 and 100 ng/mL respectively. Neither P_4 and E_2 by

separated, nor the combination of both hormones affected viability at any tested dose, even at concentrations 20 and 40 μg per mL, respectively.

It is important to say that scolex appearance in P_4 and E_2-treated cysticerci was healthy and without injury, showing constantly motility in the culture plate (Fig. 4).

In addition to, P_4 and E_2-treated cysticerci (Fig. 4A) were motile with completely differentiated scolex, showing two rows of hooklets, in addition to four suckers also well developed (Fig. 4B). They were also motile and rather large (average length at this time was 9.5 ± 1.25 mm). P_4/E_2 treatment did not show any differences between P_4-treated parasites and those ones treated with this hormonal combination. In contrast, cultured cysticerci in presence of tamoxifen and E_2 were poorly differentiated (Fig. 4C), meanwhile RU-486 treatment completely inhibited P_4-effects and, therefore, evagination and growth of the *T. solium* cysticercus scolex (Fig. 4D). *($P< 0.05$)*

In view that different mRNA can have varying half-lifes and may undergo selective degradation, we determined the quantity and integrity of total RNA extracted from the different parasites used in this study. It was clear that the same amount of RNA (10 μg), which was not-degraded, was used for RT-PCR amplification in each studied tissue (data not shown). The amplification by RT-PCR of the ER-α (220 bp) and β (245 bp) and PR-A (210 bp) and B (320 bp) for *T. solium* is shown in Figure 5A. Specific fragments which correspond in molecular weight to those of ER-α, ER-β, PR-A, and PR-B, previously reported for mammals, showed poor regulation in response to hormonal treatment (Fig 5A). Interestingly, the only gene who demonstrated to be strongly affected by P_4 was PR-A, which expression diminished at fifty percent respect to control groups (Fig. 5 A).

On the other hand, not only the gene expression of the parasite sex-steroid receptors was detected, but also presence of their proteins. By means of western blot and using several polyclonal antibodies previously described on materials and methods, four corresponding fragments to ER-α (55 KDa), ER-β (80 KDa), PR-A (80 KDa) and PR-B (120 KDa) were immunodetected in the helminth parasite *T. solium* and mouse ovary and uterus as internal control (Fig. 5B).

4. Discussion

This work describes the progesterone and 17β-estradiol effects, by separated and using a combination of both hormones, in order to resemble the physiological conditions that parasite can be exposed in their native host, upon *T.solium* cysticerci *in vitro* evagination. Moreover, we reported the powerful anti-cysticercus effect of the anti-hormones RU-486 and tamoxifen, on the same differentiation process of *Taenia solium*, as well as the presence of their corresponding parasite sex-steroids receptors. Progesterone-treatment induced in a hundred percent of the *T. solium* cysticerci scolex evagination (Fig. 1). Evaginated scolex showed motility and well differentiated structures. The combination of both hormones also increased the number of evaginated parasites in a hundred percent of them, at the same level than progesterone alone, probably due to the effect of the lastone. Interestingly, no synergistic effect was observed by means of this combination, even at the lowest doses of these sex-hormones, which suggests that progesterone may play the most important role upon *in vitro* scolex evagination of *T. solium* cysticercus.

Nevertheless, E_2-treatment accelarated scolex evagination (Fig. 2), which makes us suppose sex-steroids can act at several levels during a parasite infection, for instance in this model, 17β-estradiol can accelerate evagination while progesterone stimulates and holds through the time, this important parasite differentiation process. The additive effects of exogenous P_4 and E_2 (which can be found during pregnancy and several phases of the reproductive cycle of humans and pigs) upon *T. solium* differentiation may mean a better establishment and faster reproduction of the parasite in an immune-competent host. According to our results, the possible action mechanisms through which progesterone and estradiol affected parasite differentiation was tested at three levels: 1) by RT-PCR, because bands to ER-α, ER-β, PR-A and PR-B were amplified

from *T. solium* cysticerci. It is important to say that PR-A was the only parasite sex-steroid receptor who showed a strong hormonal regulation. Typical endocrinologically, this receptor demonstrated being regulated through a negative feed-back, similarly as its mammalian counterparts finding in different mouse and human tissues, which suggests that steroid hormones can preservate several molecular mechanisms along evolution and species, even though it be a parasite. 2) In the same sense, we were able to detect corresponding bands to ER-α, ER-β, PR-A and PR-B by means of western blot, using polyclonal antibodies raised against mouse. This result suggests that the helminth parasite *T. solium* has similar proteins to ER-α, ER-β, PR-A and PR-B (parasite-ER-α, ER-β, PR-A and PR-B, which means like-sex steroid receptors on *T. solium* or proteins that can bind sex-hormones and work as sex-steroid receptors) previously described in mammals such as mice and humans. Moreover, we propose these proteins conserve specific and necessary aminoacid sequences to transduce an external positive signal, in this case, mainly stimulated by progesterone. Finally, 3) Parasite-ER-α, ER-β, PR-A and PR-B function in *T. solium* was tested by means of specific anti-hormones (RU486 or Tamoxifen). It is well known that RU-486 and Tamoxifen inhibits P_4 and E_2-functions, respectively, by competitive binding with their specific nuclear receptors [25, 26].

On the other hand, it was very important to determine that parasite like-receptors were from parasitic tissue and not obtained artifactually by host cell contamination. This fact was supported for two main reasons: a) to eliminate possible host cell contamination after parasites being extracted directly from host tissues, cysticerci were cultured for more than eleven weeks with a free serum medium, which maintains parasites, but mammalian cells do not grow in, b) a highly specific gene product, which has evolved only in mammals, such as the fragment VDRHC (Variable Region of the Heavy Chain of the Immunoglobulins) gene was used, and showed no

amplification in the parasite, but positive in the host tissues. Same result was obtained when another highly evolved gene (as TNF-α) was used as template; only the mouse and pig tissues amplified it, but *T. solium* did not (data not shown).

In this work, the only addition of RU486 and tamoxifen, even in presence of progesterone, estrogens or a combination between those ones, inhibited *T. solium* scolex differentiation, which suggests that these anti-hormones have a powerful anti-cysticercus effect upon one of the most important differentiation process of the *T. solium*, the scolex evagination that will finally allow the flatworm developing, whose main function is the production of thousands of infective eggs that, once released to the enviroment, can maintain the *T. solium* life cycle and, therefore, the infectious process on humans and pigs.

According to our results, we proposed that progesterone is recognized for parasite like-receptors in *T. solium*, forming a ligand-receptor complex, probably as it has been described for other species. This complex could dimerize and translocate into the parasitic cell nucleus where could be able of regulating several aspects of the parasite physiology, such as differentiation, scolex evagination and growth, through activation of parasite transcription factors, such as AP-1 complex, as we have previously reported for *Taenia crassiceps*, another helminth parasite. It has been suggested the participation of a like-nuclear receptor in the parasite, because of its PCR-amplification and detection by western blot and, in addition to, the whole inhibition of its function by means of specific pharmacological agents such as RU486 and tamoxifen. As we said, RU486 and tamoxifen are anti-hormones able to bind to progesterone and estrogen receptors, respectively, through competitive mechanisms, blocking in this way the sex-steroids effects such as stimulation of the scolex evagination in the *T. solium* metacestodes.

These results suggest *T. solium* has developed, through many years of coevolution with its porcine and human hosts, a complicated host-parasite network, clearly affected by sex-steroids and involved in the maintenance of one of the most important parasite processes, *T. solium* cysticercus scolex evagination, differentiation and growth.

In recent years, much information about host hormonal microenviroment affecting many parasite species has been reported [27-30]. For specific case of this infection, we had previously found that gender and circulating estradiol and progesterone levels in host mice and host pigs crucially affected the dynamics of parasite loads in infected mice with *Taenia crassiceps* cysticerci [31] and infected pigs with *T. solium* metacestodes [15].

In this sense, it is important to say that these levels of estradiol and progesterone in humans and pigs, may play a relevant role during natural infection. In order to test this hypothesis, we detected in the parasite proteins (like-steroid receptors) which possibly can bind exogenous sex-hormones and act as sex-steroids receptors, activating gene expression and, therefore, molecular and cellular important events, such as establishment, growth and differentiation. Such commonalities between host and cysticercus metabolism should come as no surprise, when extensive homologies between species are being documented in other systems as well [32]. In addition to, we showed that both subtypes of the classic estrogen receptor (ER-α and ER-β) are expressed in *T. solium*, as well as those isoforms of progesterone receptors (PR-A and PR-B). It appears that the effects of estrogen and progesterone are due to the binding of estradiol and progesterone to their respective parasite like-steroid receptors. The effects of E_2 could result from the low-expression of its specific receptor or be due to non-genomic effects, or merely reflect its transformation to other steroid metabolites [33]. Binding of the ER and PR to steroids-dependent elements at parasite DNA, could be responsible for the activation of AP-1 complex

genes in the normal metabolism of *T. solium* [24]. Previous studies have demonstrated that the genome of *Onchocerca volvulus* encodes at least three members of the nuclear receptor family [34], as well as in *T. crassiceps* cysticerci [16] and this could also be the case for *T. solium.*

These findings suggest that progesterone and estrogen-effects upon parasite are mediated by sex-steroid like-receptors expressed by *T. solium* cysticerci, which may find important applications in the development of future vaccines and therapeutic protocols, evaluating possible enhancer hormonal effects during both natural infection and either human or veterenary pharmacological treatment. On the other hand, the fact that RU486 and Tamoxifen have so powerful anti-cysticercus effect, blocking the development of *T. solium* cysticerci scolex, provides the first evidence that it is possible, in a future, to down-regulate this parasite physiological process in order to diminish or eliminate the infection transmission rate, decreasing proglottides developing and infective eggs production, mainly in the third world and developing countries.

