UNIVERSIDAD AUTÓNOMA AGRARIA ANTONIO NARRO

SUBDIRECCIÓN DE POSTGRADO

SUPLEMENTACIÓN DE BETACAROTENO SOBRE LA EFICIENCIA REPRODUCTIVA EN CABRAS JÓVENES Y ADULTAS: ACTIVIDAD OVÁRICA Y HORMONAS METABÓLICAS

Tesis

Que presenta GERARDO ARELLANO RODRÍGUEZ

como requisito parcial para obtener el grado de
DOCTOR EN CIENCIAS AGROPECUARIAS

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Suplementación de Betacaroteno sobre la eficiencia reproductiva en cabras jóvenes y adultas: actividad ovárica y hormonas metabólicas

Gerardo Arellano Rodríguez
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Los carotenoides son un grupo de pigmentos que presenta varias actividades biológicas de importancia nutricional y fisiológica, como precursores de la vitamina A ó retinol. El ácido retinoico modula diversos productos genéticos relacionados con el rendimiento reproductivo, sin embargo, se ha propuesto que el betacaroteno podría actuar de manera directa, independientemente de la vitamina A. Sobre estas bases nos hemos planteado la hipótesis de que la suplementación con betacaroteno podría estar asociada con la eficiencia reproductiva en cabras jóvenes y adultas, midiendo la actividad ovárica total y la posible relación del betacaroteno con cambios en el perfil de hormonas reproductivas, metabólicas y metabolitos sanguíneos. Se realizaron dos experimentos para evaluar el rol del betacaroteno sobre la función reproductiva en cabras. En el experimento 1 (Exp. 1) se evaluó el efecto de la suplementación con betacaroteno sobre la actividad ovárica. Se utilizaron 22 cabras adultas (34 meses de edad) las cuales fueron asignadas aleatoriamente a dos grupos experimentales: 1) Betacaroteno (BETA, n= 10) y 2) Control (CONT, n= 12). Las cuales fueron sincronizadas con esponjas intravaginales impregnadas con Acetato de Fluorogestona (FGA). Las cabras del grupo BETA recibieron 50 mg de betacaroteno diariamente durante 35 días pre y 17 días post-ovulación. Una vez que la ovulación ocurrió, hacia el final de la fase lutea (día 18) se realizó ultrasonido a todas las cabras para evaluar el total de folículos (FT), numero de cuerpos lúteos (CL) y la actividad ovárica total (AOT). Las cabras del grupo BETA tuvieron una mayor actividad ovárica (P=0.07). En el experimento 2 (Exp. 2) se analizó el efecto de la suplementación con betacaroteno sobre el número y

volumen de cuerpos lúteos, así como la síntesis de progesterona. Como en el experimento 1, las cabras fueron asignadas aleatoriamente en dos grupos experimentales. Las cabras fueron sincronizadas con esponjas intravaginales impregnadas con Acetato de Fluorogestona (FGA). En el grupo 1 (n= 10) las cabras recibieron 50 mg de betacaroteno diariamente 35 días antes y 15 días después de la ovulación mientras que las cabras del grupo control (n= 12) no recibieron ningún tipo de suplementación. Los días 4, 8, 12 y 16 post ovulación se tomó una muestra sanguínea de las cabras para medir los niveles de P4 en sangre y el día 18 se realizó ultrasonografía para evaluar el número y el volumen del CL. Las cabras suplementadas con betacaroteno presentaron un mayor número de CL (p=0.07) y mayores niveles de progesteron sérica (p=0.05). Sin embargo, no hubo diferencias significativas (p=0.53) en el volumen de CL entre ambos grupos. Estos resultados sugieren que la suplementación con betacaroteno afecta positivamente la actividad ovárica en las cabras. El betacaroteno mejora la función ovárica al incrementar la actividad ovárica total, así como la síntesis de progesterona. Lo anterior es esencial para la implantación y sobrevivencia embrionaria durante el periodo del reconocimiento materno de la gestación. Lo anterior es relevante ya que podría ayudar a disminuir las perdidas tempranas de la gestación en los caprinos.

Palabras clave: Reproducción, Actividad ovárica, Carotenoides, Hormonas metabólicas y Metabolitos sanguíneos.

ABSTRACT

Effect of beta-carotene supplementation upon reproductive efficiency in young and adult goats: ovarian activity and metabolic hormones

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Carotenoids are a group of pigments that present several activities of nutritional and physiological importance, as precursors of vitamin A or retinol. Retinoic acid modulates various genetic products related to reproductive performance, however, studies have proposed that betacarotene could act directly, independently of vitamin A. On these grounds we have hypothesized that supplementation with betacarotene could be associated with reproductive efficiency in young and adult goats, measuring total ovarian activity and the possible relationship of betacarotene with changes in the profile of reproductive and metabolic hormones and blood metabolites. Two experiments were conducted to evaluate the role of beta-carotene on reproductive function in goats. In experiment 1 (Exp. 1) the effect of beta-carotene supplementation on ovarian activity was evaluated. Twenty-two adult goats (34 months old) were used, which were randomly assigned to two experimental groups: 1) Beta-carotene (BETA, n = 10) and 2) Control (CONT, n = 12). The goats of both groups were synchronized with intravaginal sponges impregnated with Fluorogestone Acetate (FGA). Goats in the BETA group received 50 mg of beta-carotene daily for 35 days before and 17 days post-ovulation. Once ovulation occurred, towards the end of the luteal phase (day 18) an ultrasound was performed on all the goats to evaluate the total number of follicles (FT), number of corpora lutea (CLT) and total ovarian activity (AOT). The goats of the BETA group had a higher ovarian activity (P = 0.07). In experiment 2 (Exp. 2) the effect of beta-carotene supplementation on the number and volume of corpora lutea, as well as the synthesis of progesterone, was evaluated. As in experiment 1, the goats were randomly assigned into two

experimental groups. The goats were synchronized with intravaginal sponges impregnated with Fluorogestone Acetate (FGA). In group 1 (n = 10) the goats received 50 mg of beta-carotene daily 35 days before and 15 days after ovulation, while the goats in the control group (n = 12) did not receive any type of supplementation. On days 4, 8, 12 and 16 post-ovulation, a blood sample was taken from the goats to measure the levels of progesterone in the blood and on day 18 an ultrasound was performed to evaluate the number and volume of CL. Goats supplemented with beta-carotene presented a higher number of CL ($p = 0.07$) and higher levels of serum progesterone ($p = 0.05$). However, no significant differences ($p = 0.53$) were observed in CL volume between both groups. The results suggest that beta-carotene supplementation positively affects ovarian activity in goats. Beta-carotene improves ovarian function by increasing total ovarian activity, as well as the synthesis of progesterone. This is essential for embryo implantation and survival during the period of maternal recognition of pregnancy. This is relevant as it could help reduce early pregnancy losses in goats.

Keywords: Reproduction, Ovarian activity, Carotenoids, Reproductive and metabolic hormones and Blood metabolites.

1. INTRODUCCIÓN

En los pequeños rumiantes se conoce que una inadecuada nutrición se caracteriza por perfiles metabólicos y endocrinos deficientes, por su parte, estos pueden comprometer el rendimiento reproductivo de los animales, lo que puede originar un retraso en la pubertad, irregularidad en los ciclos estrales y una caída en la efectividad reproductiva general (Popwell *et al.,* 1996; Scaramuzzi *et al.,* 2006; Meza-Herrera *et al.,* 2007). En efecto, la nutrición es considerada el principal modulador de la reproducción, además de que el crecimiento de los folículos y la maduración de ovocitos dependen principalmente de las gonadotropinas hipofisarias (FSH y LH) así como, también de algunos otros factores intraováricos (p. ej., IGF-I). Antes, la función del eje hipotalámico-hipofisario-gonadal (HHG) es modificada por las entradas neuronales, gobernada por las señales fotoperiódicas, que modulan la actividad de las terminales neuronales liberadoras de GnRH en el hipotálamo teniendo una repercusión en la homeostasis energética (Meza-Herrera *et al.,* 2013)

Se ha reportado que la influencia de la estacionalidad reproductiva puede ser alterada por el entorno nutricional, con un período de reproducción más largo en cabras con mayor nivel nutricional, lo que se refleja en mejores puntajes de condición corporal y peso vivo. Existe evidencia de que en el ganado criollo mexicano que se mantiene en las latitudes tropicales la duración del período de anovulación se modula parcialmente por el estado de energía nutricional (Gallego-Calvo *et al.,* 2014). Al respecto también existe relación entre mediadores endocrinos, el estado nutricional y la reproducción; por ejemplo, el factor de crecimiento asociado a la insulina (IGF-I), la hormona del crecimiento y su relación con la insulina, la glucosa regula la liberación de GnRH además de que participan en el control del metabolismo de energía en el cerebro (Arroyo, 2011).

Los carotenoides son pigmentos en la naturaleza, los cuales, están presentes en los organismos fotosintéticos y no fotosintéticos así como en bacterias, algas, hongos, insectos y animales y son responsables de la mayoría de los colores rojo

a amarillo de mucha hojas, frutas, flores y peces. En los animales, presentan varias actividades biológicas importantes relacionado al estado nutricional y fisiológico (Fraser y Bramley, 2004; Nozière *et al.,* 2006; Arellano-Rodríguez *et al.,* 2008).

Los carotenoides, por otro lado, son de suma importancia en los humanos y los animales como precursores de la vitamina A (Chew *et al.,* 1993; Hattori *et al.,* 2000; Schweigert *et al.,* 2003). Los β-carotenos, que están presentes en plantas verdes, pero también puede sintetizarse químicamente, ha demostrado tener un papel clave en una amplia gama de procesos biológicos (Schweigert, 1998). Aunque se sabe que muchos productos genéticos relacionados con el rendimiento reproductivo están modulados por el ácido retinoico, el producto de la oxidación del retinol (Harrison *et al.,* 2012), otros estudios han propuesto que el betacaroteno podría actuar de manera directa, independientemente de la vitamina A (Kawashima *et al.*, 2010, 2012; Kramer y Aurich, 2010). Sobre la base de tales hallazgos y estudios previos, nosotros planteamos la hipótesis de que la suplementación con betacaroteno podría estar asociada con la eficiencia reproductiva en cabras jóvenes y adultas, midiendo la actividad ovárica total y la posible relación del betacaroteno con cambios en el perfil de hormonas reproductivas, metabólicas y metabolitos sanguíneos.

2. OBJETIVO GENERAL

Evaluar la suplementación de Betacaroteno sobre la eficiencia reproductiva en cabras jóvenes y adultas, midiendo la actividad ovárica total y la posible relación del Betacaroteno con hormonas reproductivas, metabólicas y metabolitos sanguíneos.

2.1. Objetivos específicos

1. El efecto de la suplementación con Betacaroteno sobre la tasa de ovulación, número de cuerpos lúteos, el volumen lúteo total y la secreción pulsátil de LH en cabras hembras adultas.

2. Evaluar los efectos de un suplemento nutricional de Betacaroteno a corto plazo sobre la secreción de P4 y los niveles séricos de GH e IGF-1.

3. Evaluar la posible relación entre la suplementación con Betacaroteno y algunos metabolitos sanguíneos relacionados con el metabolismo de lípidos, carbohidratos y proteínas.

4. Evaluar la posible relación con respecto a la suplementación con Betacaroteno y la Triyodotironina sérica al inicio de la pubertad.

3. REVISIÓN DE LITERATURA

3.1. Estacionalidad reproductiva

La rotación de la tierra impone fluctuaciones predecibles en el medio ambiente en escalas de tiempo diarias y anuales. Estos cambios cíclicos han impactado profundamente la evolución. La capacidad de realizar oportunamente funciones específicas proporciona una ventaja adaptativa significativa que mejora el estado físico. Por lo tanto, los organismos desarrollaron mecanismos de temporización endógenos para mantenerse en sintonía con su entorno, anticipar los próximos cambios y mostrar la respuesta fisiológica adecuada en el momento adecuado (Dardente *et al.,* 2016).

Los primeros humanos fueron nómadas y se adaptaron a la disponibilidad estacional de productos animales. A lo largo del proceso de domesticación, se perdió la estacionalidad de muchos productos ganaderos. Sin embargo, los productos como la leche y la carne proporcionados por la cría de ovejas y cabras permanecieron estacionales en muchas regiones del mundo (Balaro *et al.,* 2018).

Los animales de granja muestran diferencias en algunos parámetros de producción, contribuyendo de este modo la disponibilidad de productos de origen animal frescos (carne, leche y queso). Esto es debido, a que ovejas y cabras, son reproductores de días cortos, se reproducen durante el otoño. Debido a que estos animales tienen un período de gestación de aproximadamente cinco meses, sus crías también nacen y se crían durante la primavera y el verano (Guh *et al.,* 2019) teniendo así una más o menos marcada época de nacimiento, donde aseguran la supervivencia de su progenie (figura 1), siendo esta una de las ventajas de la reproducción estacional donde los animales pueden evitar gastar energía para la reproducción en una época inapropiada del año (por ejemplo, en el invierno), y la supervivencia de la descendencia se maximizará si son paridos y criados en estaciones con un clima moderado (Balaro *et al.,* 2018; Guh *et al.,* 2019).

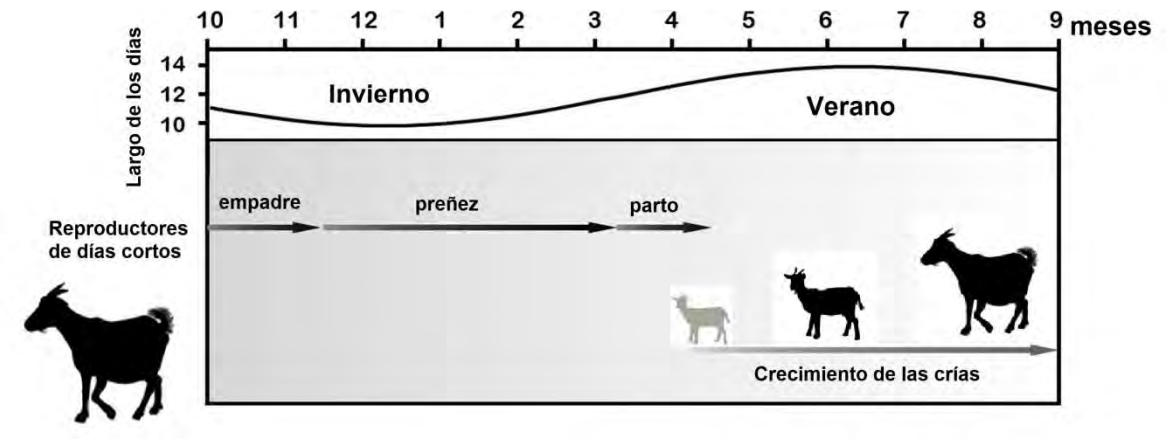


Figura 1. Calendario de reproductores estacionales de días cortos (Adaptado de Gu et al., 2019).

Algunas especies tienen variaciones estacionales sobre la frecuencia de ovulación (presencia o ausencia), la espermatogenesis (disminución a ausencia), la calidad del gameto (variaciones de fertilización y supervivencia embrionaria) y del comportamiento sexual (Chemineau *et al*., 2008). En ovejas y cabras además de ser reproductoras estacionales la actividad reproductiva es dependiente de las hormonas, en varios casos el entorno social puede ejercer alguna acción moduladora (Veliz *et al.,* 2002). En ovinos y caprinos el fotoperiodo es el principal en la regulación de la actividad reproductiva (Chemineau *et al*., 2008).

Es complicado superar la estacionalidad intrínseca del estro en las cabras, pero se han logrado cambios que son operativos, aumentando el entendimiento de la fisiología reproductiva, y la reproducción estacional de la cabra mediante el desarrollo gradual de tecnologías de la reproducción (Luo *et al.,* 2019).

En cabras, hay variables extrínsecas (los cambios estacionales) e intrínsecas (tamaño corporal, la duración reproductiva). Los principales factores que participan en la reproducción son temperatura, humedad, cantidad y distribución de lluvias, radiación solar, fotoperíodo, nutrición, manejo del sistema de producción y las interacciones sociales entre individuos dentro de la misma población (Chemineau *et al.,* 2008; Gallego-Calvo *et al.* 2014; Delgadillo *et al.,* 2015; Morales *et al.*, 2016). La asociación de todos ellos parecen ser componentes esenciales en la plasticidad cerebral (Balaro *et al.,* 2018).

3.1.1. Control fotoperiódico

Aunque el fotoperíodo es una señal importante, la naturaleza de la respuesta a un fotoperíodo determinado depende de la especie. Por lo tanto, para las ovejas y las cabras, el fotoperíodo corto se asocia con una mayor competencia reproductiva, lo que significa que la "temporada de reproducción" es durante el otoño, lo que permite que las crías nazcan 5 meses más tarde en los cálidos días de la primavera, cuando la disponibilidad de alimentos es óptima. El fotoperíodo corto también se asocia con una menor ingesta de alimentos y una disminución del peso corporal (Helfer *et al.,* 2018). Aunque estos efectos, no son permanentes, cuando están sujetos a un fotoperiodo los animales se vuelven “refractarios”, del fotoperiodo: los días largos dejan de ser inhibidores, y los días cortos dejan de estimular (Malpaux *et al.*, 1998; Chemineau *et al.*, 2010). En cabras y borregas, esto podría ser considerado conceptualmente como el primer paso n del ritmo circanual, el cual puede ser sobrepasado por animales en transferencia del fotoperiodo opuesto (Malpaux *et al.*, 1998), conocido como refracción a los días cortos, lo cual ocurre de manera natural en borregas durante finales de invierno, y se rompe por dos meses de exposición a días largos en diciembre-enero, permitiendo que la eficacia de los días cortos se restablezca (Arrebola *et al.*, 2010).

En caprinos de climas templados, el fotoperiodo y sus variaciones determinan los cambios estacionales de la actividad neuroendocrina. Por intermedio de la duración de la secreción de melatonina. Los días cortos estimulan la actividad pulsátil de LH y los días largos la inhiben. La testosterona comienza a elevarse desde la cuarta semana después de los días cortos y disminuye la segunda semana después de los días largos (Chemineau y Delgadillo, 1993).

3.1.2. Neuroendocrinología de la estacionalidad

Hay una marcada relación entre el ambiente y la conducta reproductiva, en los reproductores estacionales, de acuerdo a factores proximales, especialmente el fotoperiodo que provoca cambios fotoneuroendocrinos (Bustos-Obregón y Torres-Díaz, 2012).

En los mamíferos, la retina transmite información fotoperiódica a los núcleos supraquiasmáticos hipotalámicos en el sistema nervioso central (SNC). El SNC alberga el reloj circadiano principal, que sincroniza una miríada de relojes centrales y periféricos a través de vías neurales y humorales. En el contexto de la estacionalidad, el control diario ejercido por el SNC sobre la glándula pineal es de suma importancia ya que da forma al patrón nocturno de producción de melatonina (figura 2); por lo tanto, la melatonina lleva un mensaje diario y estacional a los tejidos que expresan sus receptores específicos MT1 y MT2. Múltiples tejidos en todo el cuerpo, incluidas varias regiones del cerebro, expresan estos receptores, particularmente el subtipo MT1. Sin embargo, una sola región es común en todas las especies y muestra invariablemente la expresión MT1 más fuerte de todos los tejidos examinados: la pars tuberalis (PT), que constituye la parte rostral de la hipófisis anterior. Debido a que el papel fundamental de la PT en el control estacional de la prolactina (PRL) y las gonadotropinas (LH y FSH) (Dardente *et al.,* 2016). Las gonadotropinas, así como la secreción de melatonina, intervienen en esta regulación (Bustos-Obregón y Torres-Díaz, 2012).

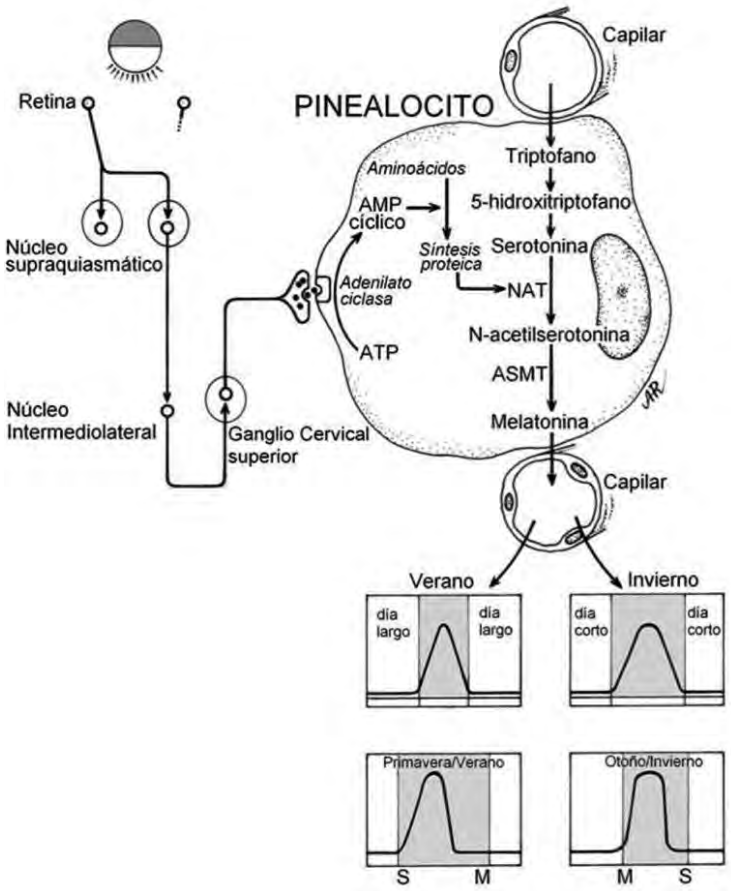


Figura 2. Esquema del tracto fotoneuroendocrino en reproductores de días largos y días cortos (Tomado de Bustos Obregón y Torres-Díaz, 2012).

Los endocrinocitos establecen un asa de retroalimentación con la adenohipofisis y el hipotálamo en un circuito de asa larga, corta y ultracorta. Los sustentocitos son importantes por su rol mecánico, trófico y metabólico respecto a la secreción de la activina e inhibina (Bustos-Obregón y Torres-Díaz, 2012).

Como reproductores estacionales, los machos cabríos también muestran grandes cambios en la actividad sexual. La temporada de reproducción de los machos cabríos comienza a principios del otoño y termina a fines del invierno. El

comportamiento sexual, el tamaño testicular, un índice de la actividad espermatogénica y la producción de esperma cuantitativa y cualitativa disminuyen dramáticamente durante la temporada no reproductiva. La evaluación de las funciones reproductivas de los machos cabríos se realiza generalmente entre los 7 y 9 meses de edad en un corral individual en 15 colecciones de semen durante el comienzo de la temporada de cría, de los machos jóvenes se recolecta dos veces por semana, mientras que de los machos adultos se pueden recolectar de 3 a 5 veces por semana (Luo *et al.,* 2019).

En el macho también se refleja la estacionalidad en testosterona y el tamaño testicular (Santiago-Moreno *et al*., 2005). La actividad sexual del macho es de mayo a diciembre (verano – otoño) seguida de un periodo de reposo sexual de enero a abril (invierno - primavera). Existe una variación marcada según la estación del año en que se encuentren (Chemineau *et al*., 2010). Durante el reposo sexual, la LH, testosterona, el peso testicular y la producción espermática reducen (Delgadillo *et al*., 2001), por lo tanto, el comportamiento sexual de los machos reduce y las copulaciones pueden desaparecer totalmente. Esta estacionalidad depende de las variaciones anuales del fotoperiodo (Duarte *et al.*, 2010); esta acción se genera a nivel de eje hipotálamo-hipofisario-gonadal mediante la glándula recibe las variaciones de horas luz (Chemineau *et al*., 1993).

La melatonina, mantiene un patrón circadiano caracterizado por niveles basales durante el día y elevados durante la noche. Las variaciones en la secreción de esta hormona reflejan el fotoperiodo (Malpaux *et al.,* 1998) regulando la secreción de la hormona liberadora de las gonadotropinas (GnRH), que a su vez regula la secreción de la hormona luteinizante (LH) y la hormona folículo estimulante (FSH) (Clarke y Caraty, 2013). El principal mecanismo de regulación de la estacionalidad por parte de la melatonina se efectúa mediante una modulación, a nivel hipotalámico. La disminución en la secreción de melatonina determina un incremento a la acción inhibitoria de los esteroides gonadales, estableciéndose una inhibición de la actividad reproductiva (Bustos-Obregón y Torres-Díaz, 2012).

El inicio del incremento del tamaño testicular y de la secreción de testosterona en el mes de septiembre, coincide con los grupos mixtos y las luchas entre machos, que establecerán el orden en la cubrición de las hembras en el mes de octubre. Este mecanismo está orientado a conseguir el nivel mayor de testosterona y producción espermática. Sin embargo, al final de la estación reproductiva hay una disminución en la producción seminal, los machos pueden mantener una capacidad de fecundación, y poder cubrir a aquellas hembras que no quedaron gestantes con las primeras ovulaciones de otoño (Bustos-Obregón y Torres-Díaz, 2012).

A nivel testicular, la FSH controla la actividad de las células de Sertolí, regulando la espermatogénesis y la secreción de inhibina (Lincoln *et al.,* 1990). El incremento de la frecuencia de los pulsos de LH durante el otoño, tiene un efecto de retroalimentación negativo de la testosterona, que permitirá un adecuado desarrollo de las glándulas accesorias (Santiago-Moreno *et al.,* 2005).

La reactivación gonadal origina un aumento en el peso testicular durante el periodo verano-otoño, a nivel celular se constata un aumento del volumen de las células de Leyding con un incremento del tamaño del núcleo (Bustos-Obregón y Torres-Díaz, 2012).