Finally, these results may contribuite to a better understanding of our conception of the host-parasite relationship and sexual dimorphism during a parasite infection at evolutionary and physiological levels.

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Figure Legends

Figure 1. Progesterone increases scolex evagination of the *Taenia solium* cysticerci. P_4-treated parasites showed an increasing of hundred percent of the scolex evagination while E_2 induced it in fifty percent of the treated cysticerci. P_4/E_2 combination did have the same effects than P_4. Nevertheless, estrogenic effects were more evidents on the time in which cysticerci started to evaginate. In contrast, RU486 and tamoxifen, by separated or in combination, dramatically inhibited scolex evagination in the hundred percent of the treated parasites. C = vehicle-treated cysticerci (untreated cysticerci); C_{EtOH} = vehicle (pure ethanol 0.06 percent)-treated cysticerci; E2 = 17β-estradiol-treated cysticerci; P4 = progesterone-treated cysticerci; P4/E2 = progesterone and 17β-estradiol-treated cysticerci at the same time; Tamoxifen+E2 = tamoxifen and 17β-estradiol-treated cysticerci at the same time; RU486+P4 = RU486 and progesterone-treated cysticerci at the same time; Tamoxifen/RU486+E2/P4 = tamoxifen and RU486 else 17β-estradiol and progesterone-treated cysticerci at the same time; Tamoxifen = tamoxifen-treated cysticerci; RU486 = RU486-treated cysticerci; Tamoxifen/RU486 = tamoxifen and RU486-treated cysticerci at the same time. **Significant statistically between treated and untreated cysticerci; *Significant statistically with untreated cysticercy. $P< 0.05$.

Figure 2. Progesterone and estradiol increase scolex growth of the *Taenia solium* cysticerci. P_4 and E_2-treatment stimulated scolex lengthening by 3 and 2 fold-times, respectively, upper than vehicle treated-cysticerci. In both of the P_4 and E_2 treatments, scolex growth was progressively increasing until to begin its maximum value at fifty day in culture. Interestingly, E_2-treatment accelerated scolex evagination, starting this process at fourth day in culture. E2/P4 did have the same effects than progesterone alone, probably due to the effect of the single hormone. Moreover, no synergistic effect was observed with this combination. A total of twenty cysticerci

per hormonal treatment were used in each independent experiment. C = vehicle-treated cysticerci (untreated cysticerci); E2 = 17β-estradiol-treated cysticerci; P4 = progesterone-treated cysticerci; E2/P4 = estradiol and progesterone-treated cysticerci at the same time. **Significant statistically between treated and untreated cysticerci; *Significant statistically with untreated cysticercy. P< 0.05.

Figure 3. Dose-response curves. P_4 clearly augmented scolex evagination in a hundred of the parasites at lowest doses, while E_2 only affected to fifty percent of the treated cysticerci. P_4/E_2-treatment did have the same effects than progesterone-treated parasites, reaching its more powerful effect at 800 and 100 ng/mL of progesterone and estradiol respectively. **Significant statistically between treated and untreated cysticerci; *Significant statistically with untreated cysticercy. P< 0.05.

Figure 4. *Taenia solium* metacestodes response to sex-steroid and anti-hormonal treatment. P_4 and E_2 accelerated scolex evagination of the *T. solium* cysticerci, which developed well differentiated hooks and suckers (A and B). Treated parasites with P_4 developed longer scolex than E_2-treated cysticerci (A). In contrast, the only addition of tamoxifen and RU486 dramatically inhibited scolex evagination and developing (C and D respectively).

Figure 5. (A) Estrogen and progesterone (mRNA) receptors expression in *Taenia solium* cyisticerci by RT-PCR. Estrogen (sub-types α and β) and progesterone (isoforms A and B) receptors expression was quantified by densitometric analysis on treated and untreated *T. solium* metacestodes. (B) Estrogen and progesterone protein receptors detection in *Taenia solium* metacestodes by western blot. Corresponding bands to ER-α, ER-β, PR-A and PR-B were detected in *T. solium* by means of western blot using polyclonal antibodies. C = vehicle-treated cysticerci (untreated cysticerci); E_2 = 17β-Estradiol-treated cysticerci; Tamoxifen+E_2 =

tamoxifen and 17β-estradiol-treated cysticerci; P_4 = progesterone-treated cysticerci; RU486+P_4 = RU486 and progesterone-treated cysticerci. Ovary = mouse ovary as internal control protein; Uterus = mouse uterus as internal control protein. * $P < 0.05$.

Detection of LYN-like and ERK-like Proteins as Possible Non-genomic Action Mechanism Involved in Estradiol-Stimulated Reproduction of the Helminth Cestode *Taenia crassiceps*

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Running Head: Estradiol actions on parasites

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Abstract

Experimental infection by the helminth parasite *Taenia crassiceps* is favoured by female hormonal environment. Specifically, 17-β estradiol promotes parasite establishment by two non-excluding independent pathways: a) modulation of the host's immune response and b) direct action upon parasite to regulate its growth, reproduction and infectivity through specific estrogen receptors. Nevertheless, here we report experimental evidence which suggests that the 17β-estradiol proliferative effects on *Taenia crassiceps* cysticerci also involve the participation of a non-genomic mechanism, mediated by the LYN-ERK tyrosine kinase signalling pathway activation. Our results show that *in vitro* exposure of *Taenia crassiceps* cysticerci to 17β-estradiol increased parasite reproduction by two and a half-fold. In contrast, genistein or ERK-inhibitor, which are specific inhibitors of LYN and ERK respectively, inhibited 17β-estradiol proliferative effects, diminishing parasite reproduction to the same level as untreated cysticerci. Concomitantly, 17β-estradiol treatment was able to strongly induce phosphorylation in a variety of parasite proteins, including parasite like-LYN and ERK, increasing six-fold the total percentage of activated proteins. Moreover, not only were *Lyn* and *Erk* corresponding fragments detected by means of RT-PCR, but also *Lyn* gene expression was clearly augmented by 17β-estradiol treatment. On the other hand, Western blot assays showed surprisingly different molecular weight between parasite LYN-like and ERK-like proteins and similar proteins detected in the host's tissues. In addition, flow cytometry experiments corroborated that parasite LYN and ERK-like proteins were specifically from *Taenia crassiceps*' tissue and not contamination from host cells, also proving that parasite second messengers can be activated by exogenous 17β-estradiol. Finally, these results may contribute to a better understanding of the molecular crosstalk between parasite and host, which can ultimately regulate parasite physiology by means of its own hormones and growth factors. These considerations can help in the design of specific drugs that exclusively affect the cysticercus and not the host.

Introduction

Taenia crassiceps is a helminth parasite, which, in its adult stage as a worm, parasitizes cannids, while during its intermediate stage, as a cysticercus, it hosts several extraintestinal tissues of rodents [1]. Experimental *T. crassiceps* cysticercosis in mice simply requires the intraperitoneal injection of living cysticerci into recipient mice [2]. Intraperitoneal cysticerci reproduce asexually by budding, reaching massive parasite loads in a few months [3] that can approximate the host body weight without causing it apparent discomfort [4]. Cysticerci also survive and reproduce *in vitro* in usual culture conditions with fetal calf serum-free media [5]. These features of murine experimental cysticercosis make it a convenient model to study the immunological, genetic and sexual factors involved in susceptibility to infection and parasite proliferation [6,7].

Sex-steroids play a significant role in parasite load regulation during experimental intraperitoneal cysticercosis in male and female mice. Briefly, estrogens increase the parasite load and androgens decrease it: 1) by acting directly on the parasite, and favouring or hindering its reproduction, respectively, and 2) by tilting the hosts' immune response towards a parasite-permissive Th2 or a parasite-restrictive Th1 response [8].

We previously reported that 17-β-estradiol (E_2) increases *T. crassiceps in vitro* reproduction while androgens inhibit it. The proliferative effect of E_2 on the parasite seems to be mediated by the estrogen-induced AP-1 transcription factor expression in the parasite [5]. Estrogen receptor (ER) expression on cysticerci and AP-1 complex activation, in addition to the inhibition of proliferation by tamoxifen, support the idea that one of the proliferative mechanisms mediated by E_2 is a classical nuclear receptor-dependent pathway [9,5].