Aunque de alguna manera, la actividad espermatogénica y el comportamiento sexual de los machos cabríos están presentes, varían con la estación (Chemineau *et al.,* 2010). Los cambios se originan por la glándula pituitaria (Rosa y Bryan, 2002), la cual, es controlada en el sistema portal-cerebral a nivel pituitario (Chemineau y Delgadillo, 1993). La frecuencia de la liberación de GnRH es un mensaje esencial para controlar todo el eje hipotálamo hipofisiario–gonadal (Thiery *et al.,* 2009*)*. Estos cambios son accionados por el fotoperiodo y la melatonina (Malpaux *et al.,* 2001), que actúa como sincronizadora del ritmo endógeno de la reproducción (figura 3) (Thiery *et al.,* 2009; Calderón-Leyva, 2017).

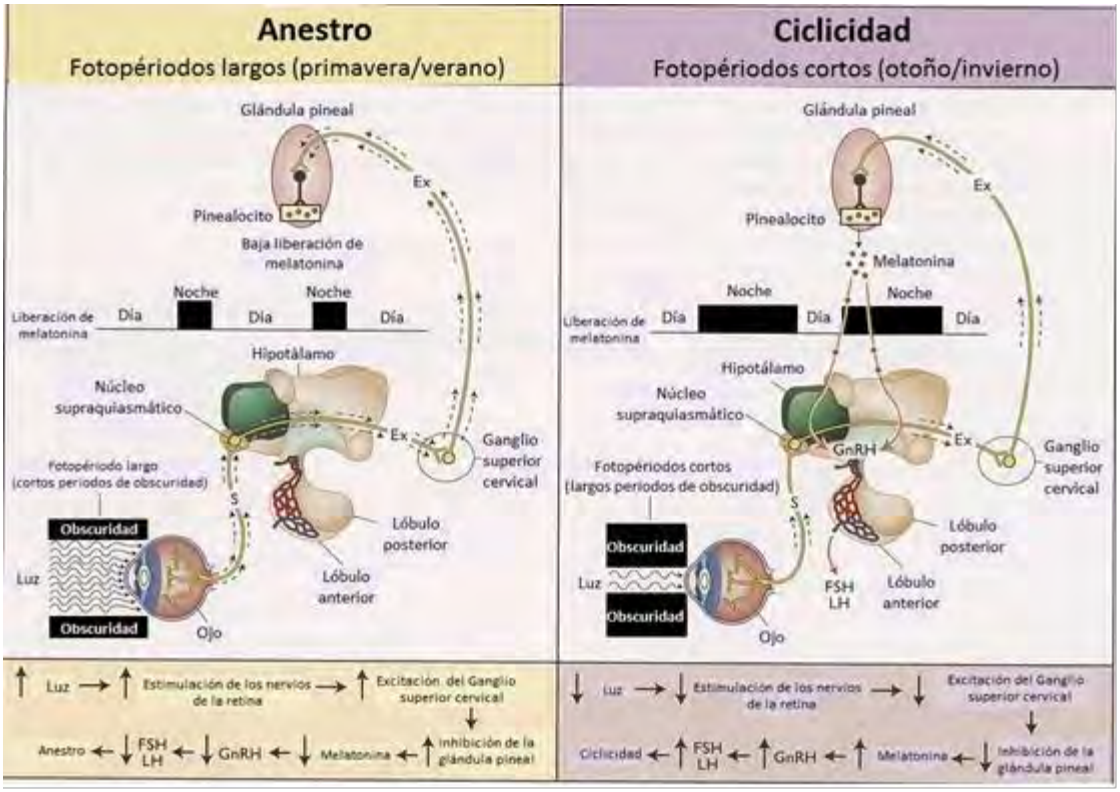


Figura 3. Estacionalidad reproductiva en reproductores de días largos y días cortos (Tomado de Calderón-Leyva, 2017).

En las razas estacionales, ejemplo, en el macho cabrío Alpino de enero a mayo, los niveles basales de testosterona son de 0.3 ng/ml en plasma, la frecuencia de los pulsos es alrededor de 1 en 8 horas, la amplitud de éstos son menores de 0.2 ng/ml y, como consecuencia, la concentración media de LH (0.4 ng/ml de plasma), es baja (Zarazaga *et al.,* 2012).

3.1.3. Regulación de la reproducción estacional por Kisspeptina

Muchas especies de animales domésticos tienen estaciones claras de actividad reproductiva con ciclicidad poliestrica y producción de espermatozoides junto con períodos de inactividad reproductiva. La comprensión de los sistemas que controlan esta reproducción estacional es esencial para gestionar la reproducción y la productividad de estas especies y dado el pronunciado papel estimulante de la kisspeptina en la pubertad y del estado reproductivo del adulto, este péptido representaba un atractivo sistema candidato responsable de controlar los cambios estacionales en la reproducción (Kriegsfeld *et al.,* 2015). Las kisspeptinas están reguladas por estación/ fotoperíodo fue discutida por primera vez cuando se observó que el número de células que contienen ARNm de KISS1 fue mayor en el núcleo arqueado en las ovejas durante la temporada de reproducción que durante la temporada de no reproducción. Un estudio posterior encontró una diferencia similar en el número de células kisspeptina en el núcleo arqueado medio y caudal en la cabra y en la yegua, a pesar de que la yegua es reproductora de día largo, mientras que las ovejas y las cabras son reproductoras de día corto (Scott *et al.,* 2019). La literatura ha demostrado la participación de estos péptidos en los aspectos de la maduración y la función del eje reproductivo. La reproducción debe ser un "buen diálogo" entre el cerebro, la hipófisis y las gónadas (Clarke y Caraty*,* 2013). Los genes de kisspeptina se expresan en una amplia gama de tejidos. En el cerebro de ovejas, las kisspeptinas se localizan en el arco y el área preóptica (POA) (Kitahashi y Parhar, 2013). En la mayoría de las especies, es mediada principalmente por una acción estimulante de GnRH (Clarke y Caraty, 2013; Kriegsfeld *et al.,* 2015).

Los aspectos en la regulación génica de la kisspeptina, los cuales estimulan la liberación de la GnRH, además, en la mayoría de las especies de vertebrados es evidente la existencia de múltiples kisspeptinas, por lo tanto, es importante aclarar los mecanismos para su regulación para entender las funciones de sus múltiples formas en el cerebro. Estos aspectos se centran en los aspectos comparativos de kisspeptina y su regulación de genes, que incluyen a los

esteroides gonadales, el fotoperíodo y las señales metabólicas (Kitahashi y Parhar, 2013; Kriegsfeld *et al.,* 2015; Scott *et al.,* 2019).

Las especies domésticas tienen una estacionalidad por función reproductiva por cambios en la secreción de GnRH. En particular, la kisspeptina, al transducir el efecto de retroalimentación de los esteroides gonadales (Clarke y Caraty, 2013).

Se conoce que las hormonas como el estradiol (E2) y la testosterona (T4), son los principales reguladores de los genes kisspeptina (Kitahashi y Parhar, 2013). Aunque la melatonina tiene un papel importante en la transducción de fotoperiodo con el sistema reproductivo, las células de kisspeptina no parecen expresar el receptor de la melatonina, por lo que los medios por los cuales la estacionalidad cambia el nivel de la actividad kisspeptina sigue siendo desconocido (Clarke y Caraty, 2013). Algunos estudios plantean que la kisspeptina es parte del mecanismo de regulación ambiental de la reproducción (Kitahashi y Parhar, 2013).

3.1.4. Aspectos nutricionales y su relación con la reproducción estacional

La nutrición y el efecto de la condición corporal (CC) y el peso vivo (PV) pueden influir en la actividad reproductiva en diferentes razas de cabras.

Se ha reportado que la influencia de la estacionalidad puede modificarse por el entorno nutricional. Existe evidencia de que en el ganado criollo mexicano que se mantiene en las latitudes tropicales la duración del período de anovulación se modula parcialmente por el estado de energía nutricional. En el área mediterránea, la mayoría de las cabras se manejan en sistemas extensivos o semiextensivos, y la producción está sujeta a variaciones estacionales en la disponibilidad de alimentos. La eficiencia reproductiva se correlaciona con los cambios en el peso corporal (Gallego-Calvo *et al.,* 2014).

Por otro lado, se ha demostrado que restringir el 25% de requerimientos nutricionales afecta la reproducción de la cabras durante el periodo de transición, pero no durante la época reproductiva (Rosales-Nieto *et al.*, 2006).

De manera general se concluyó que la secreción de GnRH se reduce en animales desnutridos. Sin embargo, se investigaron mediadores endocrinos entre el estado nutricional y los procesos reproductivos; entre ellos, el factor de crecimiento asociado a la insulina (IGF-I), la hormona del crecimiento, la colesistoquinina, el neuropéptido Y (NPY), los péptidos opioides endógenos y su relación con la insulina (Arroyo, 2011).

El hígado es el sitio del catabolismo de muchas hormonas lo cual al cambiar la dieta lo afecta directamente, mientras que un elevado plano de la nutrición podría resultar en una disminución en las concentraciones circulantes de testosterona que podrían alterar la secreción de gonadotropinas y por lo tanto, la actividad testicular en el eje (Martin *et al.,* 1992).

En carneros Merino, la inmunización activa contra la inyección de GnRH resultó en la regresión testicular (Hötzel *et al.,* 1992), lo que sugiere que el efecto de la nutrición en el crecimiento testicular es parcialmente independiente de los cambios en la secreción de gonadotropina (Martin *et al.,* 1992).

Los cambios bruscos en la nutrición tienen efectos marcados en el desarrollo testicular de los caprinos, como en el peso vivo (Walkden-Brown *et al.,* 1994).

Los machos salvajes tienen una marcada respuesta testicular a los cambios nutricionales, con incrementos testiculares de hasta el 50% la respuesta testicular es asociada muy estrechamente con aumentos en el peso vivo (Martin y Banchero, 1999).

En la latitud subtropical, el fotoperíodo es de 1336 h de luz en el solsticio de verano a 1024 h de luz en el solsticio de invierno. Esta área tiene un clima seco con una temperatura media anual de 21 °C, que varía de 37 °C entre mayo y agosto, a 6 °C en diciembre y enero. La precipitación anual promedio es de 266

mm (en un rango de 163 a 504 mm) y el período seco dura de noviembre a mayo. En México la población de cabras se distribuye en latitudes subtropicales y tropicales (<23.5° N). La región de la Comarca Lagunera es una importante área de producción de caprinos. Las cabras locales se derivan de las razas española Granadina, Murciana y Malagueña. Estos animales fueron cruzados con razas, Anglo-Nubia, Alpina, Saanen y Toggenburg durante los últimos 70 años para mejorar la producción de leche y carne. Las hembras generalmente se mantienen con los machos en condiciones naturales de pastoreo, comiendo solo vegetación natural disponible sin alimentación suplementaria en el corral. De junio a enero, la disponibilidad de alimentos (%) y el contenido de nutrientes de las dietas seleccionadas por las cabras disminuyen (Junio: 9.1% CP; 1.7 Mcal / kg; enero: 7.4% CP; 0.9 Mcal / kg; figura 4)

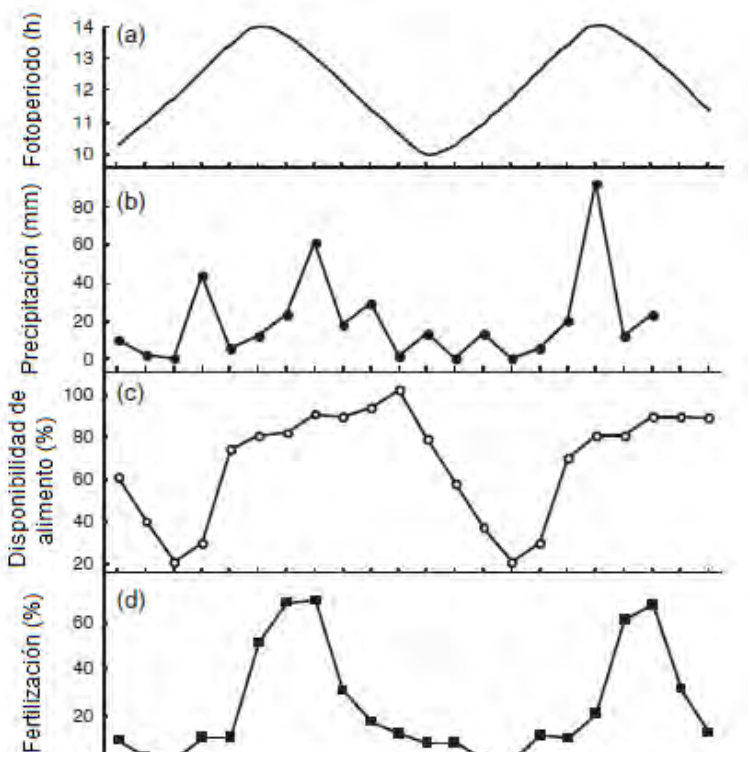


Figura 4. Cambios anuales en el fotoperiodo (a), lluvia (b), disponibilidad de alimento (c) y fertilización (d) de cabras locales del subtrópico de México mantenidas en contacto con machos en condiciones naturales de pastoreo (Adaptado de Delgadillo, 2010).

Para evaluar si la desnutrición causa la disminución estacional de la actividad sexual durante la temporada no reproductiva en machos cabríos, se determinó el peso testicular, la secreción de testosterona y la producción de esperma en machos cabríos alimentados con alfalfa ad libitum y 200 g de un concentrado comercial cuando se mantienen en corrales abiertos, sombreados, bajo fotoperiodo y cambios de temperatura naturales. En estos machos, el peso testicular, las concentraciones plasmáticas de testosterona y el semen cuantitativo y cualitativo variaron estacionalmente y fueron más altos durante la temporada de reproducción, que dura desde finales de primavera (mayo a junio) hasta finales de otoño (diciembre a enero), que durante la temporada no reproductiva (Figura 5) (Delgadillo, 2010).

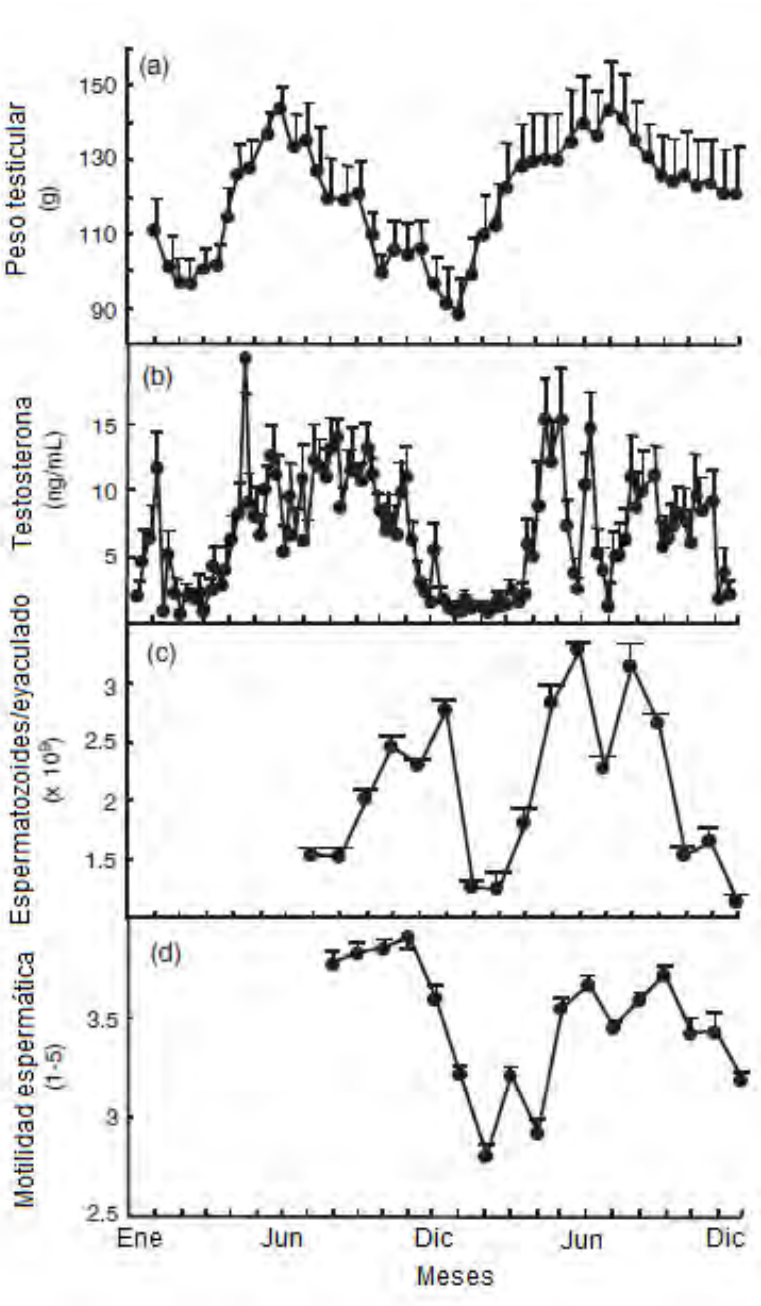


Figura 5. Variaciones estacionales en el peso testicular (a), concentraciones plasmáticas de testosterona (b), número total de espermatozoides por eyaculado (c) y motilidad progresiva de los espermatozoides (d) en machos cabríos locales bien alimentadas y confinados en el subtrópico de México mantenidos en un corral abierto y sometidas a cambios fotoperiódicos y de temperatura naturales (Adaptado de Delgadillo, 2010).

3.2. Mecanismos neuroendocrinos de la reproducción

Los animales han evolucionado diversas variaciones estacionales en fisiológicas y reproductivas para adaptar los cambios anuales en las condiciones ambientales y climáticas. La pars tuberalis (PT) de la hipófisis, que comprende el lóbulo anterior del tallo pituitario que conecta el hipotálamo y la hipófisis, se ha identificado como un sitio clave involucrado en la regulación de la estacionalidad entre aves y mamíferos. Un paso clave en la comprensión del papel de la melatonina en el mamífero como respuesta fotoperiódica fue la identificación de receptores de melatonina en la PT (Helferd *et al.,* 2019).

La reproducción exitosa requiere de la activación de vías neuronales y hormonales para sincronizar la ovulación con la actividad locomotora máxima y el estado de excitación. Las hembras mamíferas muestran ritmos de diferentes escalas de tiempo que van desde minutos (liberación GnRH pulsatil) a horas/días (aumento de LH), días/semanas (ciclo ovárico) o incluso meses (reproducción estacional). Es importante aquí reiterar la definición de pulso generador como: un grupo de células organizadas que generan períodos intermitentes de actividad síncrona suficiente para generar un pulso de GnRH (Hebirson *et al.,* 2018).

La importancia de la secreción pulsátil de GnRH y LH para la fertilidad normal está bien establecida. Los diferentes patrones de liberación pulsátil de la GnRH a través del ciclo ovárico regulan diferencialmente la biosíntesis y secreción de la gonadotropina pituitarica (Thompson y Kaiser, 2015)

La generación de la oleada de LH requiere de altos niveles de circulación de estradiol (E^2), indicativo de maduración del folículo, así como una señal diaria, para asegurar que la ovulación se produzca en el momento adecuado para optimizar el éxito de la reproducción. En las hembras adultas hay dos señales importantes al tiempo de la ovulación: el nivel circulante de las hormonas gonadales, específicamente el estradiol, que es un indicador de maduración de los ovocitos, y la hora del día en que surgen los relojes biológicos. Esta doble

regulación garantiza que el momento de la ovulación coincida con el período de máxima actividad y motivación sexual (Simonneaux y Bahougne, 2015).

3.2.1. Eje hipotálamo-Hipófisis-Gonadal

La actividad reproductiva de las cabras lecheras está controlada por diversos factores tanto *in vivo* como *in vitro*. Entre ellos, el eje hipotálamo-hipófisis-gonadal desempeña un papel importante en la regulación con el hipotálamo modulando la secreción de gonadotropinas hipofisarias o factores inhibitorios. La gonadotropina actúa sobre la gónada a través de la circulación sanguínea periférica, la gonadotropina puede controlar el desarrollo y el proceso reproductivo del individuo al afectar las glándulas exocrinas y endocrinas (Nagamalleswari *et al.,* 2004). La mayoría de las cabras lecheras son mamíferos de uno o dos fetos, con 1 a 3 folículos maduros por ciclo estral, mientras que otros folículos de las cabras se inhiben en atresia y apoptosis. La selección de los folículos dominantes depende básicamente de dos aspectos: nivel de gonadotropina en la sangre y la expresión de los receptores hormonales en los folículos (Graff *et al.,* 2000). La FSH es importante, estimula el desarrollo de los folículos y el reclutamiento de óvulos inmaduros. La LH controla el desarrollo, la selección y la ovulación del folículo dominante, y también induce la apoptosis de otros folículos pequeños. En condiciones fisiológicas, cuando la FSH se eleva hasta un umbral, los folículos de 2 a 5 mm de diámetro pueden ser reclutados periódicamente, el tiempo de duración del reclutamiento del folículo determina el número de folículos reclutados (Leyvaocariz *et al.,* 1995).

Durante el proceso de selección de folículos dominantes de los animales, debido a la diferente sensibilidad de los folículos a la elevación de la concentración de gonadotropina, se seleccionan varios folículos sensibles del grupo de folículos. A través del efecto de la FSH, estos folículos sensibles aumentan el número de células granulosas y líquido folicular, lo que resulta en el crecimiento de los folículos. El reclutamiento, el desarrollo, la selección y la ovulación de los folículos son los factores principales que afectan la fertilidad de las cabras (Leboeuf *et al.,*

2008). En el proceso de desarrollo folicular, el reclutamiento de folículos primordiales, el desarrollo de folículos preantrales, la selección de folículos antrales, la maduración y ovulación de folículos dominantes son factores clave que determinan el número y la calidad de los óvulos; estos factores juegan un papel clave en la determinación de la tasa de preñez y el tamaño de la camada en cabras. Un estudio en Israel indicó que un alto nivel de producción de leche tiene efecto adverso significativo en el rendimiento reproductivo (Gootwine y Pollott, 2000). La heredabilidad del tamaño de la camada fue muy baja (~ 0.05), y la correlación genética con la producción de leche también fue baja (0.0 a 0.2) (Hamann *et al.,* 2004).

Las cabras lecheras como reproductoras estacionales tienen naturalmente un período de anestro durante la primavera en el hemisferio norte, y los machos también muestran grandes cambios en la actividad sexual. La temporada de reproducción de los machos cabríos comienza a principios del otoño y termina a fines del invierno. El comportamiento sexual, el tamaño testicular, la actividad espermatogénica, y la producción de esperma cuantitativa y cualitativa de los machos cabríos disminuyen dramáticamente cuando no hay estacionalidad reproductiva (Leboeuf *et al.,* 2008).

La evaluación de las funciones reproductivas de los machos cabríos de razas lecheras se realiza generalmente entre los 7 y 9 meses de edad en un corral individual por 15 colecciones de semen durante el comienzo de la temporada de cría, de los machos cabríos jóvenes se recolecta dos veces por semana, mientras que en los machos cabríos maduros se pueden recolectar de 3 a 5 veces por semana (Leboeuf *et al.,* 1995). Para tener más producción de cabritos, los productores generalmente prefieren inseminar utilizando protocolos de tratamiento hormonal, aunque tales prácticas de tratamiento hormonal están restringidas en Europa (Martin *et al.,* 2004). Alternativamente, el efecto macho puede ser una forma eficiente de inducir celos en un programa de IA. La inseminación podría realizarse una o dos veces durante un período de 24 h después de que se detecte el celo, como mediante la introducción de un macho

cabrío o por la introducción del macho cabrío celador (Leboeuf *et al.,* 2008). En medio del anestro estacional, se obtuvo un alto nivel de fertilidad (71% a 78% de la IA) utilizando semen congelado en cabras sometidas a tratamiento con largos días artificiales y progestágeno, seguido de IA 52 h después de la introducción del macho cabrío equipado con un mandil (Pellicer-Rubio *et al.,* 2008).

La reproducción estacional de los machos cabríos inhibe la recolección de semen durante 6 meses cada año, lo que limita el número total de dosis de semen producidas por macho durante su vida. Los ciclos fotoperiódicos artificiales, ampliamente utilizados en la industria avícola introducida por primera vez para carneros, permiten el control de la actividad sexual de las razas estacionales. La variación de 1 a 2 meses de días largos (16 h de luz: 8 h de oscuridad) seguido de 1 a 2 meses de días cortos (8 h de luz: 16 h de oscuridad), disminuye la variación estacional en la actividad sexual de los machos cabríos (Delgadillo *et al.,* 1999; Leboeuf *et al.,* 1998; Pelletier *et al.,* 1998).

Se informó que los machos cabríos bajo tratamientos fotoperiódicos durante 3 años consecutivos y con recolecta seminal dos veces por semana dieron una tasa de fertilidad idéntica y un mayor número de espermatozoides totales que los machos cabríos de prueba, con recolecta seminal dos veces por semana durante la temporada reproductiva. En otro ensayo con un régimen de recolección más intensivo de 4 veces/semana durante el año, los machos cabríos que tuvieron un tratamiento de 2 meses de días largos seguidos de 2 meses de días cortos produjeron más dosis de semen que los machos cabríos mantenidos bajo el mismo ritmo de recolección y con luz natural de septiembre a febrero (Leboeuf *et al.,* 1998; Leboeuf *et al.,* 2004; Pellicer-Rubio *et al.,* 2007).

El conocimiento de los diferentes efectos del fotoperíodo en las vías neuroendocrinas y la actividad reproductiva en las cabras ha permitido a los productores aplicar con éxito tratamientos con luz/melatonina para controlar la actividad reproductiva estacional en condiciones de campo y también en machos cabríos criados en centros de IA (Chemineau *et al.,* 1992; Abecia *et al.,* 2012; Zaja *et al.,* 2018). Los machos cabríos tratados con fotoperíodo tienen la misma

capacidad que los machos cabríos tratados con melatonina para inducir una respuesta en la reproducción de las cabras durante la primavera (Zarazaga *et al.,* 2019). Actualmente, este esquema fotoperiódico se utiliza en Francia y otros países europeos en estaciones de ganado como parte del programa de cría de cabras. El semen congelado es sin duda beneficioso para los programas de reproducción. El semen de un macho cabrío superior se puede crioconservar para la mejora genética después de las pruebas de progenie. Además, el semen fresco que es menos costoso que el semen congelado podría usarse para el manejo reproductivo, lo cual es una práctica común en los países en desarrollo (Barrell *et al.,* 1992).

La Actividad neuroendocrina se sabe desde hace mucho tiempo que los días largos inhiben mientras que los días cortos estimulan la actividad sexual en los reproductores de días cortos, como ovejas y cabras. Sin embargo, estos efectos específicos de la duración del día no son permanentes, y cuando son sometidos a un fotoperíodo constante, los animales se vuelven 'refractarios', escapan del fotoperíodo prevaleciente: los días largos ya no son inhibitorios y los días cortos ya no son estimulantes. En cabras y ovejas, esta refractariedad podría conceptualmente ser considerado simplemente como el primer paso de la expresión de los ritmos endógenos circanuales. Puede ser superado transfiriendo animales al fotoperíodo opuesto: refractariedad a los días cortos, que ocurre naturalmente en las ovejas a fines del invierno, se rompe por 2 meses de exposición a días largos en diciembre-enero, permitiendo restablecer la eficiencia de los días cortos estimuladores (Jackson *et al.,* 1988). Por lo tanto, controlar la estacionalidad de la reproducción es posible sometiendo a los animales a fotoperiodos opuestos. Esta propiedad ahora se usa comúnmente en tratamientos fotoperiódicos aplicados en granjas o en centros de IA (Chemineau *et al.,* 2007). La definición de lo que realmente es días largos y días cortos no es sencillo: es posible definir un umbral de fotosensibilidad basada en el número de horas de luz por día, bajo el cual días largos son estimulantes y bajo del cual días cortos son inhibitorios. Ahora se acepta comúnmente que días largos son días

más largos que los anteriores, y que los días cortos son días más cortos que los anteriores.

La temporada de reproducción para una raza dada puede ser muy estable de un año a otro, con fechas fijas de inicio y fin de actividad de la ovulación y un período de máxima calidad y producción de esperma. Este momento preciso es mecanismo complejo que permite a los animales 'localizar' su temporada de reproducción durante el año y expresar su actividad sexual en el momento adecuado, sincrónicamente con factores ambientales externos y entre sexos. En estos mamíferos estacionales, de manera similar al reloj circadiano (es decir, con un período de ejecución libre cercano a las 24 h que genera ritmos circadianos endógenos), al ritmo endógeno circanual es probable que los animales generen períodos alternos de descanso sexual con períodos de actividad sexual durante todo el año. Estos períodos alternos pueden ser observados experimentalmente durante al menos dos años consecutivos, cuando los animales se mantienen bajo constantes regímenes artificiales fotoperiódicos (días cortos constantes). Las ovejas y los hámsters son dos modelos experimentales que han contribuido a una mejor comprensión de los ritmos endógenos y de refractariedad, mecanismos esenciales que rigen la reproducción estacional (Larkin *et al.,* 2002; Lehman *et al.,* 2002; Paul *et al.,* 2008) o la muda del pelaje (Paul *et al.,* 2008). En las ovejas, este ritmo endógeno se cronometra por señales discretas dadas por cambios externos en el fotoperíodo (Barrell *et al.,* 2000). Sin embargo, los mecanismos fisiológicos subyacentes a este sistema circanual permanecen en gran medida desconocidos. La entrada fotoperiódica se percibe en mamíferos exclusivamente a través de los ojos, luego es transmitida a través de una vía multisináptica a la glándula pineal, que transduce la señal fótica en una sustancia química. Se piensa que la melatonina es entregada a los tejidos periféricos por la circulación sistémica y al cerebro a través del líquido cefalorraquídeo (Thiery *et al.,* 2009).

Se ha demostrado recientemente que la tasa de rotación de LCR en ovejas cambia de acuerdo con ciclo claro-oscuro; se incrementa durante los días cortos

y se reduce en días largos, por lo tanto este fenómeno podría explicar las diferencias en las concentraciones hormonales en CSF, y podría afectar en cierta medida a los mensajes melatonérgicos de los sitios receptivos dentro del cerebro. Para controlar la actividad reproductiva, y especialmente la actividad pulsátil de GnRH en ovejas, la melatonina actúa sobre el área premamilar del hipotálamo (PMH). Se demostró claramente que implantes de melatonina insertados dentro del PMH desencadenaron actividad GnRH-LH, mientras que los implantes de melatonina que se insertan dentro de la pars tuberalis de la pituitaria no modifican la secreción de LH (aunque una fuerte densidad de receptores de melatonina se expresan en ovejas como en la mayoría de mamíferos) (Malpaux *et al.,* 1998). La melatonina se puede detectar en las transcripciones del receptor MT1, y los receptores de membrana se expresan en ovejas ILe-de-France mediada por el PMH. Aproximadamente 45 días después del inicio del día impregnación de melatonina estos es probable que los receptores participen en la estimulación de la actividad pulsátil de GnRH que, a su vez, conducirá a actividades sexuales gonadales y conductuales (Migaud *et al.,* 2005).

En ovejas la actividad dopaminérgica inhibe la pulsatilidad de GnRH. Más recientemente, el papel estimulante de kisspeptina y su receptor GPR54 se mostró en la temporada y control del fotoperiodo y la pulsatilidad GnRH/LH, sugiriendo que esta pareja juega un papel clave para la estimulación de la pulsatilidad y que las neuronas de kisspeptina, ellas mismas están sujetas a estacionalidad y control del fotoperíodo (Wakabayashi *et al.,* 2010).

La variabilidad entre razas tiene grados variables, por ejemplo, ovejas merinas y manchegas (Santiago-Moreno *et al.,* 2000) así como las ovejas Chios (Avdi *et al.,* 1993), presentan una expresión moderada de estacionalidad, mientras que las ovejas Soay y Texel son altamente estacionales (Hafez, 1952). En los trópicos, las razas locales generalmente presentan una muy baja estacionalidad o ciclo todo el año sin período anovulatorio. Los productores de los trópicos están interesados por esta baja estacionalidad, ya que pueden organizar la temporada de cría de su rebaño durante todo el año, sin tratamientos hormonales caros. Por

desgracia cuando son sometidos a las grandes variaciones fotoperiódicas y climas templados de los países del norte, una marcada estacionalidad se expresa en estas razas (Chemineau *et al.,* 2004). Esto impide su posible práctica de uso en bandadas bajo latitud templada y también perjudica su uso experimental para explorar los mecanismos involucrados en el control genético de la estacionalidad de la cría y actividad bajo latitudes templadas.

La variabilidad entre razas también existe en razas templadas mantenido bajo condiciones ambientales similares de donde se originan. Algunos rasgos reproductivos incluyendo el inicio, el final y la duración de la temporada de reproducción se encontraron repetibles y heredables, y son por lo tanto, rasgos candidatos para la selección genética. La fertilidad fuera de la temporada también demostró ser un rasgo que puede usarse para una selección divergente exitosa. Para evitar efectos nocivos de seleccionar experimentalmente contra fertilidad, rasgos indirectos como la presencia de actividad ovulatoria espontánea en principios de la primavera, lo que probablemente indica una temporada de reproducción más prolongada y está vinculada a la fertilidad potencial, se encontró que era heredable en ovejas Merinas y podría ser utilizado en la selección. En esta raza, la selección divergente en este rasgo es factible (Teyssier *et al.,* 2002). Tales líneas podrían ser utilizadas en el futuro para explorar con más detalle los mecanismos por los cuales las razas estacionales controlan su capacidad reproductiva anual y los genes involucrados para controlar esto (Sakamoto *et al.,* 2010).

Las neuronas de la GnRH en el prosencéfalo basal son la vía común final a través de la cual el cerebro regula la reproducción. La secreción de GnRH ocurre de manera pulsátil, y la evidencia indirecta sugiere que las neuronas de kisspeptina en el núcleo arqueado (ARC) sirven como el marcapasos central que impulsa la secreción pulsátil de GnRH. Los estudios genéticos en humanos han revelado que la kisspeptina y la neuroquinina B (NKB) están involucradas en el mecanismo de control central de la secreción de GnRH. Se ha demostrado que la

administración de kisspeptina o NKB (o sus análogos) aumenta la secreción de LH (Wakabayashi *et al* 2010; Oakley *et al.,* 2009).

Mientras que la administración de sus antagonistas suprime la secreción de LH (Ramaswamy *et al.,* 2010). Una población de neuronas en el núcleo arqueado (ARC) coexpresa estos 2 neuropéptidos en una variedad de mamíferos, incluidos los ratones, ratas, ovejas, cabras y monos. La evidencia emergente reciente sugiere que la población de neuronas de kisspeptina/NKB en el ARC es un candidato probable para la fuente intrínseca del generador de pulsos GnRH. Esta hipótesis está respaldada por los hallazgos de que el electrodo activo que detectó la descarga de la actividad de unidades múltiples (MUA), estaba ubicado muy cerca de las neuronas kisspeptina/NKB en la porción caudal del ARC en caprinos tanto en machos como en hembras. Por lo tanto, es razonable plantear la hipótesis de que la señal neural de la feromona masculina se transmite y procesa en neuronas de kisspeptina ARC/NKB para facilitar la actividad del pulso generador de GnRH (Sakamoto *et al.,* 2010).

3.2.2. Rol de las kisspeptinas en el proceso reproductivo

La Kisspeptina es un neuropéptido hipotalámico que es crítico para la fertilidad. En prácticamente todas las especies, las neuronas kisspeptina estimulan la secreción de la GnRH y actúan como transmisores para la retroalimentación de esteroides sexuales a las neuronas GnRH.

Las neuronas Kp se localizan dentro de dos áreas hipotalámicas, en el núcleo arqueado (ARC) y el núcleo periventricular rostral del tercer ventrículo, también llamado núcleo anteroventral periventricular (AVPV), o el área preótica (según la especie). El receptor Kp, Kiss1R (anteriormente GPR54), se expresa altamente en las neuronas de GnRH, pero también en otras áreas cerebrales (Herbison *et al.,* 2010; Simonneaux *et al.,* 2013), y en la mayoría de los tejidos endocrinos como la glándula, ovario y placenta (Kotani *et al.,* 2001); además se habla del reloj circadiano maestro ubicado en el núcleo supraquiasmático hipotalámico

(SCN) que se sincroniza con el ciclo diario principalmente a través del ciclo de luz/oscuridad y a una extensión menos por otras señales de tiempo (ingesta de alimentos y actividad de sueño/vigilia); los transmisores remiten la información diaria al eje reproductivo: la arginina vasopresina (AVP) es el transmisor diario más esencial que se proyecta a las neuronas kisspeptin (Kp) de los núcleos anteroventrales periventriculares (AVPV), que a su vez activan fuertemente las neuronas GnRH ubicadas en el área preóptica (POA); a lo largo del ciclo estral, el estradiol se retroalimenta en diferentes niveles del eje reproductivo principalmente a través del receptor de estrógeno (ER). Las neuronas Kp son los principales objetivos de estradiol con un efecto inhibitorio de bajo estradiol en las neuronas Kp del núcleo arqueado (ARC) y una acción estimulante de alto estradiol en las neuronas Kp AVPV. Además del reloj circanual maestro, los relojes periféricos se encuentran en las neuronas AVPV Kp, neuronas GnRH, gonadotrofos pituitarios, y los diferentes tipos de células del ovario (Simonneaux y Bahougne *et al.,* 2015)

Curiosamente, la retroalimentación de estradiol en roedores depende de la localización de la neurona Kp ya que el estradiol estimula la expresión Kiss1 en el AVPV e inhibe la expresión Kiss1 en el ARN (Smith *et al.,* 2005). En los mamíferos no roedores, se observa una diferencia similar por estradiol, también se observa con un efecto estimulante en el nucleo periventricular/POA rostral y un efecto inhibitorio en el ARN (Hoffman *et al.,* 2011). La mayoría de las funciones biológicas, incluida la reproducción de las hembras, son sincronizadas con la variación diaria de los factores ambientales. Entre estos factores, el ciclo de luz/oscuridad recurrente es el ambiental predecible utilizado por los mamíferos para ajustar su comportamiento y fisiología apropiadamente (Simonneaux y Bahougne *et al.,* 2015).

Aunque el AVP libera las neuronas SCN por la tarde (Kalsbeek *et al.,* 2010), el AVP puede activar las neuronas Kp por la mañana o por la tarde, indicando que el control diario de la oleada de LH no está cerrado por las Neuronas del AVPV Kp (Williams *et al.,* 2011).

Cada tipo de célula del ovario, incluyendo células lúteas, células de granulosa, y los ovocitos tienen un reloj circadiano (Sellix *et al.,* 2015). Otros análisis han reportado que los ritmos genéticos del reloj sólo se observan en la granulosa madura y células lúteas, lo que indica que estos ritmos se activan en una etapa específica del desarrollo del folículo, posiblemente bajo el control de FSH actuando como un sincronizador de las actividades de células foliculares (Chen *et al.,* 2013)

3.3. El betacaroteno

Los carotenoides son pigmentos que están presentes en todos los organismos fotosintéticos y no fotosintéticos así como en bacterias, algas, hongos, insectos y animales y son responsables de la mayoría de los colores rojo a amarillo de mucha hojas, frutas, flores y peces. Los colores característicos de muchas aves, insectos e invertebrados marinos, se han originado en la dieta debido a la presencia de carotenoides (Fraser y Bramley, 2004; Nozière *et al.,* 2006; Young y Lowe, 2018).

La principal función biológica es de servir como pigmentos en los procesos de fotosíntesis, y como sustancia fotoprotectora. Además, actúa inhibiendo la propagación de ROS y otros radicales libres (Fraser y Bramley, 2004; Nozière *et al.,* 2006).

Estos pigmentos orgánicos sintetizados a partir de unidades de isopreno en plantas y algas, representan uno de los grupos más extendidos de pigmentos naturales. Existen 1117 carotenoides conocidos y caracterizados estructuralmente (Yabuzaki, 2017). Las plantas los sintetizan de novo, pero están presentes en los animales, donde se acumulan sin cambios de la dieta o modificados metabólicamente. Los carotenoides vegetales son la fuente dietética primaria de provitaminas A, con el betacaroteno como el ejemplo más conocido. El requisito estructural esencial en la molécula de vitamina A unido a una estructura de polieno conjugada intacta, por lo que el betacaroteno con dos unidades estructurales esenciales es la provitamina A más potente. El sitio

principal de la conversión de betacaroteno a vitamina A es la mucosa intestinal y dos enzimas están involucrados: la oxigenasa que divide la molécula en el centro para producir la retina (aldehído de vitamina A) y la reductasa retiniana que convierte la retina en retinol (Goodman *et al.,* 1965). El retinol y los metabolitos como el ácido retinoico son importantes en la visión, las funciones inmunes y cerebrales la remodelación de tejidos y el metabolismo (Brossaud *et al.,* 2017).

3.3.1. Estructura y propiedades químicas de los carotenoides

Los carotenoides son moléculas lipofílicas clasificadas por estructura como carotenos y xantofilas. Ambas clases comparten una estructura común poliisoprenoide C40 que contiene una serie de enlaces dobles conjugados ubicados centralmente (Fratianni *et al.,* 2010). Algunos carotenoides son moléculas de polieno de cadena abierta, mientras que otros tienen grupos extremos cerrados, como un anillo de b-ionona. Los carotenos (por ejemplo, b-caroteno, a-caroteno y licopeno) son hidrocarburos no polares. Las xantofilas más polares (por ejemplo, luteína, zeaxantina, canthaxantina y b-criptoxantina) contienen oxígeno como un grupo hidroxilo o ceto contenido en el grupo final (Deming y Erdman, 1999).

Las funciones de una molécula de carotenoide dependen principalmente de su estructura química, se considera el factor más importante en las reacciones de transferencia de energía, que es absorber la luz durante la fotosíntesis y proteger las células de la fotosensibilización (Demmig-Adams *et al.,* 1996; Young y Lowe, 2001).

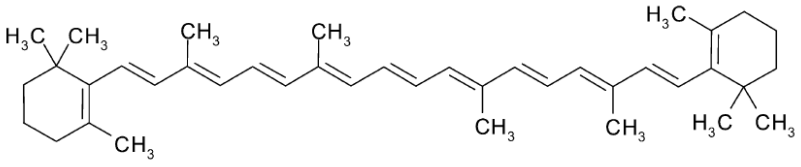
La estructura no solo determina la absorción de luz y color de las moléculas de carotenoides, sino que también puede proporcionar información sobre su absorción, metabolismo y efectos biológicos complejos, que pueden ser relevantes para procesos in vivo en especies de mamíferos, esta característica de la molécula también permite la extinción de $^1O_2$ (Young y Lowe, 2001).

El anillo de b-ionona de seis miembros de b-caroteno, a-caroteno y b-criptoxantina proporciona a estos principales carotenoides dietéticos la capacidad

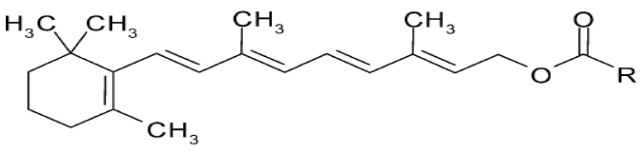
de ser metabolizados a vitamina A. La presencia de un anillo de seis miembros ubicado en el extremo de la estructura polieno se ha asociado con la estimulación de la comunicación de uniones gap intercelular (Sies, 1997). De los carotenoides de provitamina A, se ha demostrado que el betacaroteno induce las proteinas de las uniones gap, las conexinas (Deming y Erdman, 1999).

Las uniones gap son canales de membrana que permiten la transferencia de iones y moléculas pequeñas entre células contiguas (Huang *et al.,* 1998). Las conexinas (Cxs) son las proteínas que forman los canales y la comunicación intercelular de uniones gap (GJIC) juega un papel clave en la comunicación celular y la homeostasis en organismos multicelulares. GJIC puede disminuir progresivamente durante la carcinogénesis de etapas múltiples y la prevención de la baja regulación de GJIC en las células cancerosas sin comunicación podría ser un enfoque quimiopreventivo y quimioterapéutico efectivo (Trosko, 2003).

Zhang *et al.* (1992), encontraron que los carotenoides inducen GJIC a través de una mayor expresión de conexina 43 (Cx43) en la línea celular de fibroblastos de embrión de ratón. Cx43 es un componente del canal de unión GAP en muchos tipos de células, incluidas las células pulmonares humanas (Cesen-Cummings., et al 1998). La actividad de estimulación está asociada con las propiedades estructurales de los carotenoides (Sies, 1997; Stahl y Sies, 2005).

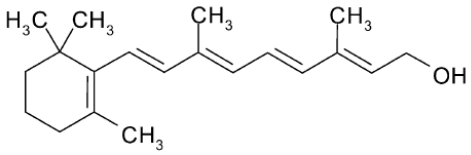


Beta caroteno

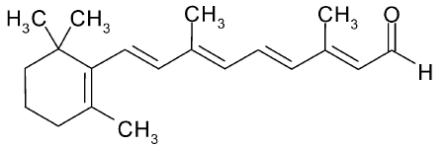


Ésteres de retinol

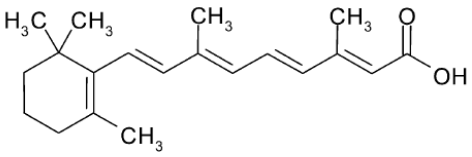
Retinol



Retinal



-trans ácido retinol



9-cis-ácido retinoico

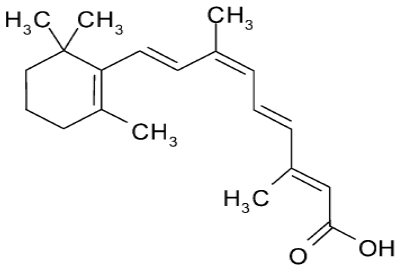


Figura 6. Fórmulas estructurales del betacaroteno y sus metabolitos

3.3.2. Contenido de betacaroteno en forrajes

El contenido de carotenoides en los forrajes depende de su síntesis y degradación en la planta, la síntesis se da a partir de la formación de isopreno y ocurre en muchos niveles de la planta (Armstrong y Hearst, 1996; Gandul y Mínguez, 1996). El betacaroteno (BC) es importante, sin embargo el contenido de betacaroteno en los forrajes es variable (Latscha, 1990). Además existen

otros factores que intervienen en la concentración de carotenoides, de acuerdo a la especie, los niveles de carotenos varían de 5-10 veces (Livingston *et al.,* 1968).

En la mayoría de los casos, incluso si la concentración de b-caroteno y a-tocoferol en el forraje es alta, los requerimientos de vitaminas A y E de los rumiantes no pueden ser cubiertos adecuadamente por su consumo diario de forraje (McDowell, 2002). Por lo tanto, varios factores pueden modificar el suministro nutricional de vitaminas A y E del forraje. El nivel de conversión de b-caroteno varía (1 mg de betacaroteno = 400 UI de vitamina A en una vaca lechera lactante) según el estado de salud y la cantidad consumida. La tasa de conversión se reduce a medida que aumenta el nivel consumido, y generalmente se acepta que este factor de conversión para rumiantes solo es válido en los niveles de ingesta correspondientes al mantenimiento (Bondi y Sklan, 1984).

Entre otros factores que intervienen en la variación del contenido de BC está la estación del año, como la alfalfa que tiene mayor concentración en marzo/abril (349 mg kg-1) *vs* mes julio (251 mg kg-1) (Williams y Morgan 1998). De igual forma, el contenido de BC varía según la edad de la planta, al encontrar mayor concentración de BC en el raygrass inmaduro *vs* maduro y la fertilización con N incrementa la concentración de caroteno en especies cultivadas; podría deberse a la actividad de los carotenos en la biosíntesis de proteínas (Park *et al.,* 1983).

3.3.2.1. Efecto de los procesos de preservación de forrajes cultivados sobre las concentraciones de carotenoides

En forrajes secos expuestos al sol, decrece fuertemente la concentración de carotenoides, especialmente cuando éstos son humedecidos por la lluvia durante el henificado. Las diferencias entre especies sobre el contenido de carotenos, son menos importantes que las diferencias del contenido de carotenos cuando éstas se someten a un proceso de secado. La degradación ocurre rápidamente por oxidación, principalmente por alta exposición a la radiación solar (Chauveau-Duriot *et al.,* 2010), ellos observaron en promedio una pérdida de 83% de carotenoides entre el corte directo y el henificado. Esta pérdida de carotenoides es principalmente por la radiación solar, debido a la exposición a los rayos UV,

que son capaces de destruir todos los carotenos (Cardinault y Noziere, 2004). Sin embargo, ocurren pérdidas moderadas de carotenoides durante el almacenamiento del heno (Bruhn y Oliver, 1978), debido probablemente a la presencia de oxígeno.

El principal factor responsable de la variación en los niveles de betacaroteno es la proporción de hoja a tallo. La formación de tallos se acompaña de un aumento en la concentración de MS. Por lo tanto, existe una correlación negativa entre el contenido de MS y el nivel de betacaroteno (Ballet *et al.,* 2000).

El ensilado de maíz es bajo en carotenoides, independientemente del marchitamiento, el proceso de ensilado disminuye en varios niveles la concentración de carotenoides. La pérdida de carotenoides es dependiente del pH y favorecida por la condición aeróbica. La pérdida de carotenoides es superior en leguminosas que en pastos cuando el pH es bajo (pH=5) y cuando esto se adiciona al tiempo en el retraso al silo y aumenta el tiempo de almacenamiento en el silo, las pérdidas de carotenos incrementan. La máxima pérdida puede estar alrededor del 80% de la concentración inicial, pero cuando se hace el ensilado, las pérdidas en la concentración son generalmente del 20% (Ballet *et al.,* 2000).

La deshidratación de alfalfa resulta en una pérdida de caroteno y xantofilas y depende de la extensión del proceso, esta pérdida es elevada especialmente de xantofilas a causa de la alta temperatura del proceso y cuando el producto final contiene una elevada humedad. Cuando la temperatura es muy elevada (más de 150ºC) y la humedad de 3%, puede resultar en una pérdida superior a 33% de caroteno y 73% de xantofilas (Burdick y Fletcher, 1985).

En general la pérdida de betacaroteno en los diferentes procesos de conservación de forrajes es evidente. Williams y Morgan (1998), determinaron los valores medios de betacaroteno en 196, 159, 81 y 36 mg kg-1 MS para forrajes verdes, forrajes deshidratados, ensilados y henos, respectivamente.

3.3.3. Metabolismo Retinoide

El metabolismo retinoide, ocurre en la porción proximal del intestino delgado tanto en la luz como en el enterocito (Blomhoff *et al.,* 1991). Para una absorción óptima del retinoide, la grasa debe consumirse junto con el retinoide recién ingerido. Esto facilita la entrada de retinoides en los enterocitos desde la luz del intestino y permite la formación óptima de quilomicrones, ya que los retinoides, como otros lípidos de la dieta, ingresan al cuerpo como un componente de quilomicrones nacientes ricos en triglicéridos.

Los carotenoides proretinoides de la dieta, como el betacaroteno, así como los carotenoides no proretinoides como el licopeno y la luteína se liberan de la matriz alimentaria y se emulsifican con ácidos grasos y ácidos biliares para facilitar su absorción en los enterocitos e incorporarse en quilomicrones e ingresar a la circulación general y al cuerpo (Blomhoff *et al.,* 1991). En intestino los carotenoides proretinoides de la dieta se absorben intactos en el enterocito, donde se convierten a retinoides o se pueden empaquetar sin modificar en quilomicrones.

3.3.4. Conversión enzimática de carotenoides proretinoides en retinoides

Las enzimas involucradas en la conversión de carotenoides proretinoides en retinoides existen esas enzimas dentro de los tejidos de los mamíferos que pueden escindir el betacaroteno, ya sea simétricamente en su doble enlace central carbono-carbono 15,15 ', formando dos moléculas de retinaldehído, o asimétricamente en otros enlaces dobles carbono-carbono, formando dos productos de longitud de cadena desigual (D'Ambrosio *et al.,* 2011).