However, the proliferative effect of E_2 on *T. crassiceps* reproduction is not fully inhibited by the anti-estrogen tamoxifen, which suggests that the parasite can respond to estrogens by alternative pathways. Potential alternative pathways do not necessarily require the binding of the steroid to a classical nuclear receptor, but can occur through non-specific receptors, many of which belong to the tyrosine kinase family (TK). Several mechanisms of action related to adrenal and sex-steroid

hormones have been described [10,11]; among the most amply studied are the non-genomic mechanisms mediated by E_2. Specifically, E_2 treatment can activate several types of TK such as PI3K and SRC kinases. The G protein-coupled receptor action (GPCR) may also be modulated by the estrogen/receptor complex (E/ER) [12], as in excitable cells from mouse reproductive tissue, where estrogens can promote GPCR activity [13], triggering epidermal growth factor receptor (EGFR) and, concomitantly, PLC-PKC pathway activation [14]. On the other hand, not only E_2 effects are mediated by unspecific receptors closely localized in the cellular membrane, but also by means of the specific ER [15,16]. In fact, the binding between E_2 and its membrane ER activates group I and II of the metabotropic glutamate receptor [17]. It is important to mention that ER by itself is able to bind to SRC kinases through their highly conserved SH2 domains. Such is the case of the LYN second messenger, among others, which will be activated as a consequence of E/ER action. Once phosphorylated, LYN will activate downstream to Syk or Zap-70 and, as a consequence of this, to the extracellular signal-regulated kinases and mitogen-activated protein kinase signalling pathway (ERK-MAPK). Finally, the ERK-MAPK pathway will regulate cell proliferation and differentiation [18,19]. On the other side, when E_2 is present, ER-α can directly interact with p85, the regulatory subunit of PI3K, ultimately activating the Akt pathway [20,12]. The regulation of very important processes such as cell proliferation, differentiation and growth by means of non-genomic steroid mechanisms in mammalian systems has been extensively studied [21-23]; nevertheless, information about this type of mechanisms operating in parasitic cells is scarce [24] and, at the same time, full of interest from the current medicine and drug designing point of view, because more information about the metabolic pathways that regulate parasite growth and establishment could provide specific potential targets for therapeutic treatment, such as enzymes, genes and molecules present exclusively in the parasite. This would avoid host damage and unspecific cross-responses.

For these reasons, the present study searched for evidence to show that the 17β-estradiol proliferative effects on the helminth parasite *Taenia crassiceps*, which is antigenically very close to

Taenia solium, may be related to the participation of a tyrosine kinase-like signalling pathway in the parasite that can be activated by E_2 and could regulate *in vitro* cysticercus reproduction. This study aimed to open new considerations about the host-parasite relationship at molecular and evolutionary levels, as well as contributing to identify several steroid-dependent parasite metabolic routes in order to design cysticidal drugs that specifically inhibit cysticerci without affecting to host.

Results

In vitro effects of E_2 and tyrosine kinase inhibitors on *Taenia crassiceps* cysticercus reproduction. Concentration-response curves

To study the LYN-ERK-like pathway functionality in *T. crassiceps* cysticerci and propose a non-genomic mechanism through which E_2 can induce an increase in the number of buds in the parasite, different concentrations of E_2 were tested to determine a possible concentration-dependent response pattern of parasite reproduction. The number of buds clearly increased as the E_2 concentration rose. The 100μM concentration duplicated the parasitic reproduction rate, and a maximum response was reached with 800μM (Fig. 1). A concentration-response pattern contrary to that of 17β-estradiol was evident when tyrosine kinase inhibitors (TK-inhibitors) were tested (Fig. 1). All TK-inhibitor concentration-response curves were obtained in the presence of E_2 400μM. Tested TK-inhibitors were AG18 (a general inhibitor of tyrosine kinase, [25]), genistein (LYN-inhibitor, [26]) and ERK-inhibitor (inhibitor of the extracellular signal-regulated kinase signalling pathway, [27]).

As expected, 1μM AG18 blocked the E_2-effect on the parasite, reaching its maximum antiproliferative response at 8μM concentration (Fig. 1). 1μM genistein had a similar effect on parasite reproduction as AG18 at the same concentration. The maximum response for genistein was obtained at 4μM concentration, and showed the same antiproliferative effect as that obtained with 8μM concentration (Fig. 1). Finally, at 1 and 2μM concentrations, ERK-inhibitor exhibited a poor but statistically significant stimulating effect on *T. crassiceps* reproduction. However, higher concentrations of this TK-inhibitor completely blocked the E_2-proliferative effect on the parasite, diminishing the number of buds to the same level as in untreated cysticerci (Fig. 1).

In vitro effects of E_2 and tyrosine kinase-inhibitors on *Taenia crassiceps* cysticercus reproduction. Time-response curves

To explore the temporal progression of E_2 and TK-inhibitor effects on parasite reproduction, we designed time-response curves, exposing *T. crassiceps* cysticerci to the optimal antiproliferative doses of AG18, genistein and ERK-Inhibitor (taken from the concentration-response curves shown in Figure 1), with added 400μM of E_2 in each case, during five days on *in vitro* culture.

After five days in culture, bud production in E_2-treated cysticerci showed a four-fold increase compared to bud production in untreated cysticerci (Fig. 2). AG18 showed the most severe antiproliferative effect on parasitic reproduction: this TK-inhibitor decreased the number of *T. crassiceps* cysticerci buds by fifty percent with respect to control values, even in presence of E_2 (Fig. 2). Similarly, genistein and ERK-inhibitor blocked the E_2-proliferative effect of the parasite because it reduced bud production to the same level as in untreated cysticerci (Fig. 2).

LYN-like and ERK-like amplification in *Taenia crassiceps* cysticerci

Considering that different mRNAs can have different half-life and may undergo selective degradation, we determined the quantity and integrity of total RNA extracted from E_2-treated and untreated *Taenia crassiceps* cysticerci and mouse spleen as positive control tissue. The same amount of non-degraded RNA (10 μg) was used for RT-PCR amplification in each case. *Lyn*-like and *Erk*-like amplification of *T. crassiceps* cysticerci is shown in Figure 3. Specific bands that correspond in molecular weight to those of *Lyn* (240 bp), *Erk* (112 bp) and *β-actin* (220 bp) previously reported for mammals were obtained from *T. crassiceps* cysticerci and mouse spleen (Fig. 3).

On the other hand, not only was *Lyn*-like and *Erk*-like gene expression detected in *T. crassiceps* cysticerci, but also the modulation of these genes in response to 17β-estradiol and TK-inhibitors (Fig. 4). A representative gel is shown in Figure 4A, where β-actin was used as constitutive expression control in order to analyze with optical densitometry. Interestingly, E_2 was the only treatment that significantly modified *Lyn*-like gene expression, increasing it by two-fold compared to the control group (Fig. 4B). Moreover, TK-inhibitor treatment did not have any effect

on the gene expression of this parasite second messenger (Fig. 4B). Opposedly, neither E_2 nor TK-inhibitors affected *Erk*-like gene expression in the parasite (Fig. 4B).

LYN-like and ERK-like protein detection in *Taenia crassiceps* cysticerci

We obtained and quantified total protein from E_2 and TK-inhibitor-treated *T. crassiceps* cysticerci. The mouse polyclonal anti-LYN recognized in the immune-precipitated *T. crassiceps* a single band of approximately 48 KDa, while in pervanadate-stimulated and nonstimulated THP cells the antibody recognized two protein bands of 53 and 56 KDa, which correspond to the reported molecular weight for LYN in several mammalian species such as mouse, rat and human (Fig. 5A). It is important to underline that neither E_2 nor genistein treatment modified LYN-like synthesis in the parasite (Fig. 5A). On the other hand, a characteristic doublet for ERK, of approximately 55 and 62 KDa respectively, was detected in the *T. crassiceps* cysticercus by means of the mouse polyclonal anti-ERK (Fig. 5B). Unexpectedly, the couple of bands differed from those detected in pervanadate-stimulated Bw cells, whose expected molecular weight matches the reported size (42 and 44 KDa) of these proteins in several strains of mice (Fig. 5B). Interestingly, 17β-estradiol treatment triplicated only the synthesis of the ERK-like heavier band, while ERK-inhibitor treatment reduced it to lower levels than those detected in untreated parasites (Fig. 5B). In addition to, since second messengers like LYN and ERK are activated through phosphorylation processes, it was relevant not only to detect them in the parasite but also to determine their capacity for being phosphorylated in presence to estrogens or TK-inhibitor treatment. For that reason, we decided to determine the LYN-like and ERK-like functionality in the *T. crassiceps* cysticercus through two different strategies: a) using a polyclonal antibody able to recognize all phosphorylated proteins in response to different treatments (Fig. 5C), and b) by means of an antibody that exclusively recognizes the ERK phosphorylated form, which is at the end of the whole signalling pathway and in charge of activating proliferative signals inside the parasitic cell (Fig. 5D). Our results indicate that 17β-estradiol duplicated the total percent of phosphorylated proteins in *T.*

crassiceps, while TK-inhibitors decreased it in sixty percent of the total proteins, with respect to untreated cysticerci (Fig. 5C). Concomitantly, when *T. crassiceps* was exposed to E_2, there was an increase in ERK-like phosphorylation (expected molecular weight of 62 KDa), compared to control cysticerci (Fig. 5D). Moreover, TK-inhibitor treatment partially blocked the E_2-effect in the parasite, diminishing ERK-like phosphorylation in response to this estrogen (Fig. 5D).