El producto del gen Bcmo1, que codifica una enzima capaz de catalizar la escisión central del betacaroteno, media la formación de retinoides a partir de los carotenoides proretinoides en la dieta (Hessel *et al.,* 2007; Fiercea *et al.,* 2008) dos proteínas estructuralmente relacionadas, betacaroteno-15,15′-monooxigenasa (BCMO1), codificada por Bcmo1, y betacaroteno-9 ', 10′-

monooxigenasa (BCMO2), codificada por Bcmo2, son las únicas enzimas mamíferas conocidas para escindir los carotenoides. BCMO1 y BCMO2 son altamente homólogas entre sí y comparten 39% de identidad de secuencia con BCMO1 y BCMO2 de ratón. Cada una tiene un alto grado de homología de secuencia con la proteína retiniana RPE65, que cataliza la isomerización de todo el retinoide trans al isómero 11-cis para su uso en el ciclo visual. La BCMO1 es una proteína soluble, que contiene Fe^{2+}, de 63 kDa. Se expresa en el intestino delgado (a niveles más altos, mientras más proximales al estómago), hígado, riñón, pulmones, piel, testículos, el epitelio pigmentario de la retina dentro del ojo y en varios tejidos embrionarios (Paik *et al.,* 2001; Wyss, 2001; Paik *et al.,* 2004; Wyss, 2004).

Los genes Bcmo1 y Bcmo2 y su expresión: varios laboratorios han estudiado el gen para Bcmo1 y la regulación de su expresión. Se ha demostrado que los genes de ratón y humano para Bcmo1 contienen elementos de respuesta funcionales del receptor activado por proliferador de peroxisoma (PPAR) [PPRE] (Boulanger *et al.,* 2003; Gong *et al.,* 2006).

3.3.5. Esterificación de retinol por enterocitos

El retinol dietético recientemente absorbido dentro del enterocito debe esterificarse antes de su envasado como éster de retinilo en quilomicrones nacientes. La literatura anterior había indicado que el intestino posee dos actividades enzimáticas distintas capaces de sintetizar ésteres de retinilo a partir de retinol (Blomhoff *et al.,* 1991). Uno de estos, la lecitina: retinol aciltransferasa (LRAT), cataliza la transesterificación del retinol empleando un grupo acilo graso presente en la posición A1 de una molécula de fosfotidilcolina de membrana. El otro, acil-CoA: retinol aciltransferasa (ARAT), cataliza la esterificación del retinol dependiente de acil-CoA graso. En resumen, LRAT representa la gran mayoría del éster de retinilo formado en el enterocito tras el consumo de niveles normales de retinoide en la dieta; DGAT1, un ARAT intestinal, representa la actividad de esterificación restante (Wongsiriroj *et al.,* 2008).

- **Proteína de unión a retinol celular, tipo II (CRBPII)**

Los retinoides son muy insolubles en agua y, en consecuencia, dentro del ambiente acuoso del cuerpo, generalmente se encuentran unidos a proteínas de unión a retinoides específicas. En el adulto CRBPII se expresa únicamente en la mucosa intestinal y facilita la absorción óptima de retinol de la dieta (Blomhoff *et al.,* 1991).

La CRBPII actúa para garantizar el suministro adecuado de retinol a un feto en desarrollo cuando el retinoide en la dieta es limitante. Investigaciones posteriores que hacen uso de ratones deficientes en CRBPII criados en el fondo deficiente en LRAT (que carecen de CrbpII y Lrat) establecieron que CRBPII canaliza metabólicamente retinol a LRAT para la síntesis de éster de retinilo (Wongsiriroj *et al.,* 2008).

3.3.6. Quilomicrones y su metabolismo en la circulación

Para la absorción del retinoide de la dieta, el éster de retinilo se envasa junto con otros lípidos en los quilomicrones nacientes, que se secretan en el sistema linfático (Blomhoff *et al.,* 1991). Así, el carotenoide dietético que no se ha convertido en retinoide también se incorpora a los quilomicrones. Después de ingresar a la circulación general, los quilomicrones se someten a un proceso de remodelación que implica principalmente la hidrólisis de triglicéridos por la lipoproteína lipasa (LpL) y la apolipoproteína E (apoE) de la circulación, lo que resulta en la formación de restos de quilomicrones. Durante mucho tiempo se ha establecido que el 66-75% del retinoide de la dieta (quilomicrón y retinoide remanente de quilomicrón) es absorbido por el hígado, donde se almacena en las células estrelladas hepáticas (HSC), y el resto es eliminado por los tejidos periféricos (Goodman *et al.,* 1965).

La lipoproteína lipasa (LpL) puede hidrolizar el éster de retinilo presente en los quilomicrones, y se ha propuesto que la hidrólisis del éster de retinilo facilita la absorción de retinol por los tejidos periféricos (Blaner *et al.,* 1994). La LpL facilita la absorción de retinoide posprandial en los tejidos y han estableciendo que LpL

actúa para modular la absorción de retinoides posprandial por el músculo esquelético, el corazón y el tejido adiposo (Blaner *et al.,* 1994; Van Bennekum y Kako, 1999), el tejido mamario y la leche (Ross *et al.,* 2004; Oliveira *et al.,* 2017), y probablemente pulmón (Ross y Li, 2007).

3.3.7. Metabolismo hepático retinoide

El hígado es el sitio principal del metabolismo retinoide y el almacenamiento en el cuerpo (Blomhoff *et al.,* 1991). Hay dos tipos de células hepáticas importantes para estos procesos: las células parenquimatosas (también conocidas como hepatocitos) y las células estrelladas (también conocidas como células de almacenamiento de grasa, adipocitos, células de Ito y/o células perisinusoidales). Los hepatocitos comprenden aproximadamente el 66% de las células en el hígado y contienen el 90% de la masa proteica total (Geerts, 2001; Friedman, 2008). Las células estrelladas hepáticas (HSC) son relativamente mucho más pequeñas y menos abundantes. Las HSC comprenden solo el 6-8% de las células en el hígado y contienen el 1% de la proteína hepática (Geerts, 2001; Friedman, 2008). Está bien establecido que los hepatocitos están involucrados centralmente en la absorción y el procesamiento de retinol en el hígado, y que las HSC desempeñan un papel central en el almacenamiento de retinoides hepáticos.

- **Captación y procesamiento de Chylomicron Retinyl Ester (éster de retinilo de quilomicrón) por el hepatocito**

Cuando el retinilo remanente que contiene éster de quilomicrones llega al hígado, pasa al espacio de Disse (ubicado entre el endotelio y el hepatocito) en un proceso denominado tamizado (Wisse *et al.,* 1985). Solo pueden pasar restos del tamaño apropiado, mientras que las partículas más grandes, incluidos los quilomicrones completos, están excluidas. Una vez dentro, el remanente es absorbido exclusivamente por los hepatocitos por una de las dos posibles vías mediadas por el receptor.

- **Hidrólisis del éster de retinilo en el hepatocito**

Al ingresar al hepatocito, el éster de retinilo se asocia con endosomas tempranos y se somete a una hidrólisis rápida (Harrison, 1995). La hidrólisis del éster retinílico se lleva a cabo por enzimas las cuales son denominadas en la literatura como REH, carboxilesterasas y / o lipasas.

- **Transferencia de retinol de hepatocitos a las células estrelladas hepáticas (HSC).**

Después de que el éster de retinilo de quilomicrón se hidroliza a retinol dentro del hepatocito, luego se transfiere a las HSC donde se reesterifica y se almacena en gotas de lípidos. Esta transferencia ocurre principalmente en tiempos de ingesta de retinoides suficiente o excesiva, de modo que estas reservas de retinoides pueden ser utilizadas y movilizadas en tiempos de deficiencia de vitamina A. Los primeros estudios sugirieron que esta transferencia está mediada por la proteína de unión a retinol (RBP). Blomhoff y col. informaron que los anticuerpos contra RBP bloquearon completamente la transferencia de retinol de los hepatocitos a las HSC (Blomhoff *et al.,* 1988).

- **Almacenamiento de retinoides en el HSC como éster de retinilo en gotas de lípidos**

El papel de las gotitas de lípidos HSC en el almacenamiento de retinoides: la característica más distintiva de HSC es la presencia de numerosas gotitas de lípidos que contienen éster de retinilo en el citoplasma, que recientemente se ha propuesto que sean orgánulos especializados para el almacenamiento de retinoides (Blaner *et al.,* 2009). El contenido único de retinoides de estas gotitas, su capacidad de respuesta al estado de los retinoides en la dieta, su dependencia de la síntesis del éster retinílico y su pérdida en diferentes tipos de enfermedades hepáticas. Estos estudios han demostrado un fuerte papel regulador de los retinoides en la fisiología de las gotas de lípidos HSC.

- **Esterificación del retinol en el HSC**

Después de la transferencia del hepatocito al HSC, el retinol se esterifica de nuevo a retinil éster para almacenarlo en las gotitas de lípidos del HSC. Una vez en el HSC, el retinol se une a CRBPI y se transfiere a LRAT para la esterificación (Boulanger *et al.,* 2003). La unión de retinol a CRBPI es necesaria para su transporte en el ambiente acuoso del citosol y se ha propuesto para evitar que las enzimas dependientes de acil CoA en el hígado catalicen la formación de éster de retinilo. Tanto LRAT como CRBPI están enriquecidos en HSC (Blomhoff *et al.,* 1991; Blaner *et al.,* 2009), y ambas proteínas son necesarias para asegurar la acumulación óptima de HSC de las reservas de retinoides.

3.3.8. Requerimientos de Vitamina A, referidos en betacaroteno en cabras lecheras

Las vitaminas son nutrientes con alta actividad biológica y esenciales para un óptimo funcionamiento de todos los tejidos y como resultado un excelente estado de salud y desempeño, tanto productivo como reproductivo, (NRC, 1981). La vitamina A puede ser suministrada en los ingredientes de la dieta, así como sus precursores. De acuerdo a la NRC (2007), un equivalente de retinol (RE) es equivalente a 1 µg de trans-retinol, 5 µg de trans β-caroteno, o 7 µg de otros carotenoides pro-vitamina A. Además, 671 UI de vitamina A equivalen a 1 mg^{-1} de betacaroteno y 436 UI de vitamina A = mg^{-1} de otros carotenoides comunes. La ecuación propuesta para los requerimientos diarios de vitamina A para el mantenimiento-crecimiento, de animales con un peso de 10-40 kg^{-1} y ganancias de peso diarias entre los 25 y 300 g^{-1} (NRC, 2007), es la siguiente:

- **A, RE kg-1 = 31,40 x PV**

Dónde:

PV = peso vivo del animal en kg^{-1}; RE = equivalentes de retinol. 1 RE = 1 µg de trans-retinol, 5 µg de trans β-caroteno, ó 7 µg de otros carotenoides pro-vitamina A. Además, 671 UI de vitamina A = mg^{-1} de β-caroteno y 436 UI de vitamina A = mg^{-1} de otros carotenoides comunes.

Ecuación para los requerimientos de vitamina A para crecimiento de cabras lecheras.

- **A, RE kg^{-1} = 100 x PV**

Dónde:

PV = peso vivo del animal en kg^{-1}; RE = equivalentes de retinol.

Cuadro 1. Requerimientos de vitamina A para el mantenimiento y suma de mantenimiento y ganancia de peso de cabras lecheras en crecimiento, expresados en RE d^{-1} y Betacaroteno mg d^{-1}, de acuerdo a la NRC, 2007.

Peso corporal (kg)	Ganancia diaria de peso (g)	Vit. A (RE d^{-1})	β-caroteno (mg d^{-1})
10	0 - 200	1 000	5
20	0 - 250	2 000	10
30	0 - 300	3 000	15
40	0 - 300	4 000	20

RE= equivalentes de retinol.

3.3.9. Betacaroteno como Antioxidante

Los radicales libres presentan un electrón desapareado de electrones o impar en el orbital externo, y se pueden formar cuando el oxígeno interactúa con ciertas moléculas. Algunos ROS son anión superóxido (O_2•), peróxido de hidrógeno (H_2O_2) e hidroxilo (OH•), otras especies reactivas importantes son el oxígeno molecular singlete ($^1O_2$), resultado de la excitación electrónica del estado basal del oxígeno molecular (triplete), y los radicales peróxidos, presentes en la peroxidación lipídica (Sies, 1997).

El estrés oxidativo es cuando la producción de estas sustancias prooxidantes supera a la capacidad de respuesta antioxidante del organismo. Si esto ocurre, las células pueden funcionar mal o morir. Para prevenir el daño de los radicales libres, el organismo tiene un sistema de defensa de antioxidantes (Barnham *et al.,* 2004).

Todas las células en los organismos eucariotas tienen enzimas antioxidantes que contrarrestan la generación de radicales libres. De estas defensas antioxidantes que posee la célula las tres clases principales de enzimas son, la superóxido dismutasa (SOD), la catalasa y la glutatión peroxidasa (GSH-px) (Sies, 1997).

Existen además una gran variedad de antioxidantes exógenos, entre los que destacan: la vitamina C o ácido ascórbico, la vitamina E, alfa tocoferol y los carotenoides, además, el selenio, un metal traza que se requiere para la función adecuada de uno de los sistemas de enzimas antioxidantes del organismo a veces se incluye en esta categoría. El organismo no puede fabricar estos micronutrientes, por lo que deben suministrarse en la dieta (Elejalde-Guerra, 2001).

Los carotenoides poseen especial habilidad para destruir directamente los radicales libres (Stahl y Sies, 2005). De hecho, estos pigmentos resultan particularmente eficientes a la hora de neutralizar los radicales peróxidos (ROO), los cuales están directamente relacionados con el mecanismo de peroxidación lipídica (Wang, 2006).

- **La oxidación lipídica no enzimática o peroxidación lipídica**

Una reacción química que generalmente tiene lugar a temperatura ambiente entre el oxígeno atmosférico y el compuesto orgánico generalmente se conoce como autoxidación. Una característica importante de la autoxidación es que es autocatalítica. La tasa de oxidación espontánea es lenta al principio y aumenta a medida que avanza la reacción (Halliwell, 1995). La autoxidación es una reacción en cadena de radicales libres que consiste en los pasos de iniciación, propagación y terminación (reacciones 1-4).

Iniciación: $X \bullet + RH \rightarrow R \bullet + XH$ (1)

Propagación: $R \bullet + O2 \rightarrow ROO \bullet$ (2)

$ROO \bullet + RH \rightarrow ROOH + R \bullet$ (3)

Terminación: ROO • + ROO • → [ROOOOR] → productos no radicales (4)

En la etapa de iniciación, los lípidos poliinsaturados (RH) pueden formar radicales alquilo (R •) que reaccionan muy rápidamente con el oxígeno para formar radicales peroxilo (ROO •). En la 3° etapa de propagación, una reacción en cadena con más lípidos produce hidroperóxidos (ROOH), es decir, productos de oxidación primaria. Estos hidroperóxidos se descomponen en presencia de metales para producir radicales alcoxilo (RO •), que se escinden en una mezcla compleja de aldehídos y otros productos, es decir, productos de oxidación secundaria (Frankel, 1984; Halliwell, 1995).

3.3.10. Respuesta a la suplementación de betacaroteno en animales

- **Las respuestas en la producción**

Se ha demostrado que los carotenoides tienen acciones biológicas independientes de la vitamina A (Arechiga *et al.,* 1988; Chew, 1995). La investigación de la Universidad de Florida indicó un aumento significativo en la producción de leche en vacas lecheras que reciben betacaroteno suplementario (Arechiga *et al.*, 1988). En este gran estudio de tres partes con manejo intensivo, las vacas suplementadas (400 mg/día) produjeron de 6.2 a 11.3 por ciento más de leche que los controles no suplementados. Además, para las vacas alimentadas con betacaroteno suplementario por 90 días, la tasa de preñez a los 120 días después del parto aumentó en (35.4% vs. 21.1%)

Algunos estudios han encontrado que la suplementación de betacaroteno afecta de manera positiva la producción de leche (Oldham *et al.,* 1990), al suplementar vacas con 300 mg kg peso-1 de betacaroteno, observaron un incremento en la producción de leche en un 6.4%. Del mismo modo ganado expuesto a estrés calórico complementado con 400 mg kg peso -1 de BC aumentó la producción láctea acumulada en un 11% (Arechiga *et al.*, 1988).

La vitamina A y el betacaroteno tienen un papel importante en la protección de los animales contra numerosas infecciones, incluida la mastitis. Los agentes patógenos potenciales están regularmente presentes en el orificio del pezón y, en circunstancias adecuadas, pueden invadir e iniciar la mastitis clínica. Cualquier estado insalubre del epitelio aumentaría la susceptibilidad de una glándula mamaria a la invasión de patógenos. Hay informes de mejoría de la salud mamaria en vacas lecheras suplementadas con betacaroteno y vitamina A durante los períodos secos y lactantes (Chew, 1995). Los neutrófilos polimorfo nucleares (PMN) son la línea principal de defensa contra las bacterias en la glándula mamaria. La suplementación con betacaroteno parece ejercer un efecto estabilizador sobre la función de PMN y linfocitos durante el período de secado (Chew, 1993). El betacaroteno aumentó la actividad bactericida de la sangre y la leche PMN, contra *S. aureus*, pero no afectó la fagocitosis. La vitamina A no tuvo efecto o suprimió la actividad bactericida y la fagocitosis. El control de los radicales libres es importante para la actividad bacteriana pero no para la fagocitosis. La actividad antioxidante de la vitamina A no es importante, no apaga ni elimina los radicales libres. El betacaroteno, por otro lado, tiene propiedades antioxidantes significativas y apaga eficazmente los radicales libres de oxígeno singlete (Elejalde-Guerra, 2001).

- **Inmunidad**

En gran parte debido al papel del betacaroteno como antioxidante, se puede incrementar la respuesta inmunitaria en el ganado lechero, reportaron que las vacas con baja concentración plasmática de vitamina A y betacaroteno tuvieron mayores puntajes en los exámenes de mastitis en la prueba California (Chew, 1993), al suplementar con 300 mg kg peso^{-1} de betacaroteno, 53 KUI vitamina A, 80 KUI vitamina A, 53 KUI vitamina A, o sin suplemento en los 30 días antes parto, los datos en porcentajes de vacas con un recuento de células somáticas (RCS) > 500.000 fueron 13, 27, 54 y 67%, respectivamente, lo que indica que el betacaroteno mostró un efecto positivo en la respuesta inmune.

Lucas (2005), suplementó con 300 mg^{-1} de betacaroteno, lo que resultó en la reducción numérica de RCS en el contenido de la leche, sin mejorar significativamente la producción de leche. Otros investigadores no han encontrado indicios de que el betacaroteno mejora la función inmune (Bindas 1984), encontraron que la suplementación de 600 mg kg-1 de BC por día no afectó el RCS, Mientras que Oldham *et al* (1990), no observaron disminución en la incidencia de mastitis al suplementar las vacas con betacaroteno, sin embargo, encontraron que cuando se produjo un incremento de 100 µg mL-1 de retinol en la concentración sérica durante la semana antes del parto, hubo una reducción del 60% en mastitis clínica en la lactancia temprana.

- **Parámetros reproductivos**

La suplementación de betacaroteno mejoró la tasa de concepción, la involución uterina y la ovulación, así como la reducción en la incidencia de quistes ováricos y muerte embrionaria temprana. Además, los niveles de betacaroteno en la dieta pueden estar relacionados con la fertilidad como se evidencia por el aumento de las concentraciones de BC en el ovario, sobre todo el cuerpo lúteo (Chew, 1995). Los beneficios de la suplementación con BC en vaca lechera puede estar relacionado con la conversión de la circulación de BC en vitamina A, específicamente en el útero y los ovarios (Schweigert, 2003).

Rakes *et al.* (1984), encontraron que la suplementación con 300 mg d-1 de BC disminuyeron los días al primer estro y el cuello uterino redujo su diámetro. El efecto de los suplementos de betacaroteno en la reproducción ha sido reportado en vacas con quistes ováricos las cuales presentaban bajas concentraciones plasmáticas de BC (11 ± 2 mg dL-1) con respecto a las vacas sin quistes ováricos (33 ± 4 mg dL-1). Asimismo, Graves-Hoagland *et al.* (1988), demostraron que los niveles de betacaroteno en plasma, se relacionaron positivamente con la síntesis de producción de progesterona en las células del cuerpo lúteo y determinaron que la concentración de betacaroteno en plasma estaba relacionado con la calidad del embrión en vacas superovuladas. Las concentraciones plasmáticas

de betacaroteno por encima de 200 mg dL-1 tendieron a aumentar el número de cuerpos lúteos y el total de embriones recuperados y de manera significativa mejorar el número de embriones transferibles normales.

Cuando el ganado vacuno se suplementó con 400 mg d^{-1} de BC, se incrementó la tasa de preñez de vacas con estrés calórico a los 120 y 90 días después del parto en 35.4% y 21.1% (Arechiga *et al.*, 1988). En el caso de las concentraciones de betacaroteno en plasma de vacas que ovularon durante la primera ola folicular posparto presentaron mayor media que vacas anovulatorias (2.97 ± 0.24 mg mL^{-1} ± 1.53 vs 0.14 mg mL^{-1} (Kawahsima, 2010). Este mismo grupo de investigadores suplementaron con 500 o 2,000 mg de betacaroteno por día durante el período seco, encontraron un aumento en el número de vacas ovulando en la primera ola folicular posparto (Kawahsima, 2010).

Estudios recientes sobre el efecto del BC en la actividad reproductiva en cabras muestran un efecto positivo sobre la función ovárica, en especial en las fases folicular, lútea, y en la síntesis de P4 (Arellano-Rodríguez *et al.,* 2007; Arellano-Rodríguez *et al.,* 2008). La suplementación en cabras adultas con 50 mg de BC por día durante un periodo de 51 días, bajo fotoperiodo natural, mostraron mayor número total de cuerpos lúteos (CLT) que el grupo control (3.4 *vs* 2.8), así como una mayor concentración sérica de progesterona, respecto del grupo control (5.6 vs 4.5 µg mL^{-1}) (Arellano-Rodríguez *et al.,* 2008).

4. ARTÍCULOS PUBLICADOS

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Short-term Betacarotene Supplementation Positively Affects Ovarian Follicular Development and Ovulation Rate in Goats

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Abstract

Arellano-Rodriguez, G., Meza-Herrera, C.A., Rodriguez-Martinez, R., Velazquez-Mendez, G., Mellado, M., Salinas, H., Perez-Razo, M.A. and Sanchez, F. 2007. Short-term betacarotene supplementation positively affects ovarian follicular development and ovulation rate in goats. J. Appl. Anim. Res., 32: 177-180.

This study evaluated the effect of short-term betacarotene supplementation in goats upon ovarian activity. Adult goats (n=22, 34 mo.) were randomly assigned to one of the two experimental groups: 1) Betacarotene (BETA, n=10) and 2) Control (CONT, n=12). Once estrually synchronized with P4-vaginal sponges, the BETA group received 50 mg of betacarotene on a daily basis during 35 d pre- and 17 d post-ovulation. Once ovulation occurred, towards the end of the late luteal phase (d 18), transrectal ultrasonographic scanning was performed in all animals to evaluate total follicles (FT), corpus luteum number (CLT) and total ovarian activity (TOA). The BETA group goats had higher ovarian activity (P=0.07). Results suggest that betacarotene supplementation positively affected ovarian activity in goat.

Key words: Goat, betacarotene, ovarian function, follicular growth, corpus luteum number.

Introduction

Ovarian activity and ovulation rate are influenced by nutrition status, supplementary feeding and body reserves (Williams *et al.*, 2001). Indeed, a short-term supplementation of both protein and energy can increase ovulation rate (Scaramuzzi *et al.*, 2006), indicating that ovarian response to nutritional supplementation is a tool that would allow to increase the reproductive efficiency for the benefit of goat farmers.

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In both herbivorous and omnivorous species, β-carotene is an important source of vitamin A, but besides this function as a systemic or local vitamin A source, β-carotene has attracted attention because of its possible importance in the reproductive performance of different species of farm animals (Schweigert *et al.*, 2001). There is increasing evidence that β-carotene may be necessary for optimal steroid production, possibly acting as an anti-oxidant (Arikan and Rodway, 2000). The aim of this experiment was to evaluate the betacarotene supplementation on reproductive efficiency as measured by total ovarian activity in goat.

Materials and Methods

Goats (n=22, 34 mo old, 46.01±5.05 kg, Criollox Alpine and Saanen) were fed on a diet based on alfalfa hay, corn silage and corn grain in order to meet 100% of their nutritional requirements adjusted to body weight (NRC, 1998). Goats received water, shades and mineral salts *ad libitum* during the experimental period (51 d). Goats were randomly distributed in two experimental groups: 1) BETA (n=10, BW=44.8±1.45 kg), with daily supplementation of betacarotene (50 mg goat^{-1}, Syntex SA. de CV.) and 2) CONTROL, without supplementation (CONT, n=12; BW=45.3±1.32 kg).

Goats were estrually synchronized with intravaginal sponges impregnated with progesterone (Intervet International B. V., Boxmeer-Holland, Intervet, S. A., France). Sponges were withdrawn nine days after the insertion of the sponges and goats were injected with cloprostenol IM (Prosolvin C®, Intervet International B. V., Boxmeer-Holland), a prostaglandin $F_2\alpha$ analog, 1.0 ml per animal (0.075 mg goat^{-1}), to provoke corpus luteum regression and to cause, in turn, follicular development and ovulation.

On day 17 after the second ovulation, ovarian activity was evaluated by ultrasonographic scanning (Toshiba Medical Systems, Ltd, Crawley, UK) with a 7.5 Mhz linear transductor for veterinarian use and according to the transrectal ultrasonographic method suggested by Griffin and Ginther, (1992) and Dickie *et al.* (1999). The number and diameter of ovarian structures (follicle and corpus luteum) were recorded and photographed.

Data were subjected to analyses of variance (ANOVA) in a completely randomised design (Morris, 1999). The PDIFF option was used to evaluate least square mean differences between experimental groups by the LSMEAN procedure of the PROC GLM. All the statistical analyses were performed using the SAS Program (Littell *et al.*, 1991; 1998). The reported values are least square means ± standard error.