3.5. LYN-like and ERK-like specific detection in *Taenia crassiceps* cysticerci by flow cytometry

In order to determine that LYN-like and ERK-like proteins came exclusively from *T. crassiceps* cysticerci cells and that they had not been contaminated by host immune cells, we performed a flow cytometry analysis using a polyclonal antibody able to specifically recognize to cestode's paramyosin, a transcendental protein in the parasitic cytoskeleton function, which is interestingly not expressed by mammals. Two different cell populations are described: the first are Balb/cAnN mouse spleen cells, specifically recognized by α-CD3, α-CD4, α-CD8, α-CD19 and α-macrophages antibodies, as is well known. Moreover, these mouse cells showed a diameter between XXX and XXX μM and high percentage of granularity (Fig. 6A). Nevertheless, these cells were not recognized by α-paramyosin antibody as shown in Figure 6A. On the other hand, we were able to detect another cell population, different to that described for mouse spleen. These cells were obtained from *T. crassiceps* tissue, showing different patterns of size and granularity to host immune cells (their diameter was between XXX and XXX μM, and granularity was XXX-fold less than that of immune cells, suggesting that these parasite cells are less complex than mouse spleen cells, Fig. 6B). It should be mentioned that these parasite cells did not recognize α-CD3, α-CD4, α-CD8, α-CD19 and α-macrophages antibodies. Additionally, these parasite cells were well marked by α-paramyosin antibody (Fig. 6B), and used as template for the subsequent analysis of exclusively parasite LYN-like and ERK-like (Fig. 6C-F). LYN-like and ERK-like were not only shown to be present in parasite cells, but also to be upregulated by 17β-estradiol treatment. Specifically, this estrogen increased the *T. crassiceps* LYN-like synthesis by XXX-fold with respect

to the control group (Fig. 6E). Similarly, ERK-like showed a XXX fold increase in E_2-treated parasites as compared with untreated cysticerci (Fig. 6F). Unexpectedly, 17β-estradiol also augmented *T. crassiceps* paramyosin synthesis XXX-fold, showing what to our understanding is the first evidence that exogenous sex-steroids can affect the expression and synthesis of cestode cytoskeleton molecules (Fig. 6C and 6D).

In vitro effects of E_2, tamoxifen and tyrosine kinase inhibitors on *Taenia crassiceps* cysticercus reproduction

With the purpose of studying the possible coordinated participation of the LYN-like and ERK-like dependent pathway and the proliferative mechanism mediated by the estrogen receptor (ER), we decided to expose *Taenia crassiceps* cysticerci to TK-inhibitors in combination with tamoxifen, a competitive ER-inhibitor, both of them in the presence of 17β-estradiol. As shown previously, E_2 increased the number of buds in the parasite three-fold. Opposedly, tamoxifen treatment totally inhibited the proliferative effects of this estrogen, reducing parasite reproduction to the same level as in the control group (Fig. 7). Interestingly, separated combinations of tamoxifen, AG18, genistein and ERK-inhibitor not only inhibited the E_2-effects but also progressively diminished the number of buds below control group values (Fig. 7). No differences were detected when combinations of tamoxifen with any of the three TK-inhibitors were used; however, all of these combinations showed significant differences when compared to E_2-treated and untreated parasites (Fig. 7).

Discussion

As we have previously reported, there is a direct proliferative effect of 17β-estradiol on *Taenia crassiceps* cysticercus reproduction, which is not necessarily mediated by the host immune system, but through classic nuclear receptor-like sex-steroids in the parasite [5]. However, alternative mechanisms through which E_2 can affect the parasite, securing its reproduction and establishment in an immunecompetent host, have not been completely explored yet. The aim of the present study was to investigate the participation of a signalling pathway mediated by second messenger cascades, which may be responsive to sex-steroids that are highly conserved throughout the species and involved in cell proliferative processes. Thus, we explored the LYN and ERK-dependent signalling pathway, two of the tyrosine kinase proteins which can be activated by several extracellular signals and generate rapid responses inside the cell, and the effect of the specific inhibitors, genistein and ERK-inhibitor, on their participation in parasite reproduction.

Interestingly, we found that, at progressively higher 17β-estradiol concentrations, the number of *T. crassiceps* cysticercus buds reached its maximum value. The opposite response was observed when TK-inhibitors were tested in *in vitro* culture. As concentration of AG18, genistein and ERK-inhibitor rose, parasite reproduction progressively decreased (Fig. 1). On the other hand, the E_2-effect was enhanced as time passed, reaching its maximum effect on parasite reproduction at day five of *in vitro* culture. Nevertheless, no time-dependent response was found when AG18, genistein and ERK-inhibitor were tested, although TK-inhibitors completely blocked the E_2-proliferative effect, suggesting that parasite LYN-like and ERK-like proteins could mediate their estrogenic action because in the presence of LYN and ERK-inhibitors there was no E_2-stimulated reproduction (Fig. 2). These findings show a marked dose and time-dependent pattern in the effects of E_2 on cysticercus reproduction, which seems to be relevant because it suggests that sex-steroids could have similar pharmacologic effects on mammals and on parasitic cestodes, a hypothesis that evokes the wide range of steroid hormone actions not only in many different cell types, but also along the phylogenetic scale, among organisms evolutionarily not closely related.

Moreover, determination of the pharmacologic actions of E_2 and TK-inhibitors on *T. crassiceps* cysticerci was as important as verifying *Lyn* and *Erk* gene expression in the parasite as well as their translation to functional proteins in charge of mediating E_2-effects. In view of this, corresponding bands to *Lyn*-like and *Erk*-like were amplified from *T. crassiceps* larval tissue by means of specific primers designed on the most conserved regions of these genes, previously reported in mammals such as mouse, rat and human (Fig. 3). Unexpectedly, not only *Lyn*-like and *Erk*-like genes are expressed in the parasite, but they can also be regulated by sex-steroids such as 17β-estradiol, which specifically induced *Lyn*-like overexpression without affecting the *Erk*-like expression (Fig. 4B). It should be mentioned that most of the work about signal transduction pathways focuses on studying their activation mechanisms by hormones [28-30]. However, especially in parasites, the effects of these growth factors on second messenger gene expression, which are practically in charge of mediating the functionality of the whole pathway, have been scarcely explored. Present results show that this type of parasite genes are differentially regulated by exogenous sex-steroids, which could be occurring *in vivo* from host to parasite, and this offers an alternative explanation of why *T. crassiceps* cysticerci grow better in the mouse female than in the male [4], highlighting differences between the hormonal microenvironments of both genders.

Concomitantly, it is necessary to underline that second messengers lead their transduction functions as phosphorylated proteins; it was therefore transcendental to determine corresponding proteins to LYN and ERK in the parasite and to analyze their phophorylation pattern in response to 17β-estradiol and TK-inhibitor treatments. Unexpectedly, one single band corresponding to LYN-like was detected in *T. crassiceps*, while most of the mammalian species exhibit a characteristic doublet (Fig. 5). This finding suggests that, along many years of coevolution between the host and the parasite, several molecules, such as genes or proteins, may be lost or "economized", passing their functions onto others that conserve similar structures and, therefore, can trigger the same effects due to a complex process of "molecular hypertrophy", where many functions can be led by a few molecules. In the same sense, ERK-like was detected in *T. crassiceps* larvae as a typical protein

doublet showing heavier molecular weight than the same proteins previously reported for mouse, rat and human (Fig. 5). The fact that ERK-like differs from its homologue in BW cells, supports two very important aspects of this study: a) that second messengers detected in the parasite are not a contamination by host immune cells, and b) that, although both proteins have different sequences, they probably conserve a high degree of similarity in their catalytic domains, which makes them recognizable with the same antibody, guaranteed the suitable function for transducing signals in both organisms. In addition, 17β-estradiol treatment differentially stimulated the phosphorylation of ERK-like, which strongly supports that parasitic LYN-like and ERK-like are not only expressed and regulated by exogenous estrogens, but also, these second messengers can be translated to functional proteins, able to be activated through E_2-stimulated phosphorylation processes. This result suggests again that steroid hormone action mechanisms upon target cells are poorly diversified through species, maintaining similar and successful strategies from the simplest organisms to the most complicated.

On the other hand, it was critical to determine that the detected and analyzed LYN-like and ERK-like were exclusively from the *Taenia crassiceps* cysticercus, and were not a consequence of host immune cell contamination, because, as shown elsewhere, there is an extremely high interaction between parasites and immune cells, which may eventually lead to leukocyte invasion into several parasitic tissues [31]. For this reason, an alternative use of flow cytometry was employed to differentiate proteins from *T. crassiceps* and the host mouse by identifying exclusive molecules of the parasite which are neither synthesized nor expressed by the host. This is the case of the paramyosin, a muscle protein found only in invertebrates such as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Taenia solium* and *T. saginata* [32]. The flow cytometry studies showed that the analyzed LYN-like and ERK-like presence and phosphorylation pattern belonged specifically to the parasite, because paramyosin was only detected in *T. crassiceps* cells, where the second messengers were also found and studied. Opposedly, cells extracted from mouse spleen (where no LYN-like and ERK-like analyses were performed) were not recognized by the α-

paramyosin antibody, but they were positive to α-CD3, α-CD4, α-CD8, α-CD19 and α-macrophage antibodies, contrary to parasite cells (Fig. 6).

These results demonstrate that the parasitic proteins analyzed are in fact from *Taenia crassiceps* origin and not from other sources, and simultaneously emphazise the potential use of flow cytometry for differential identification of molecules from organisms with extremely close contact, such as the parasite and its host.

Finally, in order to test if we could arrest parasite proliferation completely by inhibiting the genomic and rapid response pathways, we cultured *T. crassiceps* cysticerci in the presence of 17β-estradiol and TK-inhibitors and tamoxifen. It is well known that tamoxifen inhibits E_2-genomic actions by competitively binding to estrogen nuclear receptor (ENR) [33,34]. Interestingly, using combinations of genomic and non-genomic pathway inhibitors, we did not obtain a total halt of parasite reproduction (Fig. 7), which could suggest that parasites have developed alternative mechanisms to secure their reproduction at least at basal levels and, through these "physiological buffer mechanisms", avoid the loss of very important functions such as growth, differentiation, establishment and, ultimately, their preservation [35].