Results and Discussion

The results observed in the present study (Table 1) confirm a positive betacarotene effect on the main ovarian cellular groups, either in their luteal or follicular phase and coincide with the observed increased corpus luteum number as well as the increased total ovarian activity in those goats supplemented with betacarotene. According to Cassano *et al.* (1999), oxidative damage to the oocyte or

Table 1
Least square means for body weight (BW), body condition score (BCS), total follicles (FT), total corpus luteum (CLT) and total ovarian activity (TOA=FT+CLT) in goats supplemented with betacorotene (BETA, 50 mg goat^{-1} day^{-1}) and no supplemented goats (CONTROL)

	BETA	CONTROL	SE[1]
BW, kg	44.8[a]	45.3[a]	1.32
BCS, units	3.2[a]	3.3[a]	0.06
FT, units	4.6[a]	3.5[b]	0.52
CLT, units	3.4[a]	2.8[b]	0.22
TOA, units	8.0[a]	6.3[b]	0.57

[1]SE, most conservative LSM-standard error.
[a,b]Means followed by different superscripts differ (P<0.07).

follicular cells were suggested to trigger atresia. As mentioned by Schweigert *et al.* (2003), carotenoids are transferred into follicular fluid where they might serve as antioxidants and (or) precursors of retinoids which, in turn, modulate follicular or oocyte functions.

In fact, betacarotene acts against lipid peroxidation as an antioxidant molecule protecting several reproductive structures from oxidative DNA-damage (Kim *et al.*, 2007). Betacarotene seems to act as an ovarian-protective molecule, not only as luteoprotective (Haliloglu *et al.*, 2002) but also improving embryo production and quality in superovulated cows (Sales *et al.*, 2007), optimizing reproductive performance in females. Betacarotene up-take by the ovary and uterine tissues increases in a dose-dependent manner, enhancing the steroidogenic activity at ovarian level, promoting an optimal ovarian function and a better uterine environment for embryonic survival and development (Chew *et al.*, 2001). Further studies should elucidate if the positive effect of betacatorene supplementation prior to mating upon ovarian activity, is associated with changes in the hormonal profile of gonadotrophic hormones like LH or FSH and(or) other metabolic hormones like insulin, growth hormone or insulin-like growth factors.

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जी. अरेल्लानो-रोद्रीग्वेज, सी.ए. मेजा-हर्रेरा, आर. रोद्रीग्वेज-मर्टिनेज, जी.वेलाज्क्वेज- मेन्डेज, एम.मेल्लाडो, एच. सालिनास, एम.ए.पेरेज-राजो, एफ.सैन्चेज। बकरियों में बीटा-कैरोटिन के अल्प अवधि पूरक का बकरियों में डिम्बीय फालिकिल विकास और डिम्बोत्सर्ग पर सकारात्मक प्रभाव होता है।

इस अध्ययन से बकरियों की अंडाशयी क्रियाशीलता पर अल्पावधि बीटा-कैरोटीन पूरक के प्रभाव का अध्ययन किया गया। वाइस प्रौढ़ बकरियों (3-4 माह आयु) को यादृच्छिक विधि से 10 और 12 के दो वर्गों में बांट कर क्रमशः बीटा-कैरोटिनयुक्त (बीटा) या बीटा कैरोटिनहीन (नियंत्रक) आहार खिलाया गया। पी 4 योनीय स्पंज से एक बार रतिजीय तुल्यकालन के बाद डिम्बोत्सर्ग से 35 दिन पूर्व और 17 दिन पश्चात् तक प्रतिदिन 50 मि ग्रा बीटा कैरोटिन दिया गया। विलम्बित ल्युटिअसी समय में (18वें दिन) एक बार डिम्बोत्सर्ग के बाद सभी पशुओं में सकल फालिकिल, कार्पस ल्यूटियम की संख्या और सकल अंडकोषी क्रियाशीलता जानने के लिए मलाशय पथ से अल्ट्रासोनोग्राफी क्रमवीक्षण किया गया। बीटा वर्ग की बकरियों में सार्थकतः अधिक डिम्बग्रंथीय क्रियाशीलता थी। परिणाम दर्शाते हैं कि बीटा कैरोटिन पूरक से बकरियों के अंडकोषीय क्रियाशीलता पर सार्थक प्रभाव पड़ता है।

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ORIGINAL ARTICLE

Short-term intake of β-carotene-supplemented diets enhances ovarian function and progesterone synthesis in goats

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Summary

The effect of β-carotene supplementation upon luteal activity, measured as number (CLT) and volume (VLT) of corpus luteum, and P4 synthesis in goats, was evaluated. Goats ($n = 22$, 34 months) were randomly assigned to one of two experimental groups: (i) β-carotene [Beta, $n = 10$; body weight (BW = 44.8 ± 1.45 kg), body condition score (BCS = 3.25 ± 0.07)], and (ii) Control (Control, $n = 12$; BW = 45.30 ± 1.32 kg, BCS = 3.33 ± 0.06). Upon oestrus synchronization, the Beta group received 50 mg of β-carotene per day during 35 days pre- and 17 days post-ovulation. The day 4, 8, 12 and 16 post-ovulation, blood samples were collected for quantification of serum P4 concentrations by radioimmunoassay, and transrectal ultrasonographic scanning was performed at day 18 for evaluating CLT and VLT. Overall, CLT and VLT mean were 3.10 and 2211.1 mm^3 respectively. The Beta-goats depicted both the largest values for CLT ($p = 0.07$) and serum P4 levels ($p = 0.05$), with no differences ($p = 0.53$) for VLT between treatments. Results suggest a higher efficiency within the cellular-enzymatic groups defining the steroidogenic pathways in the β-carotene-supplemented goats, generating a larger P4 synthesis. The last is essential for ovulation of healthy oocytes, maintenance of uterine quiescence, nourishment and survival of the embryo around implantation; all of them of paramount significance during the maternal recognition of pregnancy process.

Introduction

In ruminants, an inadequate nutrition is characterized by deficient metabolic and endocrine profiles which can compromise reproductive performance, causing retarded puberty onset, irregular oestrous cycles and a drop in the global reproductive efficiency (Popwell et al., 1996; Scaramuzzi et al., 2006; Meza-Herrera et al., 2007). Besides, maternal nutritional and metabolic environment are also critical for compromising embryo and fetal viability (Maloney and Ress, 2005; Meza-Herrera et al., 2006) and long-term health and viability of the offspring (Symonds et al., 2007).

An important aspect that defines reproductive efficiency in ruminants is the process of maternal recognition of pregnancy (Spencer et al., 2007), which involves the establishment of a dialogue between the fetus and its mother in order to assure the synthesis and release of progesterone (P4) (Roberts

et al., 1996). In goats, a successful pregnancy depends on an adequate P4 secretion by corpus luteum; in turn, luteal function depends on an optimum balance of luteotropic (i.e. LH) and luteolytic factors (i.e. PGF2α) (Ford et al., 1996). In mammals, luteal steroidogenesis affects the function of several organs such as the uterus, ovary, mammary gland and brain; thus, P4 secretion is recognized as a key element in the normal regulation of female reproductive function (Dinny and Christine, 1999).

Carotenoids, on the other hand, are important both to humans and animals as precursors of vitamin A and retinoid (Chew et al., 1993; Hattori et al., 2000; Schweigert et al., 2003). β-Carotene, which is present in green plants but can be also chemically synthesized, has shown to have a key role in a wide range of biological processes (Schweigert, 1998). In fact, β-carotene is not only a retinol precursor, but also has gained consideration because of it functions similarly to vitamin E by acting as scavenger of free radicals, especially singlet oxygen, and, thus, as a potent antioxidant such as other carotenoids (Chew et al., 1993; Sies and Stahl, 1995; Greiwe-Crandell et al., 1997).

In addition, many gene products linked to reproduction are known to be modulated by retinoic acid, the product of retinol oxidation (Schweigert et al., 2003). Therefore, an optimal intake of β-carotene is hypothesized to have a positive effect on fertility (Arechiga et al., 1998). Most of the studies have been performed in animals, but results are contradictory; some authors reported negative effects (Folman et al., 1987), whilst others described an increased fertility (Arechiga et al., 1998). Most recent studies have shown a direct relationship between β-carotene concentration at ovarian level and size and P4 secretion by corpora lutea (Halilogˇlu et al., 2002), suggesting that β-carotene may have a positive effect in luteogenesis and luteal activity.

The purpose of this study was to evaluate whether the positive effects of β-carotene on the luteal function can be induced by exogenous supply. Thus, the effects of a short-term β-carotene nutritional supplementation on the number of corpora lutea (CLT), the total luteal volume (VLT) and the secretion of P4 were evaluated in goats.

Materials and methods

Institutional approval, animals and their feeding

The study was carried out at the Regional University Unit on Arid Lands of the Chapingo Autonomous University (URUZA-UACH), at the Southern Goat Research Unit (26°NL, 103°WL, 1117 m), during November and December. Climatic characteristics for the area are warm-dry climate and mean annual precipitation and temperature of 217.1 mm and 22.3 °C respectively. The warmest month is June, with temperatures above 40 °C whilst the coldest month is January, with the lowest temperature below 0 °C.

Experimental groups were prepared during October, under a natural short-day photoperiodic scheme. Goats (n = 22, 34 months; Criollo × Alpine-Saanen), having a mean body weight (BW) of 46.01 ± 5.05 kg were fed with a diet based on alfalfa hay, corn silage and corn grain to fit 100% of their nutritional requirements adjusted to BW (NRC, 1988). Goats had free access to water, shades and mineral salts during the whole experimental period (51 days). The experimental groups were fed twice per day, with alfalfa hay and corn silage by the morning (07:00 hours), and corn grain by the afternoon (18:00 hours); always under natural light conditions. All the methods used in this study were in accordance with accepted international guidelines (FASS, 1999).

Experimental design and treatment groups

Goats were randomly distributed in two experimental groups: (i) Beta (n = 10, BW = 44.8 ± 1.45 kg), supplemented with β-carotene (50 mg per goat per day) (Syntex Mexico, SA. de CV.), and (ii) Control, without supplementation (n = 12; BW = 45.3 ± 1.32 kg). Goats were fed with the basal diet during the whole experimental period, which consisted of 34 days before and 17 days after ovulation, from October to December, under a decreasing photoperiodic scheme.

Oestrus synchronization, blood sampling and P4 quantification

Oestrus was synchronized by using intravaginal sponges containing 45 mg of fluorogestone acetate (Chronogest®; Intervet International B. V., Boxmeer, the Netherlands) for 10 days and a single i.m. dose of 1 ml of a prostaglandin $F_{2\alpha}$ analogue (0.075 mg per goat of cloprostenol; Prosolvin-C®; Intervet International B. V.).

During the cycle following the synchronized ovulation, at days 4, 8, 12 and 16, blood samples were collected by jugular venopuncture with 0.8 × 38-mm hypodermic sterile needles (PrecisionGlide™; Becton Dickson, Franklin Lakes, NJ, USA) and 10-ml sterile vacutainer serum tubes (Becton Dickson).

Samples were allowed to clot during 30 min, and then centrifuged (1500 *g*, 15 min). Serum samples were collected in 1.5-ml polypropylene tubes (Axygen® Scientific, Union City, CA, USA) and stored at −20 °C until assayed.

In total, four samples per goat were collected (40 Beta) and 44 (Control). Serum P4 concentrations were determined in duplicate by radioimmunoassay (RIA) by using a solid-phase commercial RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA), according to the procedures described by Schneider and Hallford (1996). Intra-assay coefficient of variation was 7.5% and detection limit reached 30 pg ml^{-1}. Hormonal assays were made at the Endocrinology Laboratory of the Animal Science Department of the New Mexico State University, Las Cruces, NM, USA.

Ultrasonographic analysis of ovarian activity

To evaluate the luteal function on day 18 after the second ovulation, ultrasonographic scanning was performed by one skilled operator with a 7.5 MHz linear-array transducer for veterinarian use (Toshiba Medical Systems, Crawley, UK). The number of corpus luteum observed in each ovarian structure was recorded and measured according to the procedures outlined by Dickie et al. (1999). Ovaries were visualized at an image magnification of 1.5×, and number and size of all follicles >2 mm in diameter were registered. Ultrasonographic images were also recorded on videotapes for retrospective analysis. Once obtained the largest and lowest diameters, VLT was calculated by using the following formula: $VLT = (4/3\pi \times R^3) \times CLT$, where R is the mean ratio and CLT is the number of corpora lutea.

Statistical analyses

Body weight, body condition score (BCS), CLT and VLT were evaluated by analyses of variance (ANOVA), by using a completely random design with two treatments and 10–12 replicates by treatment (Snedecor and Cochran, 1967). Serum P4 levels were analysed by ANOVA in a completely random design with a split-plot arrangement of treatments for repeated measures across time (Littell et al., 1998). Treatment effect was included in the main plot, using the goat term within treatment to calculate the error. Sampling time and treatment by time interactions were included in the subplot and were tested using the residual mean square. When significant *F* values were observed, mean separation considered LSMEANS-PDIFF option of the PROC GLM. All statistical analyses considered the use of the SAS software (Littell et al., 1991). Reported values are defined as least square means ± SE.

Results

Least square means for BW (kg), BCS (units), CLT, VLT (mm^3) and P4-serum level (P4, ng ml^{-1}) are shown in Table 1. The overall CLT mean was 3.0 ± 0.2, with the Beta group having the largest CLT number ($p = 0.07$) in relation to the Control group. Overall, VLT mean was 2.162 ± 543 mm^3, without differences between groups ($p = 0.53$) for this variable. Overall mean serum P4 levels were 4.8 ± 0.3 ng ml^{-1}, with the Beta group bearing higher serum P4 concentrations ($p = 0.05$) than the Control group. Serum P4 levels across time, showed sustained increases ($p = 0.04$) across sampling times (Table 2). In fact, P4 levels increased from day 4 to 8, and showed a slight decrease at day 16; this P4 secretion pattern being similar between and within treatments.

Table 1 Least square means for body weight (BW), body condition score (BCS), total corpus luteum number (CLT), total luteal volume (VLT) and serum progesterone concentration (P4) in goats supplemented with β-carotene (Beta, 50 mg per goat per day) and non-supplemented goats (Control) in northern Mexico (26°NL)

	Beta	Control	SE*	OSL†
BW (kg)	44.8[a]	45.3[a]	1.32	0.83
BCS (units)	3.2[b]	3.3[a]	0.06	0.42
CLT (units)	3.4[a]	2.8[b]	0.22	0.07
VLT (mm^3)	1978.2[b]	2444.0[a]	501.1	0.53
P4 (ng ml^{-1})	5.6[a]	4.5[b]	0.37	0.05

*Most conservative LSM-standard error.
†Observed significance level.
[a,b]Different superscripts denote statistically different values within variables ($p \leq 0.07$).

Table 2 Least square means for progesterone serum concentration (P4, ng ml^{-1}) on days 4, 8, 12 and 16 post-ovulation during November-December, in goats supplemented with β-carotene (Beta, 50 mg per goat per day), and non-supplemented goats (Control) in northern Mexico (26°NL)

	Days after ovulation					
	4	8	12	16	SE*	OSL†
P4 (global)	2.0	5.2	6.7	5.5	0.4	0.04
P4 Beta	1.6	6.0	7.2	6.9	0.7	0.03
P4 Control	2.5	4.0	5.9	5.5	0.6	0.001

*SE, most conservative LSM-standard error.
†OSL, Observed significance level.

Discussion

Current results give evidences for a positive effect of β-carotene supplementation on the luteal function, in terms of P4 secretion, during the early luteal phase; these results can be extrapolated to the pre-implantational period in pregnant females. This fact suggests a better efficiency in the metabolic performance by luteal cells in the β-carotene-supplemented group. From a functional point of view, it is possible to hypothesize two mechanisms of action. First, this effect may be related to possible effects on LH stimulation via the protein kinase A (PK-A) second messenger system. Indeed, in the cytoplasmatic membrane, LH links to its receptor activating PK-A, and as a consequence, the cytochrome P450 enzyme transforms cholesterol into pregnenolone, which in turn is converted in the endoplasmic reticulum to P4 by means of $3\beta HSD/\Delta^5$, Δ^4 isomerase enzyme (Niswender et al., 2000).

On the other hand, β-carotene, acting as an antioxidant, may improve the ability of luteal cells to synthesize P4 in a scenario rich in free oxygen (Tilly, 1996). During steroidogenesis, the mitochondrial P450 system has shown to produce oxygen radicals, such as cytochrome P450 and adrenoxin, which can provoke irreversible damage to the molecules in the surrounding environment. The mitochondrial system that first catalyses P450 and the proportion that limits the steroidogenesis process is highly expressed in the corpus luteum, which is also characterized by their high antioxidant level (Rapoport et al., 1998). Antioxidants play an important role in protecting the cell from free oxygen radicals, which are harmful during steroidogenesis. Reinforcing this hypothesis, free oxygen radicals can be involved in both luteolysis and apoptosis of luteal cells during the reproductive cycle (Riley and Behrman, 1991).

According to our results, β-carotene supplementation would increase not only the ovulation rate but also the P4 synthesis. Thus, as a precursor of vitamin A, β-carotene has gained specific interest; vitamin A strongly affects cellular development, differentiation and morphogenesis through retinoic acid. In turn, by interacting with nuclear receptors, retinoic acid can influence the activation of several genes as well as the expression of numerous proteins (Zile, 1998).

The observed P4 increments in our study are similar to those reported by Weng et al. (2000), who mentioned increase in P4 levels due to β-carotene supplementation, concluding that β-carotene supplementation affected plasma P4 concentration without effects on 17β-oestradiol concentration and on total of uterine protein. In other studies, however, β-carotene was found to have no effect on the plasma P4 levels: in gilts on day 12 of gestation (Schweigert et al., 2002), in dairy cattle receiving oral administration of β-carotene (Chew et al., 1982) as well as after parenteral β-carotene administration in sows (Coffey and Britt, 1993).

In both experimental groups, serum P4 levels reached their highest value at day 12; without differences between treatments with regards to the levels of P4 secretion across time. Both dynamic secretion and synthesis of P4 in our trial coincide with the P4 secretion dynamics during the luteal phase reported by Niswender et al. (2000). No significant correlations among corpora lutea number and P4 synthesis were observed in this study. On this respect, Halilloglu et al. (2002) reported that supplemental β-carotene levels were correlated ($p < 0.05$) with CL diameter, observing a positive correlation between P4 and corpus luteum diameter. As no differences were observed between treatments for the total volume of luteal tissue, a possible explanation of our results could be related to a better enzymatic efficiency in the Beta group, which, in turn, could be related to an enhanced P4 synthesis.

During steroidogenesis, the mitochondrial P450 system has shown to produce oxygen radicals – such as cytochrome P450 and adrenoxin – that can provoke irreversible damage to the molecules in the surrounding environment. The mitochondrial system that first catalyses P450 and the proportion that limits the steroidogenesis process are expressed in higher concentrations in both suprarenal cortex and corpus luteum. These steroidogenic tissues have been characterized by their higher antioxidant levels (Rapoport et al., 1998). It is possible to hypothesize that this physiological scenario could have been presented by the Beta group, generating a better efficiency in the steroidogenic pathway and, in turn, a better level of P4 synthesis.

Antioxidants play an important role protecting the cell from free oxygen radicals, which are harmful during steroidogenesis. Free oxygen radicals can be, from a functional point of view, involved in the conduction of both luteolysis process and apoptosis of luteal cells during each reproductive cycle (Riley and Behrman, 1991). Results of this study suggest that short-term β-carotene supplementation can enhance the synthesis and secretion of P4 by luteal tissue. The last may be of significant importance both from a biological and economic stand-point. Particularly with respect to the maternal recognition of

pregnancy process, promoting a higher efficiency during embryo implantation process.

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Original Research Article

Betacarotene supplementation increases ovulation rate without an increment in LH secretion in cyclic goats

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ABSTRACT

This study aimed to evaluate the effects of betacarotene (BC) supplementation on ovulation rate (OR) and luteinizing hormone (LH) secretion in adult goats during the breeding season. Additionally, total ovarian activity (TOA) comprising the total number of ultrasonographically detectable antral follicles (AF) and corpora lutea (OR) was also assessed. In early October, adult goats [n = 22, 3.5 years of age, 7/8 Sannen-Alpine; 26°N, 103°W at 1117 m.a.s.l.] were randomly assigned to: (i) BC group (BCG), orally supplemented with 50 mg of BC/goat/day [n = 10; live weight (LW) = 45.9 ± 2.0 kg, body condition score (BCS; range: 0-emaciated to 5-obese) = 3.0 ± 0.1], and (ii) control group (CONT) [n = 12; LW = 46.2 ± 2.0 kg, BCS = 3.0 ± 0.1]. All animals received a basal diet of alfalfa hay, corn silage and corn grain, with free access to water and mineral salts. The whole experimental period spanned 34 days before and 17 days after ovulation. On day 23 of the experiment, estrus was synchronized with progestin-releasing intravaginal sponges; 36 h prior to estrus, an intensive blood sampling (every 15 min for 6 h) was performed to determine mean LH concentrations, pulsatility (LH-PULSE) and area under the curve (LH-AUC) for serial LH concentrations. Afterwards, by the end of the luteal phase (i.e., 17 days after the onset of estrus), an ultrasonographic scanning was performed to evaluate OR and TOA [AF + OR]. The average LW and BCS did not differ ($p > 0.05$) during the experimental period. BC-supplemented goats showed an increase in OR (3.4 ± 0.2 versus 2.8 ± 0.2; $p < 0.05$) and exhibited lower ($p < 0.05$) serum LH concentrations, LH-AUC and LH-PULSE compared to CONT. A positive correlation was recorded between OR and LW ($r^2 = 0.42$, $p < 0.05$) and BCS ($r^2 = 0.47$, $p < 0.05$). In addition, AF (5.0 ± 0.6 versus 3.4 ± 0.6) and TOA (8.4 ± 0.6 versus 6.2 ± 0.6) were greater ($p < 0.05$) in the BC-supplemented group than CONT. Supplementation with BC enhanced ovarian follicular development and ovulation rate in adult female goats under decreased photoperiods through LHRH-independant pathways or direct effects of BC on ovarian function.

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1. Introduction

One of the major functions of the ovary is to produce mature oocytes, a process that depends on an extremely well coordinated interactions among several components of the ovarian follicle, i.e., the oocyte, theca and granulosa cells [1–3]. Follicular growth and oocyte maturation are primarily dependent upon the hypopyseal gonadotropins (FSH and LH) as well as some other, intraovarian factors (e.g., IGF-1) [2–4]. The function of the hypothalamic-pituitary-gonadal (HPG) axis is modulated by an array of neuronal inputs, governed by either photoperiodic or thermoperiodic cues, which ultimately modulate the neuronal activity of the GnRH-releasing terminals in the hypothalamus and also have an impact on energy homeostasis. These cues provide the basis for the environmental control of the: (1) onset of reproductive function at puberty; (2) recurrent reproductive cycles observed in adult females of non-seasonal breeders; and (3) intermittent reproductive cycles observed in seasonally breeding species [5,6]. In all of the above scenarios, nutritional status and/or nutritional supplementation strongly affect the functioning of the HPG axis [4,7–10].

Betacarotene (BC), a bioactive component of green plants, is a precursor of vitamin A and retinoids, while an important chemical for both the human beings and animals [11,12]. BC has been involved in multiple actions at cellular and tissue levels that promote key events in an ample range of biological processes [13,14]. In addition to being a precursor of retinol, BC exerts biological effects similar to those of vitamin E by acting as a potent scavenger of free radicals, especially the singlet-state oxygen, and therefore as a potent antioxidant, just as other carotenoids [11–14]. An optimal BC intake has been shown to have positive effects on ruminant reproductive outcomes [15], although there have been contradictory reports of either negative effects [16], a lack of effect [17], or positive effects of BC on reproductive [18–20] and metabolic [21] processes. Even though many gene products linked to reproductive performance are known to be modulated by retinoic acid, the product of retinol oxidation [12,14], some studies have proposed that BC may act independently of vitamin A, particularly in increasing both follicular and luteal steroidogenesis in ruminant species [19,20,22,23]. Despite the positive action of BC on ovarian steroidogenesis, studies of the possible role of BC on LH secretion and ovarian function are scarce. This study examined the effect of BC supplementation on ovulation rate and pulsatile LH secretion in adult female goats.

2. Materials and methods

2.1. Location, environmental conditions and animal management

The present study was carried out at the Regional University Unit on Arid Lands, Chapingo Autonomous University (URUZA-UACH), the Southern Goat Research Unit (latitude: 26°N, longitude: 103°W; 1117 m.a.s.l.). Adult goats [$n = 22$, mean live weight (LW) = 45.35 ± 1.35 kg, age: 3.5 years, 7/8 Sannen-Alpine] were employed in this study conducted under short-day photoperiodic conditions during October and November (i.e., natural breeding season at 26°N). Both the LW and body condition score (BCS) were recorded weekly prior to feeding. BCS was determined on a five point scale (from 1 = emaciated to 5 = obese) [24] by an experienced technician. All the methods used in this study were in accordance with accepted international guidelines [25].

2.2. Experimental design and treatment groups

In early October, goats were randomly placed in individual pens and allocated to two experimental groups: (1) betacarotene group (BCG; $n = 10$, LW = 45.9 ± 2.0 kg, BCS = 3.0 ± 0.1) and (2) control group (CONT; $n = 12$; LW = 46.2 ± 2.0 kg, BCS = 3.0 ± 0.1), with no differences ($p > 0.05$) for LW and BCS between experimental groups. Goats in BCG, were orally supplemented with betacarotene (50 mg/goat/day, mixed with mineral salts) (Syntex-Roche de Mexico; Guadalajara, Jalisco, Mexico) during the entire experimental period, which lasted from 34 days before estrus to 17 days post-estrus. Both groups received a basal diet of alfalfa hay (14% crude protein (CP), 4.7 net energy for maintenance (NEm) MJ/kg), corn silage (8.1% CP, 6.7 NEm MJ/kg) and corn grain (11.2% CP, 9.9 NEm MJ/kg) in a mixed-ration twice a day (0700 and 1600; 1 kg/goat/day) formulated to cover their nutritional requirements [26]. Animals had free access to water, shaded areas and mineral salts. Composition values of the ingredients of the basal diets as dry matter basis (DM%) were obtained from representative samples taken throughout the experimental period and analyzed according the previously described procedures [27] (Table 1).

2.3. Estrous synchronization, blood sampling, and LH measurements

On day 23 of the experimental period, estrus was synchronized with intravaginal sponges containing 45 mg of fluorogestone acetate (Chronogest®; Intervet International B.V., Boxmeer, Holland) left in place for 10 days; 9 days after insertion of the

Table 1 – Chemical composition of alfalfa hay, corn silage and corn grain samples which conformed the basal diet of adult crossbred goats ($n = 22$) under natural photoperiodic conditions (October–November, 26°N).[a]

Item	Alfalfa hay (%)	Corn silage (%)	Corn grain (%)
Nutrient composition[b]			
Dry matter[c]	92.0	35.8	86.3
Crude protein[c]	15.8	8.5	9.5
Neutral detergent fiber[c]	59.9	40.6	9.9
Acid detergent fiber[c]	42.1	25.0	4.0

[a] Mineral block offered ad libitum contained (%, w/w): NaCl 95; Fe 0.2; Cu 0.033; I 0.007; Zn 0.005; Co 0.0025.
[b] Composition values (% of diet DM) represent values from five samples taken throughout the experimental period. Samples dried in a forced air stove at 60 °C until constant weight.
[c] Determined according to the procedures outlined by AOAC (1990).

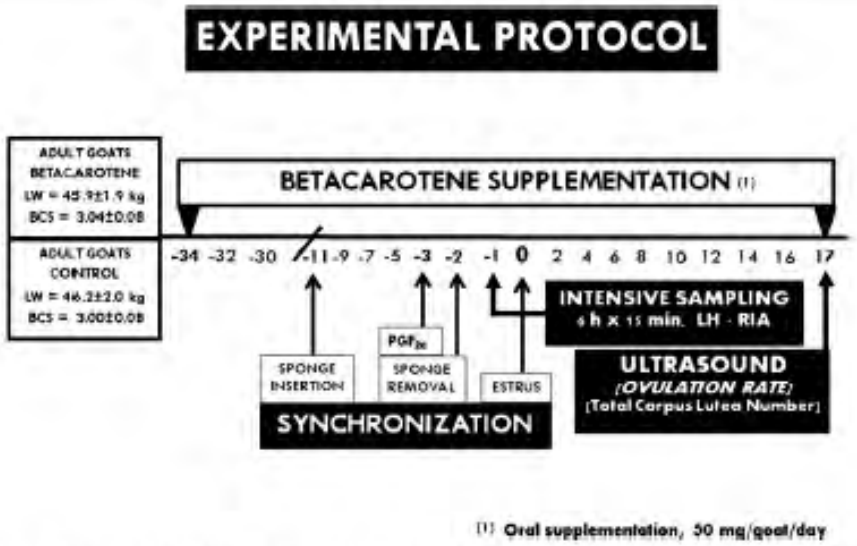

Fig. 1 – A schematic representation of the experimental protocol, including the duration of long-term betacarotene supplementation and estrous synchronization. An intensive blood sampling (every 15 min for 6 h) for LH measurements was performed 36 h prior to the estrus day (day 0). Thereafter, an ultrasonographic scanning was performed on day 17 post-estrus to relate the LH secretion pattern and ovulation rate (OR), measured as number of corpora lutea present in each ovary. Additionally, the antral follicle number (AF) was also recorded to define total ovarian activity (TOA = OR + AF) on day 17 post-estrus, in adult crossbred goats (n = 22; 45.3 ± 1.3 kg), supplemented with betacarotene or serving as controls and kept under natural photoperiodic conditions (October–November, 26° N).

sponges (day −3; day 0 = estrus), goats received a single i.m. dose of 1 ml of prostaglandin $F_{2\alpha}$ analog (0.075 mg of d-cloprostenol/goat; Prosolvin-C®, Intervet International B.V.; Boxmeer, Holland). Thereafter, on day −2, sponges were removed and 24 h later (day −1) five goats from each group were randomly selected to undergo an intensive blood sampling. Blood samples (10 ml) were collected every 15 min for 6 h, starting 3 h after the morning feeding, by jugular venipuncture into sterile vacuum tubes (Corvac; Kendall Health Care, St. Louis, MO, USA) and allowed to clot at room temperature for 30 min. Serum was separated by centrifugation (1500 × g, 15 min), decanted and transferred to polypropylene microtubes (Axygen Scientific; Union City, CA, USA) for storage at −20 °C until hormonal analysis. Peripheral serum LH concentrations were determined in duplicate in a single radioimmunoassay (RIA) [28]. The value of an intra-assay coefficient of variation (CV) for LH quantification was 10% and the assay detection limit was 0.2 ng/ml. The area under the curve (LH-AUC) was calculated using a trapezoidal summation procedure, while the pulsatility of LH (LH-PULSE) was determined using a 10% CV, 0.95 standard deviation (SD), and a detection limit of 0.2 ng/ml [29]. A detailed scheme of the experiment is shown in Fig. 1.

2.4. Ultrasonographic monitoring of ovarian activity

To evaluate ovarian activity, on day 17 post-estrus, by the end of the luteal phase in early November, an ultrasonographic scanning was performed by an experienced operator using a 7.5-MHz linear-array transducer (Toshiba Medical Systems Ltd.; Crawley, UK). The total number of corpora lutea in both ovaries (OR) and the number of all visible antral follicles (AF) were recorded, and the structures were measured [30]. Lastly, the total ovarian activity (TOA) was defined as the sum of AF and OR recorded in each animal.

2.5. Statistical analysis

The ovarian variables and LH-AUC were compared by analysis of variance, in a completely random design; due to the non-parametric nature of LH pulse frequency, LH-PULSE was analyzed using the Kruskal–Wallis test. Live weight and body condition score of animals as well as serum LH concentrations over time were analyzed by split-plot analysis of variance for repeated measures [31]. The models included treatment in the main plot, which was tested using animal within treatment as the error term. Time and the time × treatment interaction were included in the subplot and were tested by the residual mean square [32]. When significant F values were observed, mean separation was done using the LSMEANS-PDIFF option of the PROC GLM. All statistical analyses were computed using the GLM procedures of SAS (SAS Inst. Inc. V9.1; Cary, NC, USA). Pearson's correlations were used to evaluate the associations among LW, BCS and the number of luteal structures. All results are expressed as least-square means ± SE; the most conservative SE is presented.

3. Results

The general average live weight (LW) and body condition score (BCS) for both groups of animals at the beginning or end of the study period were 45.3 ± 1.4 kg and 3.0 ± 0.1 or 45.0 ± 1.4 kg and 3.3 ± 0.1, respectively. There were no differences for both parameters either at the beginning ($p < 0.05$) or during the entire ($p > 0.05$) experimental period. Nonetheless, while the BC-supplemented goats showed an increase in ovulation rate (3.4 ± 0.2 versus 2.8 ± 0.2; $p < 0.05$), they had lower serum mean LH concentrations (1.7 ± 0.8 ng/ml versus 5.3 ± 0.8 ng/ml; $p < 0.01$), area under the curve (LH-AUC; 642 ± 316 versus 1925 ± 316; $p < 0.01$), and pulse frequency (LH-PULSE; 2.2 ± 0.5 pulses/6 h versus 3.8 ± 0.5 pulses/6 h; $p < 0.05$) as compared with control goats. Least-square means for serum LH concentrations over time according experimental group as well as serum LH concentrations from representative goats in each experimental group are depicted in Fig. 2. A positive correlation between OR and LW ($r^2 = 0.42$; $p < 0.05$) and BCS ($r^2 = 0.47$, $p < 0.05$) in all ewes studied was recorded. Additionally, number of antral follicles (5.0 ± 0.6 versus 3.4 ± 0.6, $p < 0.05$) and TOA (8.4 ± 0.6 versus 6.2 ± 0.6, $p < 0.05$) were greater in the BC-supplemented compared with control group.

4. Discussion

Nutritional supplementation may modulate the functioning of hormonal systems, regulate the seasonal shifts in reproduction, and impinge on the ovulation rate and fertility in domestic livestock. Therefore, nutritional supplementation may be an alternative to hormonal treatments used to increase reproductive efficiency. Certainly, positive effects of nutrition upon reproductive yields are commonly obtained through increases in body weight of animals, both in the long-term ("static effect") and short-term ("dynamic effect" of an increased feeding over 3–4 weeks before mating); heavier females have greater ovulation rates as compared to their lighter counterparts. In addition, reproductive efficiency can be enhanced by supplying nutritional boost in a very short time period (i.e., less than 10 days), without any effects on body weight; this phenomenon is referred to as "immediate nutrient effect", "acute effect" or "focus feeding" [1–3,5,6,33,34].

The results of the present study partially support our working hypothesis since betacarotene (BC) supplementation increased the total number of antral ovarian follicles and ovulation rate in cyclic goats. However, there was no change in LH secretion in the BC-supplemented goats of the present study. According to our experimental protocol, BC supplementation up to the day of estrus should yield the dynamic effect upon LH secretion and ovulation rates. Additionally, since BC-supplementation continued up to day 17 post-estrus, the effect of the present dietary supplementation on antral follicle numbers can be considered an acute effect of BC on the phenotypic expression of antral follicular development.

In different animal species, several environmental and genetic factors can affect the activation of GnRH neurones and establishment of estrous cyclicity including, but not limited to,

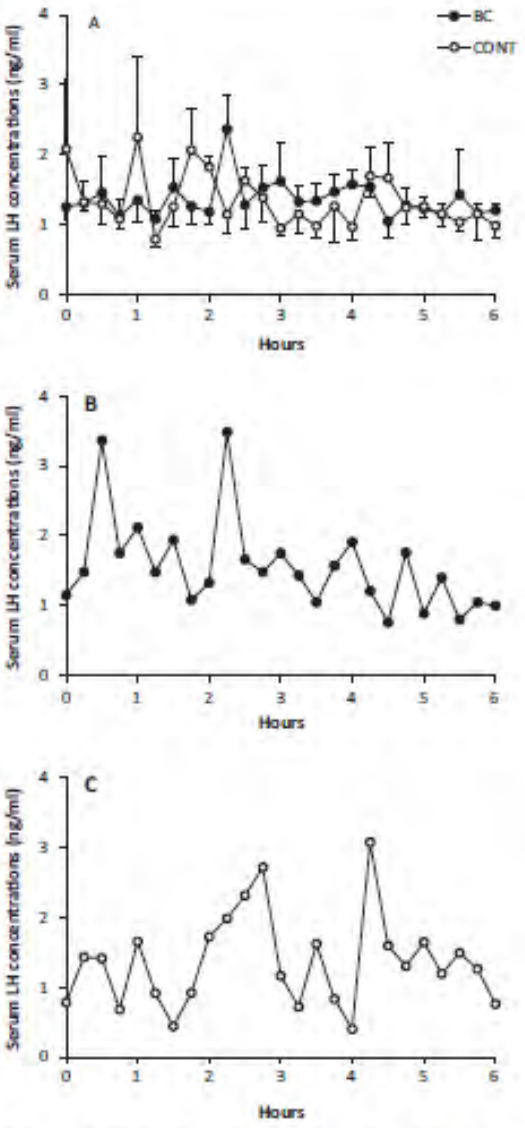

Fig. 2 – (A) Least-square means of serum LH concentrations (ng/ml) for betacrotene-supplemented (BC) and control (CONT) groups (n = 5 goats/treatment); (B) serum LH concentrations over time in one representative betacarotene-supplemented goat and (C) one representative control goat. An intensive blood sampling (every 15 min for 6 h) for LH measurements was performed 36 h prior to the estrus day. All animals were kept under natural photoperiodic conditions (October–November, 26°N).

photoperiod, endocrine profile, season of the year, nutrional status, age, stress, live weight as well as body condition score [1,4]. In evaluating the effects of short-term nutritional supplementation in ewes with low body condition scores, an increased concentration of glucose, insulin and leptin one day before ovulatory wave emergence was observed and positively correlated with the number of follicles growing from 2 to 3 mm in diameter onwards and the duration of growth of the largest antral follicles of waves. Short-term nutritional supplementation in ewes affected antral follicular development in the absence of significant changes in circulating FSH concentrations, but elevated levels of glucose, insulin and leptin may have acted directly at the ovarian level [33]. Such results are supported by a proposed mechanistic model to explain the influence of nutrition upon follicular growth and ovulation rate, in which a set of metabolic–endocrine systems (i.e., the glucose–insulin–leptin and GH–IGF-1 systems) act as the main modulators [2,3]. Therefore, not only the live weight and body condition score are highly influenced by metabolic changes which occur prior to the onset of ovarian activity [35]; the ovary itself can be a direct target of such metabolic influences. Since no differences ($p > 0.05$) were observed in live weight or body condition score between experimental groups of animals in this study, other endocrine and/or metabolic pathways are likley to be involved in the control of ovarian outcomes.

A direct relationship between serum BC concentration and reproductive performance has been shown in different species. In ruminants, BC supplementation seems to be strongly related to an increased steroidogenesis in both luteal and follicular ovarian tissues [19,22]. In a previous study, BC increased litter size and the number of live births in sows; interestingly, these authors concluded that the most likely underlying mechanism was BC-specific and independent from its role as a vitamin A precursor [36]. In beef cattle, BC supplementation exerted a positive effect on corpora lutea size and progesterone secretion, acting at the ovarian level [22]. Also, a positive effect of short-term intake of BC-supplemented diets on ovarian follicular development and ovulation rate has been demonstrated in adult goats under short-day photoperiods [18] as well as upon ovarian and luteal function in goats kept under controlled photoperiodic conditions [19]. A positive relationship between serum BC concentrations and ovarian activity during the first follicular wave of the estrous cycle was observed in cows [20]. In addition, a positive relationship among BC-supplementation, serum retinol concentration, serum gammaglutamyl transpeptidase concentration and luteal activity was reported in dairy cattle [23]. As previously mentioned, in certain animal species BC may be an important signaling molecule directly stimulating ovarian function.

The reduction in the parameters of pulsatile LH secretion in the BC-supplemented group is in contrast with previous results in heifers where LH concentrations increased after BC-supplementation [37]. However, in other earlier studies in ewes [38] and rabbits [39], BC supplementation did not affect the release pattern of LH, suggesting that the stimulatory effect of BC on ovarian function could be exerted through an LHRH-independent mechanism. Several possibilities could be proposed to explain this phenomenon. Firstly, some intra-ovarian factors (i.e., IGF-1) could support an increased ovarian activity even if in face of diminished serum LH concentrations. Secondly, BC could directly act at the ovarian level by increasing the affinity and/or numbers of LH receptors, allowing a greater ovarian response to luteotropic stimuli [2,3]. The latter is in agreement with the previously described direct effects of BC exerted at cellular and tissue levels [13,14]. Studies to determine the precise role of BC upon gonadotropin secretion and intraovarian autocrine or paracrine systems are therefore warranted.

The current study demonstrates that BC supplementation augmented the follicular development and ovulation rate in cyclic goats. Notably, such effects of BC were observed in spite of a reduction in the pulsatile LH release. Whether the effects of BC-supplementation are primarily mediated by modulating the influence of LH on ovarian tissue or the ovary itself is a direct target for a BC-dependent regulatory pathway remains to be determined. Further studies aimed at elucidating the specific gonadotropic or metabolic effects of BC at the ovarian level would contribute to a better understanding of the hypothalamic–hypophyseal–gonadal axis activation in the adult female goat, the process of clinical relevance and crucial significance in the small ruminant industry.

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Long-term betacarotene supplementation positively affects serum triiodothyronine concentrations around puberty onset in female goats

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ABSTRACT

The effect of betacarotene (BC) supplementation on serum triiodothyronine (T3) levels over time in prepubertal goats was evaluated. Goats ($n = 17$; 3 months old; 7/8 Saanen-Alpine; 26° NL) were randomly assigned to one of the following two groups: 1) the betacarotene group, supplemented daily with 50 mg of BC ($n = 9$; live weight [LW]: 17.3 ± 1.0 kg; body condition score [BCS]: 3.34 ± 0.12), or 2) the control group (CC; $n = 8$; LW: 16.1 ± 1.0 kg; BCS = 3.17 ± 0.12). The initial mean LW (16.7 ± 1.0 kg) and BCS (3.31 ± 0.12) were similar ($p > 0.05$) in both groups. Whereas BC supplementation did not affect the onset of puberty (215.7 vs. 226.7 ± 6.6 days; $p > 0.05$) for the BC and CC, respectively, increases in serum T3 during the second half of the experiment were observed in the BC supplementation group ($p < 0.05$). As the LW and serum T3 levels increased, the natural photoperiod decreased, revealing a negative correlation ($p < 0.05$) between the variables; the observed values were $r = -0.94$ for LW and photoperiod and $r = -0.41$ for T3 and photoperiod. Long-term BC supplementation was not associated with a precocious onset or an increased percentage of goats reaching puberty. Long-term BC supplementation positively affected the release pattern of triiodothyronine over time, suggesting a potential role of BC as a thyroid-activating molecule; these results might possess clinical significance.

1. Introduction

The activation of reproductive function in peripubertal stages and the cyclicity of reproductive capacity in adult stages are critical to the survival of a species, and physiological homeostasis dictates the optimal conditions for reproductive success; any disturbance of this balance might affect the function of the gonadotropin releasing hormone (GnRH) neurons (Meza-Herrera, 2008, 2012; Meza-Herrera and Tena-Sempere, 2012). This transit toward complete activation of the hypothalamic-hypophyseal-gonadal (HHG) axis could be compromised by different disruptors, such as signals dictated by stress, nutritional imbalance, body weight decreases and neurological alterations, which, in addition to the photoperiod, might directly influence the HHG axis through modifications of the GnRH secretion pattern (Scaramuzzi et al.,

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2006, 2011; Meza-Herrera, 2008, 2012; Maffucci and Gore, 2009; Meza-Herrera et al., 2004, 2007, 2008, 2010; Urrutia-Morales et al., 2009; Meza-Herrera and Tena-Sempere, 2012). The activity and functionality of this neuronal circuitry is, in turn, controlled through different neurotransmitters and metabolic hormones. Whereas activation of the complex KiSS-1/kisspeptin/GPR54 system augments the glutamatergic neurotransmission, increases in the metabolic status and in the adipocyte-derived hormone leptin are excitatory events, stimulating not only the onset of puberty but also the reproductive cyclicity in adult stages (Maffucci and Gore, 2009; Meza-Herrera et al., 2010; Meza-Herrera, 2012; Meza-Herrera and Tena-Sempere, 2012).

Betacarotene (BC) is an important bioactive substance in green plants that is a precursor of vitamin A and retinoid, which has been defined as an important molecule in humans and animals (Schweigert, 1998). At the cellular and tissue level, BC is involved in multiple actions, such as promoting key events in a range of physiological processes (Schweigert et al., 2002, 2003). Although many gene products linked to reproductive performance are known to be modulated by retinoic acid, the product of retinol oxidation (Schweigert, 1998), other studies have proposed that BC might act independently of vitamin A, particularly in increasing follicular and luteal steroidogenesis in ruminant species (Arellano-Rodriguez et al., 2007, 2009; Halilioglu et al., 2002; Kawashima et al., 2009, 2010, 2012; Meza-Herrera et al., 2013a,b).

Thyroid hormones have been found to be fundamental for the normal development of mammals; they modulate metabolic activity in several tissues, regulate reproductive outcomes, provide neuroprotection and modulate cardiovascular function (Krassas et al., 2010) as well as affecting the establishment of seasonal reproduction (Dardente, 2012). Triiodothyronine (T3) is the main active thyroid hormone, and it binds to several products of two genes, the nuclear thyroid hormone receptors alpha and beta, and regulates gene expression (Braun et al., 2010); T3 has been identified as an important modulator of steroidogenic acute regulatory protein (StAR) expression and gonadal steroidogenesis (Manna et al., 2009). Whereas thyroid hormone receptors have been detected in gonadal tissue, T3 has shown a positive correlation with a number of proliferating Sertoli cells per seminiferous tubule area, as well as a positive relationship to circulating FSH concentrations in sheep (Oluwole et al., 2013). In addition, a positive relationship between serum T3 levels and the onset of puberty in goats has been proposed in males (Gunnarsson et al., 2009) and females (Meza-Herrera et al., 2011a). There is no data demonstrating a possible relationship regarding BC supplementation and serum T3 around the onset of puberty in goats; this study aimed to evaluate such a possible relationship in peripubertal female goats.

2. Materials and methods

2.1. Location, environmental conditions, animals, feeding and experimental design

This study was conducted at the Southern Goat Research Unit (26° NL, 103° WL, 1117 m) of the Regional University Unit of Arid Lands-Chapingo Autonomous University (URUZA-UACH), Bermejillo, Durango, Mexico. The climate of the area is warm and dry, and the mean annual precipitation and temperature are 217.1 mm and 22.3 °C, respectively. The warmest month is June, with temperatures above 40 °C, whereas the coldest month is January, with the lowest temperature below 0 °C.

Prepubertal female goats ($n = 17$; live weight [LW]: 16.7 ± 1.0 kg; body condition score [BCS]: 3.31 ± 0.11; 3 months old, 7/8 Saanen-Alpine) were fed a diet of alfalfa hay, corn silage and corn grain to meet 110% of their nutritional maintenance requirements (NRC, 1998). The goats were fed twice a day, with alfalfa hay (14% crude protein; 1.14 Mcal/kg, net energy for maintenance (NEm), and corn silage (8.1% CP; 1.62 Mcal/kg, NEm) in the morning (07:00), and corn grain (11.2% CP; 2.38 Mcal/kg, NEm) in the afternoon (18:00). In early June, the goats were randomly distributed in two groups: 1) the betacarotene group (BC, $n = 9$; LW = 17.3 ± 1.0 kg; BCS: 3.34 ± 0.12), and 2) the control group (CC; $n = 8$; LW = 16.1 ± 1.0 kg; BCS: 3.17 ± 0.12). The BC group was supplemented with BC (50 mg/goat/day; orally; Syntex Mexico S.A. de C.V.) during the entire experiment (150 days, from early June to early November). The goats were kept under natural photoperiod conditions from June to November (26° NL) and had free access to water, shade, and mineral salts during the entire experiment.

Allotments of food and betacarotene were individually fed to each goat. The basal diets were entirely consumed by all the goats, and it may be assumed that each goat received the same level of BC in the basal diet. The only difference in the BC consumption between the examined groups was the BC oral supplementation provided to the BC group. The LW and BCS were recorded weekly, always prior to feeding. The BCS was determined by palpation of the goat transverse and vertical processes of the lumbar vertebrae (L2 through L5) as well as upon sternal subcutaneous adipose tissue on a five-point scale (1: emaciated to 5: obese; Aumont et al., 1994) by the same experienced technician. All the methods used in this study were in accordance with accepted international guidelines (FASS, 1999).

2.2. Blood sampling, progesterone determination and evaluation of the onset of puberty

The schedules for the blood sampling collection and determination of the onset of puberty have been previously outlined (Meza-Herrera et al., 2011b); the main activities will be briefly considered. From early June to November, blood (10 ml) was collected by jugular venipuncture twice per week, prior to feeding. The blood was collected into sterile vacuum tubes (Corvac, Kendall Health Care, St. Louis, MO) and allowed to clot at room temperature for 30 min. The serum was separated by centrifugation (1500 × g; 15 min), decanted and collected in duplicate in polypropylene microtubes (Axygen Scientific, Union City, CA, USA) and stored at −20 °C until the hormonal analysis. The serum P_4 concentration was determined by radioimmunoassay (RIA) using a commercial RIA kit (Diagnostic Products, Los Angeles, CA, USA) validated for ruminant serum (Schneider and Hallford, 1996). The intra- and inter-assay coefficients of variation (CV) were 9.9 and 12.4%, respectively. Whereas the average recovery was 94%, the sensitivity of the assay was 0.1 ng/ml. The onset of puberty was confirmed in both experimental groups based on the P_4 serum profiles; for each goat, a serum P_4 level ≥1 ng/ml in two consecutives samples were considered indicative of ovulation as well as the onset of puberty (Cushwa et al., 1992).

2.3. Intermittent blood sampling and triiodothyronine quantification

Blood (10 ml) was collected monthly by jugular venipuncture from all the goats; because several environmental factors such as temperature, season and circadian rhythm cause fluctuation in thyroid hormone levels, all the samples were collected prior to feeding at 07:00 throughout the experimental period. The serum T3 concentrations were determined in duplicate by solid-phase RIA using components of a commercial kit; the kit utilized antibody-coated tube technology, and the assay was performed without prior extraction of T3 from the serum (Diagnostic Products, Los Angeles, CA, USA (Head et al., 1996)). Whereas the intra- and inter-assay CV values for T3 quantification were 0.55% and 6.98%, the sensitivity of the assay was 0.1 ng mL^{-1}. The sources for T3 iodination and the standards were NEX 110 (New England Nuclear, Boston, MA) and T2877 (Sigma, St. Louis, MO), respectively. Fig. 1 shows a schematic representation of the experimental protocol, considering the birth of the animals, the adaptation period (Mar–May), the experimental period (Jun–Nov) and the intermittent blood sampling (Jun–Nov: progesterone (P4), triiodothyronine (T3) throughout the experimental period (Fig. 1).