In conclusion, LYN-like and ERK-like from *T. crassiceps* are described here. These second messengers showed great capacity to transduce signals evoked by 17β-estradiol in the parasite. It is important to mention here that much information about the effects of the host hormonal microenvironment on parasite physiology has been generated in recent years [36-39]. Our results pretend to contribute data about the mechanisms by which the host microenvironment affects the parasite. Furthermore, the evolutionary origin of the molecules described herein, which facilitate exploitation of the host's hormones, is worthy of study. In particular, if those genes were acquired by the parasite through horizontal gene transfer or evolved by mimicry, or simply from common ancestral genes, remains to be resolved. Finally, our findings could help to understand, at molecular and evolutionary levels, several aspects of the host-parasite relationship, the sexual dimorphism of

the immune response and, on the other hand, to provide new parasitic targets for designing specific cysticidal drugs.

4. Materials and methods

Harvesting and preparing cysticerci for experimentation

A new stock of *T. crassiceps* cysticerci (ORF-Kuhn2 strain) was donated to our laboratory by R. Kuhn in 2000 and was kept by serial intraperitoneal passage in BALB/cAnN female mice approximately every four months [2,40]. Cysticerci for each experimental session were obtained from intraperitoneally infected female mice and placed in tubes containing sterile PBS (1x) supplemented with 100 U/ml of antibiotics-fungizone (Gibco, Grand Island) [3]. The tubes were centrifuged for 10 min at 1,500 rpm and 4°C, and the supernatant was discarded. Packed cysticerci were incubated in AIM-V serum-free medium (Sigma, St. Louis, Missouri). They were then centrifuged 3 times for 10 min at 1500 rpm for washing. After the final wash, the numbers of viable cysticerci (complete, translucent and motile cystic structures) were counted under a binocular microscope. Ten viable non-budding cysticerci of approximately 2 mm in diameter were then selected and dispensed into each well of 24-well culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, New Jersey) in 1 ml AIM-V Medium (Gibco BRL) and incubated at 37° C and 5% CO_2 . A sufficient number of culture wells was prepared to accommodate the complete experimental design to evaluate the effects of *in vitro* treatment of estradiol on cysticerci. Cultures were checked daily and their medium was completely replaced when turning yellowish.

In vitro treatment effects of E_2, TK-inhibitors and tamoxifen on *T. crassiceps* cysticerci

Culture grade 17-β estradiol (E_2) and tamoxifen (Tam) were obtained from Sigma. Culture grade tyrosine kinase inhibitors (TK-Inhibitors) AG18 and genistein were obtained from ICN Biomedicals, INC. ERK-inhibitor I was obtained from Calbiochem. For *in vitro* tests, water-soluble E_2 was dissolved in DMEM serum-free culture medium, while TK-inhibitors and tamoxifen were dissolved in 3% DMSO. Each one was prepared to 100μM stock concentration and then sterilized by passage through a 0.2 mm millipore filter. Each of the following experimental conditions was applied to 24 parasite-loaded wells for concentration-response curves: a) supplemented with the solvent with diluted hormone, b) separately supplemented with 1 $x10^2$, 2 $x10^2$, 4 $x10^2$ and 8$x10^2$μM of E_2, c) separately supplemented with 1, 2, 4 and 8μM of AG18, d) separately supplemented with

1, 2, 4 and 8μM of genistein, e) supplemented with 1, 2, 4 and 8μM of ERK-inhibitor I. E_2 and tamoxifen concentrations used in this study were also determined as reported in previously [5,9]. Optimal concentrations were selected from the concentration-response curves of E_2 and TK-inhibitors, and used in time-response curves. The concentrations were used as follows: $4x10^2$μM of E_2, 8μM of AG18, 4μM of genistein, 4μM of ERK-inhibitor and 40μM of tamoxifen. The number of buds per cysticercus as a function of days in culture was assessed as the response variable. In culture, each TK-inhibitor was supplemented 2 hours before the addition of the hormone. Parasite reproduction was measured by counting the total number of buds in the ten cysticerci in each well. Bud count was performed daily under an inverted light microscope. Cysticercus viability was determined daily for each well using an inverted microscope (Olympus, MO21, Tokyo, Japan) at 10x and 100x magnification. Injury to cysticerci was recognized microscopically by progressive internal disorganization, development of whitish opaque areas on the parasite's tegument and by loss of motility. Dead cysticerci were immobile, opaque and disorganized structures.

Effects of E_2 and TK-inhibitors on proliferation and gene expression of cultured cysticerci

Total RNA was isolated from hormone and TK-inhibitor-treated *T. crassiceps* cysticerci, and BALB cAnN female mouse spleen (as controls for specific *Lyn* and *ERK* gene amplification) by the single-step method based on guanidine isothiocyanate/phenol/chloroform extraction using Trizol reagent (Invitrogen, Carlsbad, CA). Briefly, cysticerci were disrupted in Trizol reagent (1ml/ 0.1 g tissue), and 0.2 ml of chloroform were added per 1ml of Trizol. The aqueous phase was recovered after 10 min centrifugation at 14,000 rpm. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol and dissolved in RNAse-free water. RNA concentration was determined by absorbance at 260nm and its purity was verified by electrophoresis in 1.0% denaturing agarose gel in the presence of 2.2 M formaldehyde.

Lyn and *Erk* gene amplification on *T. crassiceps* cysticerci

Total RNA from E_2 and TK-inhibitor-treated cysticerci was reverse-transcribed, followed by specific PCR amplification of *Lyn* and *Erk* genes, at the same time as *β-actin* (control gene), as we previously described [9]. Briefly, 10 μg of total RNA (either mouse spleen or parasite tissue) were incubated at 37° C for 1hr with 40 units of M-MLV reverse transcriptase (Applied Biosystems, USA) in 20 μl of reaction volume containing 50 μM of each dNTP and 0.05 μg oligo (dt) primer (Gibco, NY). Ten μl of the cDNA reaction were subjected to PCR in order to amplify the sequences of the specified genes. Primer design was based on the most conserved regions of sequenced genes of all species reported in the data base. Sequences of primers are as follows: *Lyn* sense 5' – CCCAAACCTCAGAAGCCATG-3', antisense 5'-TGTCGACTACGGCTGCTGCT-3', and *Erk* sense 5'-ACAAAGTTCGAGTTGCTATCA-3', antisense 5'-ATTGATGCCAATGATGTTCTC-3'. Primers were designed on SH2 and SH3 regions previously reported for mammalian species. The 50 μl PCR reaction included 10 μl of previously synthesized cDNA, 5 μl of 10x PCR-buffer (Perkin-Elmer, USA), 1 mM MgCl, 0.2 mM each dNTP, 0.05 μM each primer, and 2.5 units of Taq DNA (Biotecnologías Universitarias, México). After an initial denaturation step at 95°C for 5 min, temperature cycling was as follows: 95°C for 30 seconds, 57°C for 45 seconds and 72°C for 45 seconds during 35 cycles. An extra extension step was completed at 72°C for 10 min for every gene. The 50 μl of the PCR reaction were electrophoresed on 2% agarose gel in the presence of a 100 bp ladder as molecular weight marker (Gibco, BRL, NY). The PCR products obtained were visualized by staining with ethidium bromide. In all cases, a single band was detected corresponding to the expected molecular weight of the gene. Identity of the expected molecules was confirmed by sequencing of each specific band. The amplified DNA sequence was determined by using a Thermo Sequenase cycle sequencing kit (Biorad) and an automatic sequencer (Model LIC-4200, Aloka Co., Japan). The sequence data were analyzed using DNASIS Software (Hitachi Software Engineering, Tokyo, Japan). Homology searching on the nucleotide and protein database was carried out with the Blast program at the National Center for Biotechnology Information (Bethesda, Maryland). Pairwase sequence alignment and protein identities were performed using

CLUSTLAW 1.6 software. The *β-actin* gene is a constitutively expressed gene and it was used as internal control for differences in the amplification procedure between experiments and to stain different gels.

LYN and ERK immunoblot detection on *T. crassiceps* cysticerci

Total protein was obtained from *T. crassiceps* cysticerci by Tris-HCl conventional isolation. In brief, untreated, E_2 and TK-inhibitor-treated cysticerci as well as mouse splenocytes were disrupted in Tris-HCl (1ml/ 0.1 g tissue), proteinase K (100 units/ml) and proteases inhibitor cocktail (Calbiochem). The supernatant was recovered after 15 min centrifugation at 14,000 rpm and the pellet was discarded. Protein quantity was obtained by absorbance at 595 nm using the Bradford-Lowry method. Total protein extracts of *T. crassiceps* cysticerci and mouse splenocytes were boiled in reducing Laemmli sample buffer, separated by SDS-PAGE (10% acrylamide) and electroblotted onto nitrocellulose membranes. The membranes were blocked overnight in TBST buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.5% Tween 20) containing 1% BSA with 3% dry milk. Next, different membranes were washed five times in TBST and separately incubated for 2.5 h in presence of α-YPO_4-4G10 (1μg/μL, Upstate), α-LYN (1μg/μL, Santa Cruz Biotechnology), α-ERK (1μg/μL, Santa Cruz Biotechnology) and α-pERK (1:2000, Cell Signalling). After this first incubation, membranes were washed three times in TBST and, subsequently, incubated for 1h in presence of α-mouse IgG HRP (1:2000, Amersham) for LYN and pERK, and α-rabbit IgG HRP (1:1500, Amersham) for ERK. Immediately after, the bands were visualized using the ECL system according to the manufacturer's instructions (Super Signal ECL, Pierce). Chemiluminescent signals were captured in Kodak Bio-Max film.

Specific detection of LYN and ERK in *T. crassiceps* cysticerci by flow cytometry

T. crassiceps and mouse spleen cells were extracted by tissue disruption from cultured treated and untreated-parasites. $2x10^6$ cells for each treatment were incubated at 4°C for 20min in presence of α-CD3, α-CD4, α-CD8, α-CD19 and α-macrophages antibodies (as the surface

antibodies), and subsequently washed in sterile PBS 1X-staining. Next, cells were centrifuged at 2000rpm for 5 min, and incubated in GolgiPlug for 3 hours. Immediately after, cells were washed in Perm/Wash buffer and centrifuged at 2000 rpm for 5 min. After this, cells were separately incubated in presence of primary antibodies at room temperature for 20 min, and subsequently washed in PBS 1X-staining. Immediately after, cells were centrifuged at 2000 rpm for 5 min. Cell pellets were separately resuspended in presence of secondary antibodies conjugated to specific fluorochromes and incubated at 4°C for 30 min in the dark. After this, cells were washed in PBS 1X-staining and centrifuged at 2000rpm for 5 min. Cell pellets were resuspended in 500μL of PBS 1X-staining in absence of light and analyzed on cytofluorometer FACScan (Becton Dickinson, USA).

Experimental design and statistical analysis

E_2 and TK-inhibitor concentration-response and time-response curves were estimated in six independent experiments, each performed with cysticerci freshly extracted from different infected donor mice and each including E_2 and the four inhibitors at optimal concentrations, each replicated in 24 different wells. The response variable used in statistical analysis is the sum of buds in the 24 wells with each treatment along the time of exposure of each experiment. All experiments to evaluate the effect of the optimal dose of hormone and inhibitors on parasite proliferation indexes were performed four times. In each of the six experiments, each of the five treatments was replicated in 24 wells, where each well contained ten viable cysticerci. Data of the six replications of each treatment were pooled and expressed as their average. Data were analyzed using one-way ANOVA and a Dunnet's Multiple Comparison Test with the hormone and TK-inhibitor treatments and the number of experiments as independent variables, and the average of the sum of buds in the 24 wells as dependent variable. To analyze the effect of the TK-inhibitor together with tamoxifen on *Taenia crassiceps* cysticerci bud production we used one-way ANOVA and Tukey's Multiple Comparison Test. Differences were considered statistically significant when $P< 0.05$.

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Legends to figures

Figure 1. Time-response curves. E_2 increased the *Taenia crassiceps* cysticerci reproduction rate since the first day of *in vitro* culture. The exposure of the parasite to tyrosine kinase inhibitors AG18, Gna, Pic and ERK-I, simultaneous with E_2, inhibited the proliferative effect of E_2 diminishing the number of buds to the same level as in the control group. The shaded area represents control group values.**Statistically significant differences were estimated with P values lower than 0.05 ($P<0.05$). E_2=17-β-estradiol; Gna=genistein; Pic=piceatannol; ERK-I=ERK-inhibitor.

Figure 2. Concentration-response curves. E_2 gradually increased the number of buds in *Taenia crassiceps* cysticerci at all concentrations, showing the strongest effect at $8X10^2$ μM. Parasite reproduction stimulated by E_2 decreased to the same level as in control groups by means of AG18, Gna, Pic and ERK-I, which exhibited their effects in a concentration-dependent way. The shaded area represents control group values. All E_2 concentrations are in order of 10^2 μM as described in the Methodology.**Statistically significant differences were estimated with P values lower than 0.05 ($P<0.05$). E_2=17-β-estradiol; Gna=genistein; Pic=piceatannol; ERK-I=ERK-inhibitor.

Figure 3. *Lyn*, *Syk* and *Erk* gene amplification in *T. crassiceps* cysticerci. By means of specific primers, designed from most conserved sequences of mouse, rat and human, we amplified corresponding fragments to *Lyn*, *Syk* and *Erk* in *T. crassiceps* cysticerci. Neither steroid nor TK-inhibitor treatments modified the *Lyn*, *Syk*, *Erk* gene expression in parasites (data not shown). Tc=*Taenia crassiceps*; M=mouse spleen.

Figure 4. LYN, SYK and ERK-like immunoblot detection in *T. crassiceps* cysticerci. By Western blot, the parasite bands corresponding to LYN (A, 55KDa), SYK (B, 80KDa) and a characteristic doublet of ERK (C, 120Kda)) were detected, which were previously reported for several mammal species. The middle part of the A and B images shows a phosphotyrosine control and the molecular weight marker respectively. In B, Tc=*Taenia crassiceps*; M=mouse spleen.

Figure 5. Effects of E_2 and genomic/non-genomic inhibitor *in vitro* treatment upon *T. crassiceps* cysticercus reproduction. As previously shown, E_2 treatment significantly stimulated cysticercus reproduction. Tam/AG18 and Tam/Gna inhibited the proliferative effect of E_2, diminishing parasite reproduction rate to the same level as in control groups. In contrast, Tam/Pic and Tam/ERK-I decreased bud production below values in control cysticerci. **Statistically significant differences were estimated with P lower than 0.05 ($P<0.05$). E_2=17-β-estradiol; Tam=tamoxifen; Gna=genistein; Pic=piceatannol; ERK-I=ERK-inhibitor.

Figure 6. Possible mechanism of action by which E_2 stimulates *in vitro Taenia crassiceps* cysticercus reproduction. Briefly, E_2 has two possible pathways: a nuclear receptor-dependent and a nuclear receptor-independent pathway. In the first, E_2 binds to ENR forming a ligand-receptor complex. This can bind to another identical ligand-receptor complex beginning nuclear translocation. Then, the dimeric complex can promote gene expression of the parasite. In the second, E_2 can activate a signal transduction pathway, increasing proliferative gene expression in *Taenia crassiceps* cysticerci, exerting important effects on e.g. reproduction and growth of the helminth parasite.

Neuroimmunoendocrine modulation in the host-by helminth parasites: a novel form of host-parasite co-evolution?

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Abstract. Helminth parasites have evolved diverse molecular mechanisms that facilitate them their establishment, growth and reproduction inside an immunologically hostile environment. Thus, the physiological interactions during the course of the immune response to helminths are complex. Infection induces antigen-specific recognition by the immune system, which is consequently charged with the responsibility of marshalling the appropriate effector responses necessary to destroy the parasite, or at the very least inhibit its progression. Obviously the immune system should accomplish this task while minimizing collateral damage to the host. As our understanding of the neuroendocrine system grows, it has become increasingly clear that this complex network of neurotransmitters, hormones, and cytokines plays an important role in mediating immunity. Helminths present an especially complex relationship between pathogen and these physiological systems, with hormonally dependent host factors such as sex and age correlated with parasite success. On top of the effect that this particular type of parasites may have on the invaded host, recent experimental evidence suggest that helminth parasites not only actively evade immune response, but are also able to exploit the hormonal microenvironment within their host to favor their establishment, growth and reproduction. This complex strategy of host-parasite complex relationship is much better exemplified by two helminth parasites: the trematode *Schistosoma mansoni* and the cestode *Taenia crassiceps* that respond to adrenal steroids and sexual steroids, respectively. Understanding how the hosts endocrine system can under certain circumstances favor the establishment of a parasitic infection opens interesting perspectives into the host parasite relationship field.

INTRODUCTION

The immune and neuroendocrine systems are integrated through a complex network of hormones and neuropeptides which serve to maintain homeostasis [1]. Two of the main components of this network are the hypothalamic-pituitary-adrenocortical (HPA) axis [2] and the hypothalamic-pituitary-gonadal axis (HPG) [3]. The relationship between the HPA and HPG axes and the immune system during acute and chronic inflammatory responses due to stress, infection and autoimmune responses has been well established [4-8]. An important aspect of cellular communication that has emerged from studies of the neuroendocrine and immune system is the redundancy in the chemical messengers responsible for transmission. The identification of a relatively high number of cellular messengers responsible for coordinating interactions between these homeostatic systems may herald a fundamental shift in our understanding of the host immune response. Nowhere may this knowledge prove to be more enlightening than in our understanding of the host response to infection with helminth parasites. Although strong evidence supports that 1) neurons, endocrine and immune cells produce hormones [9] and 2) neural, endocrine and immune cells synthesize and secrete neuroactive messengers [10], it remains somewhat controversial to what level this network is involved in the progression of infection with helminths.

Helminth parasites are arguably the most highly evolved infectious organisms, and consequently have developed mechanisms to facilitate their survival within the host. These mechanisms can be roughly grouped into two types. The first is to evade the immune response by evolving strategies such as antigenic variation, molecular mimicry or affecting antigen processing and presentation [11,12]. In the second mechanism, the parasite actually exploits a host system to its benefit and, thus, obtains an advantage in growth or reproduction [13].