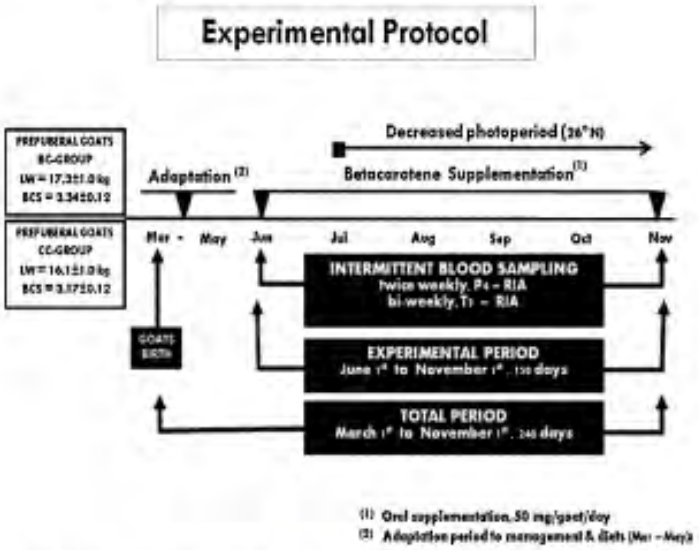

Fig. 1. A schematic representation of the experimental protocol, considering the birth of the animals, the adaptation period (Mar–May), the experimental period (Jun–Nov) and the intermittent blood sampling (Jun–Nov; progesterone (P4), triiodothyronine (T3) across time) in peripuberal crossbred female ($n = 17$; 3 months old, 7/8 Saanen–1/8 Criollo) supplemented with betacarotene (BC-group; $n = 9$) or control without betacarotene (CC-group; $n = 8$); goats were kept under natural photoperiod (26° N).

2.4. Statistical analyses

The response variables LW, BCS and serum triiodothyronine levels in the examined groups were analyzed across time by split-plot ANOVA for repeated measures (Morrel, 1999). The model included treatment in the main-plot, which was tested using animals in the treatment as the error term. The time and the time × treatment interaction were included in the subplot and were tested by using the residual mean square (Littell et al., 1998). When the significant F values were observed, the mean separation was considered the LSMEANS-PDIFF option of the PROC GLM. The correlation analysis was conducted by Pearson's product-moment test. Whereas the age at puberty was compared considering a CRD-ANOVA, the proportion of goats showing puberty or not puberty was compared with a chi-square test. The statistical analysis was computed using the SAS GLM or FREQ procedures (SAS Inst. Inc. Cary, NC, USA). The differences were considered to be statistically significant at $p < 0.05$. The reported values are defined as the least square means ± SEM.

3. Results and discussion

The LW and the BCS did not differ ($p > 0.05$) between the experimental groups during the entire experiment (Fig. 2); the same finding was true regarding the mean serum triiodothyronine (1.45 vs. 1.39 ± 0.04 ng/ml $p > 0.05$; BC vs. CC, respectively). The age at which the goats reached puberty (215.7 vs. 226.7 ± 6.6 days; $p > 0.05$) and the percentage of the goats reaching puberty (44.4 vs. 25.0 ± 17.0% $p > 0.05$) were not different between the BC and the CC, respectively. A treatment by time interaction was detected ($p < 0.05$) with respect to the serum T3 concentration across time; increases in the serum T3 during the second half of the experiment were observed in the BC group (Fig. 3). The serum T3 was positively correlated with LW ($r = 0.72$; $p = 0.04$). As the LW and serum T3 levels increased, the natural photoperiod decreased, revealing a negative correlation ($p < 0.05$) between such variables. The onset of puberty occurred by the end of the experimental period, coincident with the beginning of the natural breeding season; the natural breeding season occurrence was regardless of the treatment. The observed values among these variables were $r = -0.94$ for LW and photoperiod and $r = -0.41$ for T3 and photoperiod (Fig. 4).

Our working hypothesis stated that BC supplementation should promote an increase in serum triiodothyronine at the approximate time of the onset of puberty. This hypothesis was supported by our results. Supplementation by BC promoted increased mean serum T3 concentrations at specific points of the 2/3 and 3/3 experimental period, suggesting a potential role of BC as an activating molecule of the hypothalamic-hypophyseal-thyroid axis. This BC-dependent increase in T3 was not related to an increased attainment of puberty. The onset of puberty implies reactivation of the GnRH neurons, which causes the establishment of ovarian cyclicity (Meza-Herrera, 2008; Meza-Herrera et al., 2010). Several environmental and genetic signals affect the reactivation of the GnRH neurons and the onset of puberty, including the endocrine profile, the season of the year, the nutritional status, age, LW, stress, and BCS (Meza-Herrera et al., 2011a,b; Meza-Herrera and Tena-Sempere, 2012). A positive effect of BC supplementation upon ovarian function and insulin release in the adult goat has been previously reported by our group (Meza-Herrera et al., 2013a), whereas retinoic acid has been shown to modulate the gonadotropin receptor promoter in ovine granulosa cells (Xing and Sairam, 2002). The BC supply during the close-up dry period positively induced ovulation during the first follicular wave in postpartum dairy cows, highlighting the important nutritional role of BC upon ovarian activity (Kawashima et al., 2012). As previously stated, although BC supplementation

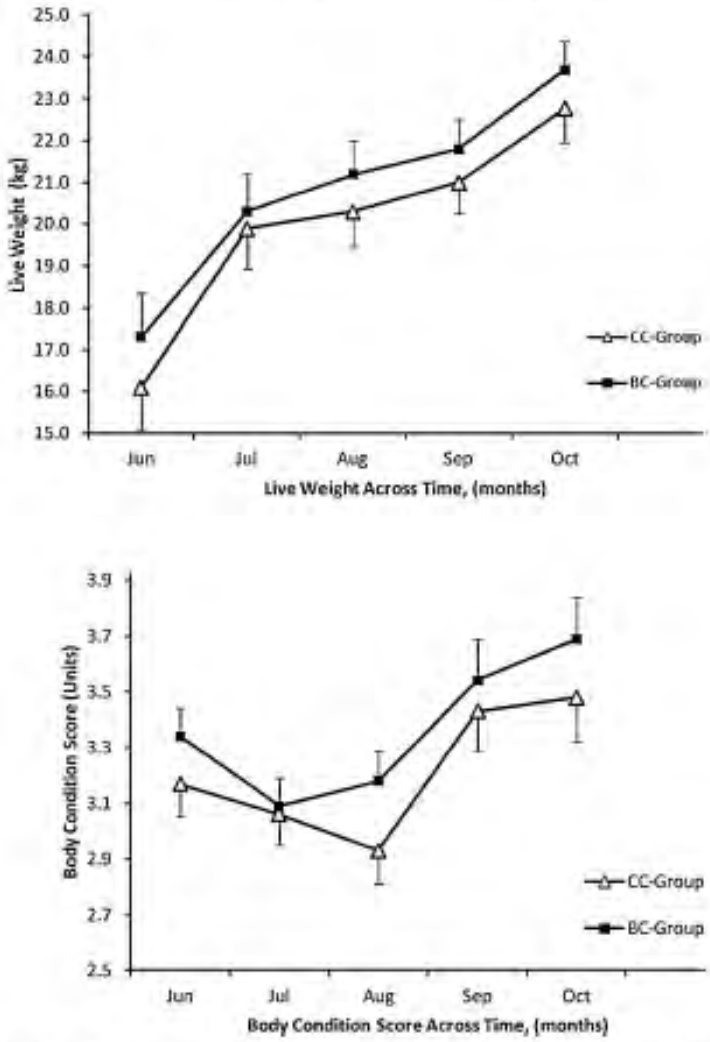

Fig. 2. Live weight (kg; upper panel) and body condition score (units, 1–5 scale; lower panel) across time of prepuberal crossbred female goats (n = 17; 3 mo. old, 7/8 Saanen-1/8 Criollo) supplemented with betacarotene (BC-Group) or without betacarotene (CC-group) under natural photoperiod (June to November, 26° N). *Note*: No statistical differences ($p > 0.05$) were observed between treatments within month at either stage of the experimental period.

is positively affected by the T3 serum concentrations across time, such an endocrine profile was not related to an increase in anticipated puberty, a physiological scenario in agreement with Kaewlamun et al. (2011) and Kuhl et al. (2012), who reported that BC supplementation had no positive effects upon reproductive outcomes.

In mammals, the thyroid hormones (TH; T4 & T3), are essential for the development and maintenance of normal physiological processes, especially those related to the functions of the central nervous system. Hypophyseal thyroid stimulating hormone (TSH) induces the thyroid to synthesize T4, which is 5′-monodeiodinated to the more biologically active T3. The rate of TSH release is controlled by the amount of the thyrotropin-releasing hormone secreted by the hypothalamus, as well as modulated by the circulating concentrations of T3 and T4 (Patel et al., 2011). In the early neonatal period, decreased TH concentrations induce alterations in sexual maturation and gonadal and reproductive tract development as well as gonadotropin and steroid concentrations. TH facilitates reactivation of the pulsatile GnRH release, whose site of action might reside at the brain level to interact directly with the hypothalamic GnRH pulse generator or at a specific somatic site(s), which communicates to the hypothalamus by endocrine cues, which convey information upon TH-dependent development. Under this scenario, the achievement of a particular live weight and a defined fat-lean body mass index are essential for pubertal development (Mann and Plant, 2010).

Although conclusions about the direct effects of thyroid hormones upon ovarian function have been contradictory, the cells from the ovarian surface epithelium express

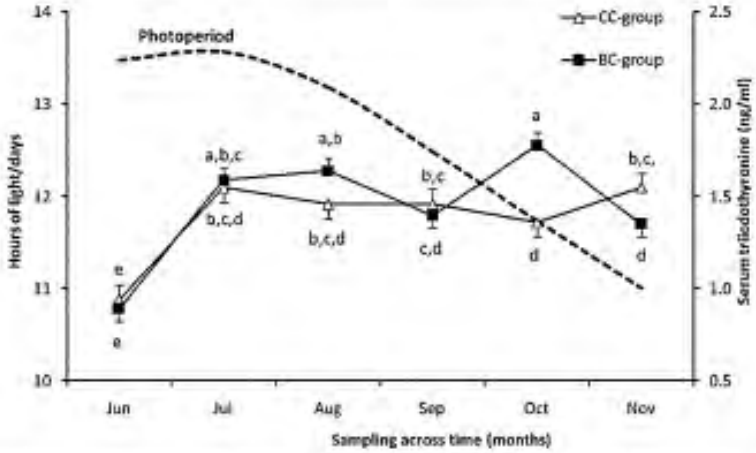

Fig. 3. Mean serum triiodothyronine concentration (ng/ml) and photoperiod across time of prepuberal crossbred female goats (n = 17; 3 mo. old, 7/8 Saanen–1/8 Criollo) supplemented with betacarotene (BC-group; n = 9) or without betacarotene (CC-group; n = 8); goats were kept under natural photoperiod (26° N). *Note:* Different superscripts indicate differences between the BC and CC groups ($p < 0.05$).

multiple nuclear hormone receptor genes including those encoding the thyroid hormone receptors (Rae et al., 2007). Previous studies have shown that T3 synergizes with FSH to induce granulosa cell differentiation in porcine follicles (Maruo et al., 1987). In ruminants, T3 concentrations have been positively correlated with the diameter of the largest follicle, whereas anestrous, low-BCS females showed reduced T4 concentrations, which were associated with a smaller dominant follicle (Flores et al., 2008). Activation of the HHG axis at puberty was accompanied by increases in TSH and T3, whereas they were positively related to the body mass index (Kratzsch et al., 2008). In addition, the FSH-increased preantral follicle growth under in vitro conditions was markedly enhanced by T3, in a concentration-dependent fashion (Zhang et al., 2011).

Whereas body condition was shown to alter the serum thyroid hormones in ruminants, which mediates the ovarian effects of nutrition, females receiving high energy diets depicted increased levels of glucose, total cholesterol, insulin, leptin and T3 and tended to have oocytes of increased quality (Campanile et al., 2010). The results of these studies demonstrate that TH plays a positive and permissive action upon reproductive function. Whether these actions of TH mediate the hypothalamic centers directly by regulating the pulsatile release of GnRH or indirectly by circulating cues reflecting TH action on somatic development should be carefully addressed. Whereas reduced thyroid hormone levels during human pregnancy have been related to decreased neuropsychological fetal development (Li et al., 2010), increased BC dietary intake can lower the risk

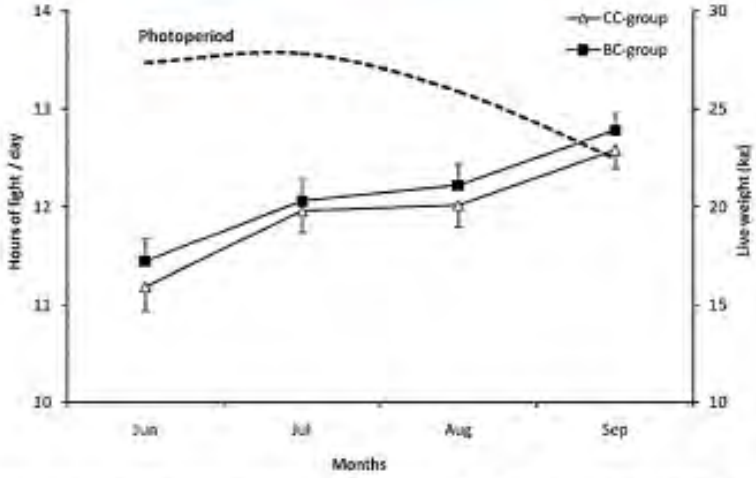

Fig. 4. Mean live weight (kg) and photoperiod across time of prepuberal crossbred female goats (n = 17; 3 mo. old, 7/8 Saanen–1/8 Criollo) supplemented with betacarotene (BC-group; n = 9) or without betacarotene (CC-group; n = 8); goats were kept under ambient photoperiod (26° N). *Note:* No live weight differences across time ($p < 0.05$) occured between experimental groups.

of Alzheimer's neurodegeneration (Li et al., 2012). Low thyroid hormone status in pregnant woman has been linked to negative effects on the neuropsychological fetal development. Although interspecies differences might exist, this positive relationship between BC-supplementation and increased serum T3 levels warrants careful evaluation.

4. Conclusions

This study reports, for the first time, the positive action of BC supplementation upon serum T3 concentrations over time. This result suggests a potential role for BC as an activating molecule in the hypothalamic–hypophyseal-thyroid axis in goats. These results might have physiological and clinical significance.

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Beta-Carotene Supplementation Positively Affects Selected Blood Metabolites Across Time Around the Onset of Puberty in Goats

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ABSTRACT

Meza-Herrera C.A., Pacheco-Alvarez P., Castro O.E., Macias-Cruz U., Avendaño-Reyes L., Mellado M., Veliz-Deras F.G., Contreras-Villarreal V., Abad-Zavaleta J., Rodriguez-Martinez R., Arellano-Rodriguez G. (2017): **Beta-carotene supplementation positively affects selected blood metabolites across time around the onset of puberty in goats.** Czech J. Anim. Sci., 62, 22–31.

The possible effect of beta-carotene supplementation upon peripubertal changes in serum concentrations across time for total protein (TP), urea (UR), cholesterol (CHOL), and glucose (GLU) around puberty onset was evaluated. The experiment was carried out from June to November and prepubertal goats (n = 17, 3 months old, 7/8 Saanen-Alpine, 1/8 Criollo) were randomly assigned to: (1) beta-carotene group (BC) (n = 9; 17.3 ± 1.0 kg live weight (LW), 3.3 ± 0.12 body condition score (BCS), oral supplementation with 50 mg beta-carotene per day per goat) and (2) control group (CC) (n = 8; 16.1 ± 1.0 kg LW, 3.1 ± 0.12 BCS). Serum blood samples were collected along the experiment to quantify progesterone concentrations (P4) through radioimmunoassay, while TP, UR, CHOL, and GLU through spectrophotometric analyses. No differences ($P > 0.05$) occurred between treatments regarding LW and BCS, and TP (67.6 ± 2.4 g/l), UR (3.8 ± 0.17 mmol/l), GLU (5.06 ± 0.09 mmol/l), and CHOL (1.62 ± 0.07 mmol/l) concentrations. However, while a treatment × time interaction occurred between treatments for TP, GLU, CHOL ($P < 0.05$) favouring the BC group, an increased serum UR levels occurred in the CC group. Nonetheless, such general serum metabolite profile was related neither to the age

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(215.7 vs 226.5 ± 6.6 days; $P > 0.5$) nor to the percentage (44.4 vs 25.0 ± 17.0%; $P > 0.05$) of goats reaching puberty in the BC and CC groups, respectively. Results should help speed-up goat productivity while may also engender key management applications to different animal industries.

Keywords: reproduction; nutritional supplementation; focus feeding; glucose; cholesterol; pubertal activation

Puberty onset is a complex process regulated by several environmental cues, in which the neuroendocrine system must convey external information to align different internal mechanisms and promote anatomical, physiological, and behavioral changes (Meza-Herrera et al. 2010). The last is accomplished throughout activation of an intricate circuitry which involves diverse endocrinological messengers and neurotransmitters, whose core endeavour is to reinitiate the activation of GnRH neurons at peripubertal stages (Dupont et al. 2014).

Chronological age was originally involved as a key modulator of puberty onset (Meza-Herrera et al. 2010). Later on, other internal signals such as the acquisition of a critical body mass, serum increases in various metabolic hormones (e.g. insulin, IGF-1, T3, and leptin) along with augments in some blood analytes (e.g. glucose, cholesterol), have been closely linked to the reactivation of the hypothalamic–pituitary–gonadal (HPG) axis (Meza-Herrera and Tena-Sempere 2012; Meza-Herrera et al. 2014). Whereas body energy reserve status has been defined as an important modulator of puberty initiation, other studies highlight the rate of muscle and fat accumulation across time in the activation of GnRH neurons (Rosales-Nieto et al. 2013). Interestingly, both approaches denote the pivotal role that, not only the adipose tissue but also the adipocyte-derived molecules adipokines (e.g. leptin, adiponectin, resistin, omentin, vaspin) may exert upon both energy balance and reproductive function (Meza-Herrera and Tena-Sempere 2012).

Beta-carotene (BC), which is present in green plants, has a paramount role in mammals as precursor of vitamin A and retinoid, demonstrating to be a key bioactive micronutrient involved in multiple biological actions while promoting strategic events in a range of physiological processes at cellular and tissue level both in humans and animals (Harrison et al. 2012; Eroglu and Harrison 2013). Although many gene products linked to reproductive performance are known to be modulated by retinoic acid, the product of retinol oxidation (Harrison et al. 2012), other studies have proposed that BC might act in a direct fashion, independently from vitamin A (Kawashima et al. 2010, 2012; Kramer and Aurich 2010).

Two different types of BC-metabolizing enzymes have been identified in several tissues from different species: (*i*) BCMO1 (BC-15',15'-monooxygenase-1) which right through a central cleavage converts BC to all-trans retinal, acting at cytosol level, and (*ii*) BCDO2 (BC-9',10'-dioxygenase) which throughout an eccentric cleavage generates β-ionone and β-apocarotenal, acting at mitochondrial level (Eroglu and Harrison 2013). Besides liver being the main site of BC accumulation (Eroglu and Harrison 2013), the mRNA expression of both types of BC oxygenases has also been detected at hepatocyte level (von Lintig et al. 2005).

Building on such findings and previous studies of our group, we hypothesized that BC supplementation could be associated with changes in the profile of blood metabolites around the onset of puberty in goats. The blood analytes evaluated included total cholesterol (CHOL), glucose (GLU), total protein (TP), and urea (UR). The final seek was to gain insights regarding a possible relationship between BC supplementation and some blood analytes related to lipid, carbohydrate, and protein metabolism.

MATERIAL AND METHODS

General. All the methods and management of the experimental units used in this study were in strict accordance with accepted guidelines for ethical use, care, and welfare of animals in research at international (FASS 2010), national (NAM 2002), and institutional levels (reference number UACH/DGIP/URUZA/11-510-405).

Location, environmental conditions, animals, feeding, and experimental design. This study was conducted at the Southern Goat Research Unit (26°N latitude, 103°W longitude, 1117 m a.s.l.) of the Regional University Unit of Arid Lands, Chapingo Autonomous University (URUZA-UACH),

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Bermejillo, Durango, Mexico. The climate of the area is warm and dry, and the mean annual precipitation and temperature are 217.1 mm and 22.3°C, respectively. The warmest month is June, with temperatures above 40°C, whereas the coldest month is January, with the lowest temperature below 0°C. Prepubertal female goats (n = 17; live weight (LW) 16.7 ± 1.0 kg; body condition score (BCS) 3.31 ± 0.11, 3 months old, 7/8 Saanen-Alpine) were fed a diet of alfalfa hay, corn silage, and corn grain to meet 110% of their nutritional maintenance requirements (NRC 2007). The goats were fed twice a day, with alfalfa hay (14% crude protein (CP), 1.14 Mcal/kg net energy for maintenance (NEm), and corn silage (8.1% CP, 1.62 Mcal/kg, NEm) in the morning (7:00), and corn grain (11.2% CP, 2.38 Mcal/kg, NEm) in the afternoon (18:00). In early June, the goats were randomly distributed in two groups: (1) beta-carotene group (BC) (n = 9, LW = 17.3 ± 1.0 kg, BCS 3.34 ± 0.12), and (2) control group (CC) (n = 8, LW = 16.1 ± 1.0 kg, BCS 3.17 ± 0.12). The BC group was orally supplemented with 50 mg beta-carotene (Syntex Mexico S.A. de C.V.) per goat per day during the entire experiment (150 days, from early June to early November). The goats were kept under natural photoperiod conditions from June to November (26°N latitude) and had free access to water, shades, and minerals during the entire experiment.

Chemical composition of the basal diet was determined from representative samples taken throughout the experimental period (Table 1) and analyzed according to the procedures outlined by AOAC (1990). Allotments of food and BC were individually fed to each goat. Since the basal diets were entirely consumed by all the goats, it may be assumed that each goat received the same BC level from the offered basal diet. Therefore, the only difference in BC consumption between the experimental groups was the oral BC supplementation provided to the BC-supplemented group. Thus, the effect to offer or not supplemental beta-carotene in both experimental groups was evaluated.

Both LW and BCS were recorded weekly, always prior to feeding. The BCS was determined by palpation of the goat transverse and vertical processes of the lumbar vertebrae (L2 through L5) as well as upon sternal subcutaneous adipose tissue on a five-point scale (1: emaciated to 5: obese; Aumont et al. 1994) by the same experienced technician. The health status of all the experimental units was controlled by an experienced veterinarian during the whole experimental period; no health problems were observed during the trial. Besides, efforts were made to minimize any possible discomfort in the experimental units.

Blood sampling, progesterone determination, and evaluation of the onset of puberty. The schedules for the blood sampling collection and determination of the onset of puberty have been previously outlined (Meza-Herrera et al. 2011); the main activities will be briefly considered. From early June to November, blood (10 ml) was collected by jugular venipuncture twice per week, prior to feeding. The blood was collected into sterile CORVAC® vacuum tubes (Kendall Health Care, USA) and allowed to clot at room

Table 1. Composition and nutrient content (feed basis and DM basis) of daily basal diet for peripubertal crossbred female goats (n = 17, 3 months old, 7/8 Alpine-Saanen and 1/8 Criollo) supplemented with betacarotene (BC) or non-supplemented (CC) exposed to a naturally decreasing photoperiod (June–November, 26°N latitude)[a]

Item[b]	Feed basis	% DM
Fresh matter (kg)	1.00	0.91
DM (kg)	0.91	90.78
CP (kg)	0.14	15.62
Degradable protein (% CP)	76.89	76.89
Non-degradable protein (% CP)	23.11	23.11
Digestible protein, degradable nitrogen (kg)	0.08	9.12
Digestible protein, fermentable energy (kg)	0.06	6.98
Crude fibre (kg)	0.29	32.32
Starch (kg)	0.11	12.37
Crude fat (kg)	0.12	1.66
Carotene (mg)[c]	56.58	51.48
Betacarotene (mg)[c]	33.22	30.23

DM = dry matter, CP = crude protein

[a]mineral block offered *ad libitum* contained (%, w/w): NaCl 95, Fe 0.2, Cu 0.033, I 0.007, Zn 0.005, Co 0.0025

[b]composition values (% of diet DM) represent values from five samples taken throughout the experimental period and dried in a forced air stove at 60°C until constant weight; DM, CP were determined according to the procedures outlined by AOAC (1990)

[c]1 mg carotene = 400 IU vitamine A; IU vitamine A = 1.46 µg betacarotene (NRC 2007)

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temperature for 30 min. The serum was separated by centrifugation (1500 *g*, 15 min), decanted and collected in duplicate in polypropylene microtubes (Axygen Scientific, USA) and stored at –20°C until the hormonal analysis. The serum progesterone (P4) concentration was determined by radioimmunoassay (RIA) using a commercial RIA kit (Diagnostic Products, USA) validated for ruminant serum (Schneider and Hallford 1996). The intra- and inter-assay coefficients of variation (CV) were 9.9 and 12.4%, respectively. Whereas the average recovery was 94%, the sensitivity of the assay was 0.1 ng/ ml. The onset of puberty was confirmed in both experimental groups based on the P4 serum profiles; for each goat, a serum P4 level ≥ 1 ng/ml in two consecutive samples was considered indicative of ovulation as well as the onset of puberty (Meza-Herrera et al. 2011).