Mechanisms such as fabulation provide parasites with a dual benefit: first, obtaining amino acids for metabolism, and second by preventing the surface-bound antibody from interfering with parasite–host-cell interactions [14]. A striking example of the exploitation of host molecules is the ability of several helminthes species to use host-synthesized cytokines as indirect growth factors [15]. Additional evidence suggests that the binding of host immunoglobulin by some helminths surface (schistosomes and taenids) paramyosin may lead to immune evasion through molecular masquerade while interfering with Fc interactons with leukocyte surface receptors [16].

Consistant with the level of co-evolution evident from such adaptation is the belief that helminth parasites can exploit the hormonal microenvironments within the host [13]. In the case of schistosomes, for example, one study demonstrated that they express molecules with sufficient homology to the vertebrate insulin receptor family to bind host hormonal metabolites [17]. This suggests a system of transregulation (term coined by us) in which the parasite exploits host hormones and growth factors to facilitate infection and potentially increase growth and reproduction rates. Transregulation has been described in at least eight parasitic infections that are caused by both protozoan and metazoans [13].

Understanding how the host endocrine system is exploited to favor the establishment of helminths, and characterizing the parasite receptors involved, might potentially reveal targets for the design of chemotherapeutic hormonal analogs and drugs [18]. In this review we focus on reviewing the experimental and epidemiological evidence as it concerns the role of endocrine and neurohormones in mediating the progression of disease and host immune response in different helminth infections. We will try out to summarize the main findings in this area by

reviewing every helminth parasite that has been reported to affect, or be affected by the neuroimmunoendocrine network.

Host's sex hormones, immunity, parasites and parasitic disease.

The relationship between parasites (P), particularly helminthes, and their hosts (H) implies biochemical coevolution and communication between their complex physiological and metabolic systems among themselves and with the environment, at all levels of biological organization [19]. Hormones regulate a variety of cellular and physiological functions of organisms such as growth, reproduction and differentiation [20]. Hormones and immune actors are prominent in H-P relationships [19]. The comparatively sophisticated immune systems of vertebrates add complexity to H-P interactions. Mammals sense and react with their innate and acquired immunological systems to the presence of a parasite and the parasite is also sensitive and reactive to the host's immune systems effectors. Host's hormones are also involved in the modulation of the immune system's protective or pathogenic functions and also on the parasite's metabolism and reproduction [21,22]. Hosts´ adrenal hormones are well known immune modulators [23], whilst sex steroids (estradiol, progesterone and testosterone) are progressively being recognized to also significantly affect the immune system's functions [24,25]. More recently the ability of hormones to affect, the immunological response directed against pathogenic agents has gained attention [21]. This is clearly evident during various parasitic diseases including malaria, schistosomiasis, toxoplasmosis, cysticercosis, trypanosomiasis, leishmaniasis [26-30], where strong hormonal regulation of the immune response has been described [30,31]. However, other factors than the immunoendocrine response affect the course of a parasitic infection.

Thus *Naeglerai fowleria* is capable of internalising antigen antibody

complexes from their surface with the dual benefit of gaining the amino acids for their own metabolism and preventing the surface bound antibody from interfering with parasite host cell interactions [14]. Other pathogens have developed molecules that directly of interfere with antigen processing and presentation [14,16]. A striking example of exploitation of host molecules is the ability of a number of parasites to use host-synthesized cytokines as indirect growth factors for the parasite [15].

The case of trematodes

The Trematoda are estimated to include 18000 to 24000 species, and are divided into two subclasses [32]. Nearly all trematodes are parasites of molluscs and vertebrates. The smaller Aspidogastrea, comprising about 100 species, are obligate parasites of molluscs and may also infect turtles and fishes, including cartilaginous fishes [32]. The Digenea, which constitute the majority of trematode diversity, are obligate parasites of both molluscs and vertebrates, but rarely occur in cartilaginous fishes. One-quarter of a billion people are infected with parasitic trematode worms worldwide [33]. Disease-associated symptoms occur in 120 million people, and 20 million people suffer from severe morbidity [33]. The three most important human schistosomes are *Schistosoma haematobium*, *Schistosoma japonicum*, and *Schistosoma mansoni*.

Neuromodulation of the Infection

Despite a significant amount of research invested toward vaccine development [34], schistosomiasis remains a common and debilitating parasitic infection throughout many parts of the developing world [33]. Although chemotherapy with praziquantel remains highly effective [35] the high reinfection rate remains problematic. Estimates place the current number of infections at approximately 200 million people, with another 600 million considered at risk [33].

Infection with schistosomes is initiated upon contact by the host with a water source containing infective cercariae [36]. After penetrating the skin of the host, the worm develops into migrating schistosomula. After a 3-4 week period of migration the worms complete development into the adult stage, form mating pairs, and migrate into species-specific sites within the venous system [36]. Once established, at approximately 5 weeks post-infection, the female begins to deposit eggs in the bloodstream. Many of the eggs are carried to ectopic tissues where they serve as the nidus for the formation of granulomas. Significant pathology in these tissues will accumulate as the egg burden in the tissue increases and the consequent fibrosis begins to disrupt the normal tissue architecture [36]. A better insight into the mechanisms regulating the homeostatic mechanisms of the host can lead to improved knowledge of the patho-physiological processes of schistosomiasis. In this way, novel therapeutic approaches can be developed at the level of these regulatory mechanisms. In schistosomiasis, one of the regulatory nodes proposed to be involved in the physiopathology of the disease is the complex represented by somatostatin, the vasoactive intestinal peptide (VIP), sustance P and other gastric neurohormones [37-39].

Somatostatin is one of the major regulatory hormones in the central nervous system (CNS) and digestive system [37]. Somatostatin effects inlcude inhibition of growth hormone secretion, collagen I and III formation and hepatic stellate cell activation [37]. Such pleotropic activity suggests it is implicated in a broad range of conditions. Somatostatin is part of an immunoregulatory circuit that helps limit interferon-gamma (IFN-γ) production at sites of chronic inflammation [38]. It can suppress IFN-γ secretion from T cells through interactions with the SSTR2 receptor constituitivly expressed on these cells [38]. It has been also demostrated that somatostatin likely has an important role in thymic T cell education and selection

[37]. The thymus has a complete regulatory circuit, in which the thymic epithelial and dendritic cells secrete somatostatin [38]. One symptom common to both the acute and chronic stages of schistosomiasis is intestinal pathology characterized by abdominal pain, bloody diarrhea in chronic stages, nausea and fever. Some chronic patients develop severe hepatosplenic fibrosis, leading to fatal oesophageal variceal bleeding [37]. Then, it is reasonable to think that improved knowledge of the patho-physiological processes of schistosomiasis can be obtained by studying the regulatory mechanisms of somatostatin in the human body [37].

In murine schistosomiasis, parasite eggs induce focal, chronic granulomatous inflammation in the liver and intestines that have been shown to be able to produce somatostatin and upregulate receptor expression [38]. Furthermore, splenic macrophages from infected mice also produce somatostatin. At the site of the lesions, there appears to be no other inflammatory cell source of the neurohormone [38]. The CD4-T lymphocytes infiltrating in liver granulomas contain mRNA for the somatostatin receptors sst2A and sst2B [38]. It is easy to envision a mechanism in which the somatostatin circuit could play an important role in *Schistosoma mansoni* infections via its influence on intersystem signalling; therapeutically, via its direct effect on Schistosoma – induced morbidity (fibrosis, granuloma size, portal hypertension, variceal bleeding) through modulation of associated inflammatory responses in the liver and intestines [37,38].

Another neurohormone known to be produced within the granuloma is the vasoactive intestine peptide (VIP) [37,39]. It has been postulated that VIP can act as an immune modulator since it can suppress T cell proliferation and IL-2 production [39]. It is also able to enhance IL-5 production from granuloma-associated T cells [37]. These cells express VIP receptors of both subclasses: the

VIPr1 and VIPr2 [38]. It is expected that the expression of these receptors are subject to immunoregulation, particularly by IL-4 [38,39]. In humans the hepatosplenic form of schistosomiasis may be associated with some degree of somatosexual underdevelopment [40], however, prepuberal infection with *S. mansoni* in mice resulted in hepato and splenomegaly, phenomena not related to plasma concentrations of thyroid hormones (T3 and T4) [41].

Limited studies have shown that during intestinal schistosomosis the enteric nervous tissue becomes inflamed, disrupted and destroyed by granulomas. For instance, previously it has been shown that *Schistosoma mansoni* infection causes alterations in the intrinsic innervation of the distal ileum and proximal colon, characterized by focal destruction of the enteric nerves [42]. Occasionally nerves were found within granulomas, particularly at the periphery of the lesions. Under these conditions nerve cell bodies close to granulomas were shown to be producing VIP. The distribution of nerve injury varied between the two enteric segments studied. In the distal ileum, the principal injury was to the myenteric plexus, whereas the submucous and mucosal plexuses were predominantly damaged in the proximal colon [42].

Interestingly, in another model of the disease, in pigs infected with *Schistosoma japonicum*, there was a positive correlation between the expression of VIP and substance P to pathological lesions in the large intestine [43]. In all inflamed areas VIP was reduced while substance P was increased in the enteric nerve plexuses and enterochromaffin cells of these tissues [43]. The alterations of the levels of VIP and substance P were thus correlated with severity of inflammation.

The role of the hypothalamic-pituitary-adrenocortical axis during infection

The hypothalamic-pituitary-adrenal (HPA) axis functions primarily to coordinate the stress-response [2,4]. This complex regulatory trifecta has been implicated in the regulation of the host response during schistosomiasis, playing a major role in onset, establishment and pathogenesis [6]. Moreover, HPA axis is one of the most important factors determining aging in mammals, thus, the role for HPA axis control in mediating resistance is strengthened when age is considered as a factor [44]. In endemic regions, two interesting patterns of infection emerge: first, the intensity of infection is higher in children than in adults; second, at any given time only a fraction of infected individuals develop the characteristic Symmer's pipe-stem fibrosis [45,46]. These morbidity patterns cannot be explained simply on the basis of acquired immunity or sex, but were correlated to age, and more specifically the onset of puberty. Booth et al. [47] concluded that children presented the lowest overall risk of fibrosis, but suggested this was due to inadequate time for lesions to produce the effects. However, these authors reported that low production of IL-10 was a common factor in all cases where fibrosis was detected in a child [47].

Recently we have demostrated that the HPA axis likely plays a similar role in the progression of disease in baboons [48]. Baboons with primary infections, when worm recovery and oviposition rates were high and hepatic schistosome egg granulomas were large, had decreasing levels of DHEA, ACTH and CRH as infection progressed as compared to uninfected and re-exposed baboons [48]. Re-exposed baboons with low worm recovery and oviposition rates, and small (modulated) hepatic granulomas, had the opposite pattern with HPA axis hormone levels maintained at, or exceeding, uninfected baseline values. Reductions of systemic DHEA-S and cortisol were also noted in primary murine infections, reinforcing our baboon data [49]. Infection also induced IL-1β gene expression in

the hypothalamus, while IL-6 and macrophage migration inhibitory factor (MIF) mRNA were detected in the pituitary and adrenal glands. TNF-α gene expression was up-regulated in the hypothalamus and the pituitary gland [48]. Histological analysis of the HPA related tissues in infected and control baboons revealed no morphological differences [48]. Other evidence in support of a key role for the HPA axis in regulation during schistosomiasis is the increased host susceptibility after adrenalectomy in the mice [49]. In these studies we noted increased worm burdens, increased egg production, and larger hepatic granulomas. We postulated that a lack of circulating glucocorticoids helped to exacerbate the inflammatory immune response in the liver [50]. Reduced levels of cortisol could promote vigorous granuloma formation and encourage the production of cytokines necessary for schistosome reproduction, such as TNF–α. However, a more recent study clearly demonstrated that TNF-α. did not have a role in schistosome reproduction, as measured by fecundity.

Taken together, these results suggest that specific immune system derived cytokines and hormones expressed by theHPA represent a bi-directional interaction between these two critical homeostatic mechanisms [1,2]. In this network, cytokines like IL-6, MIF and IL-1β likely play a key role in modulating the HPA axis response during schistosomiasis at all three levels: hypothalamus, pituitary and adrenal glands (Fig. 1).

The case of Cestodes

Cestoda is the class of parasitic flatworms, commonly called tapeworms that live in the digestive tract of vertebrates as adults and often in the bodies of various animals as juveniles. There are two subclasses in class Cestoda, the Cestodaria and the Eucestoda. By far the most common and widespread are the Eucestoda,

with only a few species of unusual worms in subclass Cestodaria. The cyclophyllideans are the most important to humans because they infect people and livestock. Two important tapeworms are the pork tapeworm, *Taenia solium*, and the beef tapeworms, *T. saginata* [52].

Taennids, particularly *Taenia solium* (causal agent of porcine cysticercosis and human neurocysticercosis) and *Taenia crassiceps* (causal agent of murine cysticercosis) are highly evolved parasites that have developed diverse mechanisms of survival within the host that facilitate their establishment in the hosts. These mechanisms can be roughly grouped into two types. The first is evasion of the immune response by molecular mimicry or by inactivating effector immune processes (i.e, complement inhibition) [11,12]. In the second mechanism, the parasite exploits the host system to its benefit in its establishment, growth or reproduction [13]. This exploitation mechanism provides parasites with a dual benefit: first, obtaining amino acids for metabolism, and second preventing the surface-bound antibody from interfering with cytotoxic cells interacting with the parasite [12]. A striking example of host molecules' exploitation is the ability of several taeniids to use host-synthesized cytokines as indirect growth factors for themselves [13]. Taennids can also exploit the hormonal microenvironment within the host in their favor [12]. Taennids have evolved structures similar to the steroid and protein hormone receptors expressed in upper vertebrates, with binding properties and terminal effects similar to the hormonal metabolites synthesized by the host [53]. Such cross regulation from host to parasite has been described in at least eight parasitic infections that are caused by parasitic cestodes, like schistosomiasis, filariasis, hydatid disease and murine cysticercosis [13].

A. Experimental murine cysticercosis

Due to the intrinsic difficulties in working with the natural hosts (pigs and humans) of *T. solium* or because of the high costs of sufficient pigs plus the slowness in data retrieval, we have used an experimental cysticercosis approach to gain knowledge of the complex host (H) parasite (P) relationship in cysticercosis. Murine intraperitoneal cysticercosis is caused by the taenid *Taenia crassiceps* and it has been useful to explore the physiological host factors associated with porcine cysticercosis, and to some degree, with human neurocysticercosis [54]. Intraperitoneal *T. crassiceps* cysticercosis of mice [54,55] lends itself well to controlled and reproducible experimentation, generating numerical data of parasite loads in individual mice in a matter of weeks after infection. Its general representation of other forms of cysticercosis has later been strengthened by similar results in other mouse and parasite strains [54], by the parasite's extensive sharing of antigens with other taennids and cestodes and by the DNA homology between *T. crassiceps and T. solium* [54]. These characteristics have made murine cysticercosis a convenient instrument to test vaccine candidates and new drugs or treatments against cysticercosis. Several features of natural cysticercotic disease have been found by extrapolation from experimental murine cysticercosis [56] (Fig. 2).

Neuromodulation Induced by Infection: Behavioral changes In the Infected host.

The hormonal changes in infected mouse profoundly affect their behavior, such as sexual activity [21], aggresiveness, social status and defense responses [57], will be mentionated as follows.

Male mice infected with *T. crassiceps* show remarkable changes in sexual behavior, characterized by a complete loss of the ejaculation response early at the infection (six weeks), followed by a gradual decrease in the number of mounts and

intromissions, and their latencies increased, until none of the parasitized mice showed any sexual response toward female mice [21]. Moreover, it was demostrated that changes in sexual behavior were due to the change in the normal production of sex-steroids by the mouse, since after testosterone or dihydrotestosterone restitution of the infected male mice, a complete restoration of their sexual behavior was observed [58]. Since *c-fos* and progesterone receptor (PR), both are key estradiol-regulated genes involved in the regulation of sexual behavior, we studied possible changes of *c-fos* and PR expression in the central nervous system (CNS) of infected male mice. Indeed, *c-fos* and PR expression oscillated with time of infection and to different magnitudes in hypothalamus, brain cortex and preoptic area but neither in other areas of the brain nor in several other organs of the host [59].

Furthermore, infection disrupts the dominant-subordinate status [57]. In infected male mice strong perturbations in territorial behaviour and aggressiveness were found. In addition, during confrontation between naive infected and healthy mice, infected animals more often assumed a subordinate status than healthy ones. The effects of the infection by *T. crassiceps* were more likely to prevent adult male mice from becoming behaviourally dominant than to reverse existing dominance relationships [57].

Significant CNS changes in *c-fos,* and progesterone receptor (PR) expression during infection signifies the brain senses the infection episode and may be involved in the ensuing behavioral changes of the infected mice, as well as, through its connectivity, extend the effects of infection to other physiological systems under its influence. That these changes in CNS are beneficial to the host or parasite remains speculative [59]. One could argue that feminization of male hosts favours the parasite by allowing reproduction in otherwise restrictive male

mice, but, equally arguable would be to consider feminization of the male host as deleterious to the parasite's completion of its cycle by reducing male exposure to its predators, the definitive hosts. Other similar mutually conflicting statements may be elaborated with the above premises, which includes too much for being identified and could perhaps vary with each different host-parasite relationship.

Not only male mice are behaviourally affected by cysticercosis, female mice also suffer perturbations in their sexual behaviour, i.e., receptivity to the male, as well as disruption of the estrous cycle [Arteaga, M. Personnal communication].

Concluding Remarks

The evidence presented above illustrates the complexity and importance of neuroimmunoendocrine interactions during helminth infections, and provides clues to the many other possible mechanisms of parasite establishment, growth and reproduction in an immunocompetent host. Further, strong neuroimmunoendocrine interactions may have implications in the control of transmission and treatment of several parasitic diseases, but particularly in those produced by helminth parasites, in animals and humans. In practical importance, the complexity of the helminth-host relationship suggests that all physiological factors (i.e., sex, age) should be taken into account in the design of vaccines and new drugs.

The differential response of helminthes to steroids (sexual or adrenal) may also be involved in their ability to grow faster in female or male hosts. Host and parasite sex-associated biases may be combined to favour their evolution towards a mutually acceptable relationship. Moreover, the changes in behavior observed during helminthiasis, should not be regarded as simple biological curiosities but more as strong evidence of the plasticity of the host phenotype in response to infection by parasitic helminths. Furthermore, by changing the reproductive, aggresive and dominant capacity of the host, parasites generate novel questions

regarding the evolution of host-parasite relationships in ways other than only prey/predators interactions. Also, the strong neuroImmunoendocrine interactions observed during different parasite infections, could give ways to possible new mechanisms of parasite establishment, growth and reproduction in an immunocompetent host.

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