Intermittent blood sampling and blood analytes quantification. Blood samples (10 ml) were collected biweekly by jugular venipuncture from all goats to evaluate blood metabolite concentrations; the analytes were all measured throughout spectrophotometric analyses (Coleman 15 Junior II; Coleman Intruments Division, PerkinElmer, USA). Serum total protein (TP) concentrations were determined in duplicate by using a commercial kit based on the bicinchononic acid reagent considering the bovine serum albumin 16 as standard and performed as described in the manual kit (Pierce Chemical Co., USA). Serum glucose (GLU) analyses were also conducted in duplicate throughout spectrophotometer techniques, following protocols supplied by the kit manufacturer (Roche Diagnostic Systems, Inc., USA). In addition, urea (UR) and cholesterol (CHOL) analytes were also measured in duplicate; serum UR concentration was quantified using the 640-A kit, based on the urease-18 (Sigma-Aldrich, USA), while serum CHOL concentrations were analyzed using the EnzyChrom™ kit ECCH-100 (Bioassay Systems, USA); assays were carried out following the protocols outlined by the manufacturer. Figure 1 shows a schematic representation of the experimental protocol, considering the birth of the animals, the adaptation period (March–May), the experimental period (June–November), and the intermittent blood sampling (June–November;

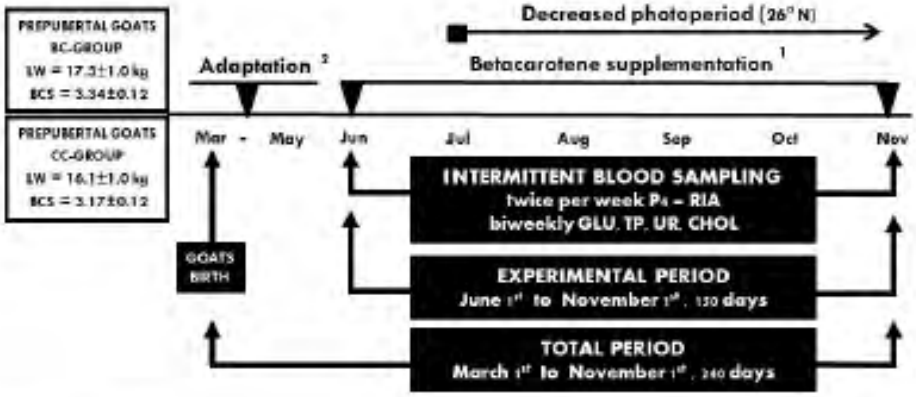

Figure 1. A scheme of the experimental protocol including data on animals' birth, adaptation period (March–May), and 150-day experimental period

Intermittent blood sampling (twice a week) for serum quantification of progesterone (P4), and every two weeks for total protein (TP), urea (UR), glucose (GLU), and cholesterol (CHOL) was performed during the whole experimental period in peripubertal crossbred female goats (n = 17, 3 months old, 7/8 Alpine-Saanen and 1/8 Criollo) supplemented with betacarotene (BC) or non-supplemented (CC), and exposed to naturally decreasing photoperiod (June–November, 26°N latitude)

LW = live weight, BCS = body condition score

[1]oral supplementation, 50 mg/goat/day

[2]adaptation period to management and diets (March–May)

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progesterone (P4)), and the blood analytes GLU, TP, UR, CHOL throughout the experimental period.

Statistical analyses. The response variables LW, BCS, serum TP, UR, GLU, and CHOL concentrations throughout the experimental period were determined by split-plot ANOVA for repeated measures across time. Previously, all blood analytes were log transformed because they were not normally distributed. The models included treatment in the main plot, which was tested using animal within treatment as the error term. Time and the time × treatment interaction were included in the subplot and were tested by using the residual mean square (Littell et al. 1998). In the case of a significant treatment effect, mean separations were achieved using the PDIFF option of the GLM Procedure. While age at puberty was compared using ANOVA for Completely Randomized Design, the proportions of either pubertal or non-pubertal goats were compared with a chi-square test. All the analyses were computed using the procedures of the SAS software (Statistical Analysis System, Version 9.1, 2004). Results are expressed as Least Squares Means and standard errors and evaluated at the significance level of $P \leq 0.05$.

RESULTS

Initial LW and BCS were 16.7 ± 1.0 kg and 3.2 ± 0.12 units, with respective values at the end of the experimental period of 23.5 ± 0.8 kg and 3.4 ± 0.11 units. No significant differences between treatments for LW and BCS were observed along the experimental period. In addition, no significant differences were observed regarding the serum concentrations for TP: 67.6 ± 2.4 g/l, UR: 3.8 ± 0.17 mmol/l (22.83 ± 1.05 mg/dl), GLU: 5.06 ± 0.09 mmol/l (91.15 ± 1.77 mg/dl), and CHOL: 1.62 ± 0.07 mmol/l (62.32 ± 2.75 mg/dl). Interestingly, however, while a treatment × time interaction was observed between treatments for TP, UR, GLU, CHOL across time, such differences favoured the BC group, except the UR-analyte the serum level of which increased in the CC group (Figures 2–5). Yet, such general serum metabolite profile was related neither to the age (215.7 vs 226.5 ± 6.6 days; $P > 0.05$) nor to the percentage (44.4 vs 25.0 ± 17.0%; $P > 0.05$) of goats reaching puberty in the BC and CC groups, respectively. According to the natural photoperiod observed during the experimental period, the longest photoperiod occurred

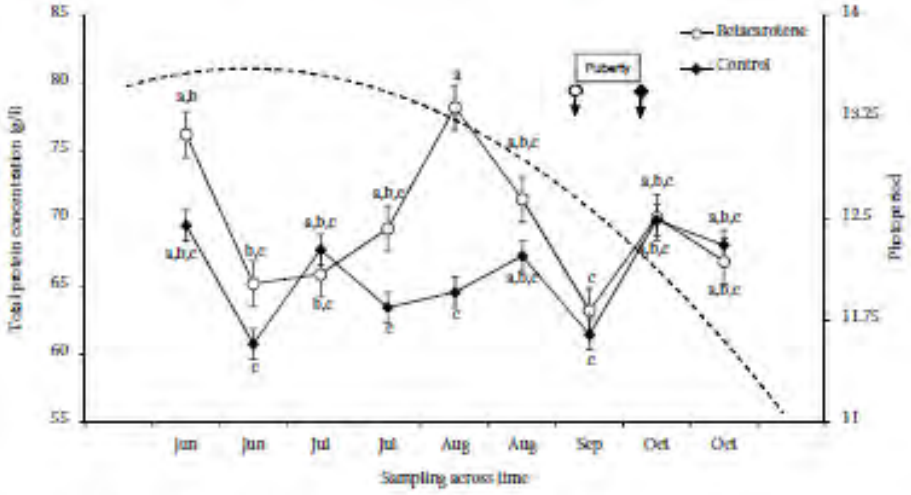

Figure 2. Serum total protein concentrations (g/dl) of experimental animals

Peripuberal crossbred female goats (n = 17, 3 months old, 7/8 Alpine-Saanen and 1/8 Criollo) were supplemented with betacarotene (BC) or non-supplemented (CC) and exposed to a naturally decreasing photoperiod (June–November, 26°N)

$^{a-c}$statistical differences ($P < 0.05$) observed between the BC and CC treatments across the 150-day experimental period; average dates for puberty onset were September 5th (BC group) and September 26th (CC group)

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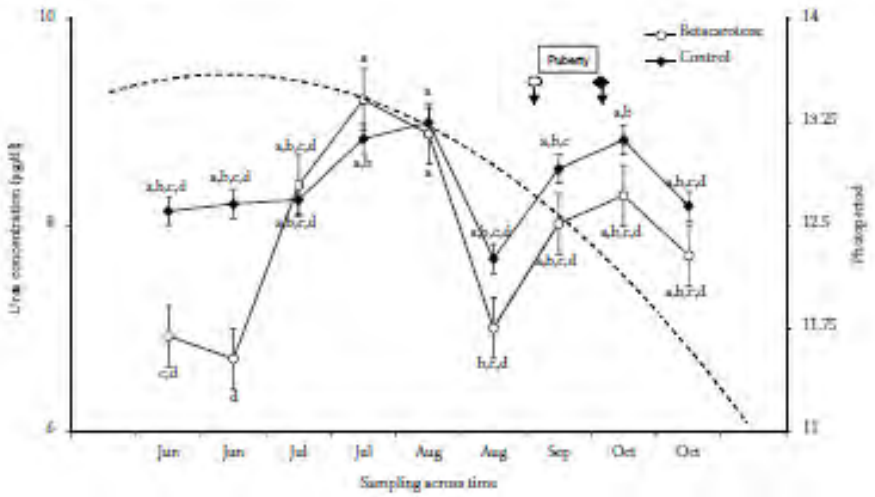

Figure 3. Serum urea concentrations (mg/dl) of experimental animals across time
Peripuberal crossbred female goats (n = 17, 3 months old, 7/8 Alpine-Saanen and 1/8 Criollo) were supplemented with betacarotene (BC) or non-supplemented (CC) and exposed to a naturally decreasing photoperiod (June–November, 26°N)
$^{a-d}$statistical differences ($P < 0.05$) observed between the BC and CC treatments across the 150-day experimental period; average dates for puberty onset were September 5th (BC group) and September 26th (CC group)

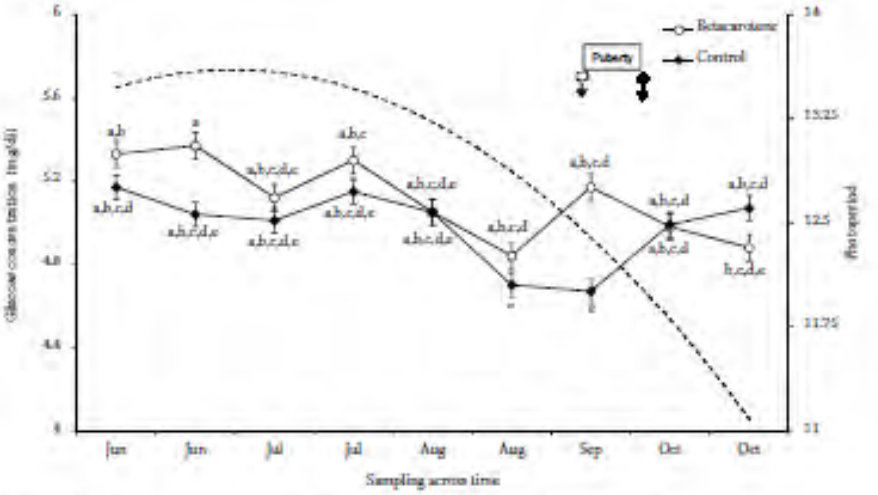

Figure 4. Serum glucose concentrations (mg/dl) of experimental animals across time
Peripuberal crossbred female goats (n = 17, 3 months old, 7/8 Alpine-Saanen and 1/8 Criollo) were supplemented with betacarotene (BC) or non-supplemented (CC) and exposed to a naturally decreasing photoperiod (June–November, 26°N)
$^{a-e}$statistical differences ($P < 0.05$) observed between the BC and CC treatments across time, although especially manifested during the 3/3 of the 150-day experimental period, coinciding with the onset of puberty in the BC group; average dates for puberty onset were September 5th (BC group) and September 26th (CC group)

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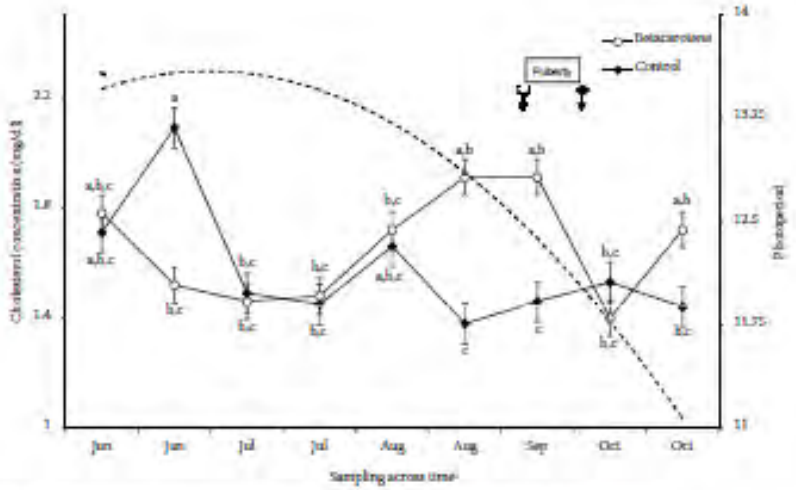

Figure 5. Serum cholesterol concentrations (mg/dl) of experimental animals across time

Peripuberal crossbred female goats (n = 17, 3 months old, 7/8 Alpine-Saanen and 1/8 Criollo) were supplemented with betacarotene (BC) or non-supplemented (CC) and exposed to a naturally decreasing photoperiod (June–November, 26°N)

$^{a-c}$statistical differences ($P < 0.05$) observed between the BC and CC treatments across time, although especially manifested during the 3/3 of the 150-day experimental period, coinciding with the onset of puberty in the BC group; average dates for puberty onset were September 5th (BC group) and September 26th (CC group)

in July (13.57 h) with a gradual decrease until early November (11.3 h). Besides, and as observed in the figures, a negative relationship was perceived between photoperiod and the onset of puberty in both experimental treatments.

DISCUSSION

Our working hypothesis stated that BC administration would promote increases across time in blood metabolites, specifically TP, GLU, and CHOL, while expecting a decrease in UR around the onset of puberty in goats. According to the observed results in our study, such hypothesis is supported by our main outcomes. This was particularly true regarding the blood analytes GLU and CHOL, the main increases of which were observed towards the final part of the experimental period, also coinciding with the onset of puberty in the BC treated group. Despite our fragmentary knowledge regarding the mechanisms modulating the intermediate metabolism (Meza-Herrera and Tena-Sempere 2012), results of our study suggest that such neurophysiologic scenario observed in the BC-supplemented peripubertal goats may potentially involve BC as an acting molecule involved in the intermediate metabolism, specially upon protein, carbohydrate, and lipid metabolism.

While an optimal intake of BC is hypothesized to have positive effects upon reproductive outcomes both in ruminant (Arellano-Rodriguez et al. 2007, 2009) and monogastric (Krammer and Aurich 2010) species, results have been contradictory, with studies reporting both positive (Kawashima et al. 2009, 2012) and negative (Folman et al. 1987) effects. Nonetheless, a positive relationship between BC supplementation, metabolic and endocrine status as well as reproductive outcomes has been previously proposed. Short-term BC supplementation positively affected ovarian follicular development and ovulation rate in adult goats (Arellano-Rodriguez et al. 2007), increased both corpus luteum diameter as well as progesterone synthesis (Arellano-Rodriguez et al. 2009). Besides, short-term BC supplementation in the adult goat increased ovarian activity and enhanced serum concentrations of insulin (Meza-Herrera et al. 2013a), although without increases in serum luteinizing hormone (LH) concentrations,

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LH-pulse or LH-area under the curve (LH-AUC) (Meza-Herrera et al. 2013b). In addition, long-term BC supplementation in peripubertal goats increased serum insulin (Meza-Herrera et al. 2011) and triiodothyronine (Meza-Herrera et al. 2014).

The precise site of BC action upon the increased release of blood metabolites observed in this study cannot be established without additional research. Yet, a clear relationship has been reported between energy balance, metabolic fuel availability, and reproductive outcomes (Meza-Herrera and Tena-Sempere 2012). As a consequence, changes in the levels not only in metabolic hormones but also in blood metabolites are dreadfully important cues that convey information to the central nervous system (CNS) regarding to the nutritional status of animals (Dupont et al. 2014). In turn, goats have to align such metabolic status to a corresponding extent of reproductive function activating, if it is the case, the hypothalamic-pituitary-gonadal (HPG) axis (Meza-Herrera and Tena-Sempere 2012). Certainly, not only the endocrine but also different neural systems are activated in response to the metabolic status as well as in reply to the circulating levels of specific blood metabolites (Meza-Herrera and Tena-Sempere 2012; Dupont et al. 2014). As stated by Krammer and Aurich (2010), BC has elicited positive effects upon reproductive outcomes in different species, and such BC action has been suggested to be most likely BC specific and independent from its role as a precursor of vitamin A. Regarding the general concentrations of the quantified blood analytes observed in our study, they are within the range with respect to TP, yet urea is slightly above the upper limit, the average concentration of glucose is higher, while the reference value for cholesterol is below the reference values reported in goats: glucose 2.78 to 4.16 mmol/l (50–70 mg/dl), cholesterol 2.08–3.38 mmol/l (80–130 mg/dl), urea 2.09–3.65 mmol/l (12.6–22 mg/dl), and total protein 61.1–70.1 g/l (Kaneko et al. 2008). Besides, the present general concentrations of blood analytes are in close agreement with the average blood metabolite concentrations reported in goats by Ye et al. (2014).

Glucose, as chemical moiety, has been involved as a key regulator of reproductive function. Its serum levels either above or below physiological range generate deleterious effects upon reproductive outcomes (Dupont et al. 2014). A synergic effect of glucose and insulin has been reported at both CNS and ovarian level (Dupont et al. 2014). Despite most mammal tissues use glucose and fatty acids as energy source, glucose is undoubtedly the principal energy source at ovarian level, denoting that its effects upon reproductive outcomes are mainly related to its sole feature as metabolic fuel (Meza-Herrera and Tena-Sempere 2012). At ovarian level, the follicle seems to possess well defined sensing systems to intuit both glucose levels and nutritional status (Dupont et al. 2014). For that reason, the follicle is able to discern information regarding the glucose level and facilitate, if this is the case, growth and development, throughout modulation in the follicle stimulating hormone (FSH) actions upon the ovarian steroidogenic pathway exerted by the theca-granulosa cell complex. Interestingly, such glucose influence is also extended upon the biological quality and competence of the oocyte (Dupont et al. 2014).

In mammals, significant differences have been described regarding serum cholesterol concentrations at different windows within the prepubertal-to-pubertal transition period as well as upon other reproductive outcomes such as fertility, which has been positively related to metabolic status (Dupont et al. 2014). Liver X receptors α and β (LXRα, LXRβ) are nuclear receptors activated by oxysterols, which are oxidized derivatives from cholesterol. They control ovarian endocrine and exocrine function; such physiological role denotes the LXRs as important molecules linking cholesterol and reproductive function (Lobaccaro et al. 2013; Urlep and Rozman 2013).

Serum total proteins, mainly composed by albumin (60%) and globulin (40%), are synthesized in hepatocytes, while gamma globulin is synthesized by plasma cells of the immune system; all of them show a wide variety of physiological functions (Kaneko et al. 2008). Regarding the observed profile of this analyte, quite high values were quantified during the first sampling date, a situation that can be related to a dehydration status which could potentially occur since the weaning of the experimental units took place prior to the onset of the experiment. As suggested by Boldt (2010), albumin possesses excellent binding capacities, especially water, calcium, sodium while it is very important in the transport of fatty acids and hormones, principally steroids. In addition, albumin acts as a free radical scavenger while it is able to bind toxic substances (Arasteh et al. 2014), sug-

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gesting a beneficial effect in the animal wellbeing. Serum albumin is also very sensitive to glycation, which involves the attachment of glucose, galactose and fructose, among other sugars, to the free amine groups of albumin; the extent of glycation depends on the glycemic status of individuals (Arasteh et al. 2014). This feature highlights its role as an important glycemic marker under both physiological and pathological scenarios (Koga and Kasayama 2010). According to Rondeau and Bourdon (2011), glucose is a vital nutrient required for cellular oxygen metabolism, hence albumin glycation should have important implications for cellular function. The last is of particular importance in that glucose has been defined as a key energy source to both the reproductive brain and the reproductive gonads (Meza-Herrera and Tena-Sempere 2012; Dupont et al. 2014).

CONCLUSION

The current study is the first report demonstrating that BC supplementation generates serum increases of total protein, glucose, and cholesterol, while decreases urea concentrations across time, around the onset of puberty in the female goat. Because of our fragmentary knowledge, further studies are needed to better understand the precise physiological actions of beta-carotene upon different reproductive outcomes and physiological scenarios; such efforts may also engender important practical applications in other animal industries

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The key role of targeted betacarotene supplementation on endocrine and reproductive outcomes in goats: Follicular development, ovulation rate and the GH-IGF-1 axis

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ABSTRACT

The possible effects of betacarotene (BC) supplementation on the secretion pattern of growth hormone (GH) and insulin-like growth factor-1 (IGF-1), and their possible relationship with total ovarian activity (TOA), was evaluated in adult goats during the breeding season. In October, goats [n = 22, 3.5 y. old, 7/8 Saanen-Alpine] were randomly assigned to: a) Betacarotene group (BC, n = 10; 45.9 ± 1.97 kg live weight (LW), 3.04 ± 0.08 units, body condition score (BCS), supplemented with 50 mg of BC goat day^{-1}), and b) Control group (CONT, n = 12; 46.2 ± 2.04 kg LW, 3.0 ± 0.08 units, BCS). An ultrasonographic scan was performed to evaluate corpus luteum number (CR) and antral follicle number (AF); TOA = CR + AF. Average LW and BCS did not differ ($p > 0.05$) during the experimental period, yet BC-goats reflected an increased CR (3.4 ± 0.2 vs. 2.8 ± 0.2), AF (5.0 ± 0.6 vs. 3.4 ± 0.6) and TOA (8.4 ± 0.5 vs. 6.2 ± 0.6). Regarding the endocrine profile, the lowest ($p < 0.05$) serum GH average concentrations (10.0 vs. 14.3 ± 1.0 ng mL^{-1}; $p = 0.01$) and GH-AUC (3670.4 vs. 5235.7 ± 369.8 units; $p = 0.01$), were observed in the BC-supplemented group. Neither serum IGF-1 concentrations (254.6 ± 28.9 ng mL^{-1} $p > 0.05$) nor GH-PULSE (1.4 ± 0.5 pulses 6 h^{-1} $p > 0.05$) differed between treatments. We document a potential role of BC as modulator of somatotrophic function, decreasing mean serum concentration and the area under the curve of GH, while also noting a positive action upon ovarian function with increases in ovulation rate and antral follicular development; such outcomes may embrace not only physiologic significance but also potential translational applications.

1. Introduction

Reproductive and productive success is closely aligned to metabolic status and food availability (Scaramuzzi et al., 2011; Meza-Herrera and Tena-Sempere, 2012). Supplementation of either vitamin A or its precursor betacarotene (BC) promotes an ample range of biological processes such as cellular development, differentiation and morphogenesis through the action of retinoic acid (RA) (Amann et al., 2011). BC is a potent scavenger of free radicals, especially singlet oxygen (Schweigert et al., 2003). Since RA interacts with nuclear receptors, it has the ability to modulate many gene products linked to reproductive performance (Schweigert et al., 2003; Amann et al., 2011).

In herbivorous ungulates, the largest BC accumulation occurs in the liver with cattle and horses reflecting the highest BC liver content, followed by goats, buffalo and sheep; goats' BC liver concentration is around 3.4 μg/g tissue (Darwish et al., 2016). Even though an optimal intake of BC is hypothesized to affect ruminant reproduction, both negative (Polman et al., 1987) and positive effects have been reported (Kawashima et al., 2009). BC supplementation has been linked to an increased steroidogenesis in both luteal and follicular tissues (Halilloglu et al., 2002; Kawashima et al., 2009, 2010). In addition, BC appears to be a modulating molecule involved in the intermediate metabolism

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(Attia, 2009), while also inhibiting the estrogen-induced transactivation of the estrogen receptor (EREα, β) (Hirch et al., 2007), delineating an interesting role of BC as a modulating molecule for the hypothalamic-pituitary-gonadal (HPG) axis.

In addition, while the gonadotropic system is the key driver of reproductive function (Meza-Herrera, 2008; Scaramuzzi et al., 2011), the somatotropic system (GH/IGF-1) modulates not only metabolic processes but also reproductive function. Since GH and IGF-1 receptors are present on the pituitary gonadotrophs, the ovary, the granulosa cells and the oocytes (Bartke et al., 2013), a possible role emerges for the GH/IGF-1 system as a modulator of the HPG axis in females. GH actions along the reproductive tract may promote effects in a dichotomic fashion, in that stimulation of both synthesis and release of GH could relate to inhibition of the hypothalamic discharge of GnRH (Scaramuzzi et al., 2011). The actions of GH may be direct in target organs or could be mediated by IGF-1 released from hepatocytes in response to GH stimulation (Bartke et al., 2013).

Whereas BC has been found to be an important signaling molecule for positive activation of the HPG axis (Halliugla et al., 2002; Kawashima et al., 2010; Salem et al., 2015), along with its role as an insulin (Meza-Herrera et al., 2011) and triiodothyronine (Meza-Herrera et al., 2014) modulating molecule, BC also has demonstrated a positive action upon selected blood metabolites (Meza-Herrera et al., 2017). Yet the possible effect of BC supplementation upon the GH/IGF-1 system, modulating in turn the HPG axis, has been elusive. The aim of this study was to evaluate the effect of BC supplementation upon ovarian function in adult female goats as well as to obtain possible evidence of BC influences on serum levels of GH and IGF-1.

2. Materials and methods

The methods of this study and the management of the experimental units used in this study were in strict accordance with accepted guidelines for ethical use, care and welfare of animals in research at international (FASS Federation Animal Science Society. 2010) and national (NAM National Academy of Medicine, 2002) levels, with institutional approval reference number UACH-DGIP-REBIZA/11-510-466.

2.1. Location, animals and feeding

The study was carried out at the Regional University Unit on Arid Lands, Chapingo Autonomous University (URUZA-UACH; 26° N, 103° W, at 1,117 m) in northern Mexico. Adult goats [n = 22, LW = 45.35 ± 1.35 kg, 3.5 years old, 7/8 Sannen-Alpine] were fed twice per day to meet their net energy requirements for maintenance (NEm) (NRC National Research and Council, 2007), with alfalfa hay [14% CP, 4.77 net energy for maintenance (NEm MJ kg^{-1})] and corn silage [8.1% CP, 6.78 NEm MJ kg^{-1}] in the morning (0700) and corn grain (11.2% CP, 9.9 NEm MJ kg^{-1}) in the afternoon (1800). Goats had free access to water, shade and mineral salts during the entire experimental period, from October to November. Both LW and BCS were recorded weekly prior to feeding. BCS was determined in all animals by palpation of the transverse and vertical processes of the goats lumbar vertebrae (L2 through L5) on a five- point scale (1 = emaciated, 5 = obese; Aumont et al., 1994) by the same experienced technician.

2.2. Experimental treatments

In early October, goats were randomly distributed in individual pens to form two experimental groups: a) Betacarotene [BC, n = 10; 45.9 ± 1.97 kg live weight (LW), 3.04 ± 0.08 units, body condition score (BCS)], and b) Control, [CONT, n = 12; 46.2 ± 2.04 kg LW, 3.0 ± 0.08 units BCS]. Goats in the BC group were orally supplemented with betacarotene (50 mg $goat^{-1}$ day^{-1}) (Syntex-Roche, Guadalajara Jalisco, Mexico) during the entire experimental period (52 d). Both groups received the same base diet in a mixed-ration offering of 1.0 kg $goat^{-1}$ day^{-1}. Since at the offered level the base diet was completely consumed and because both experimental groups consumed the same quantity of the base diet per goat, the expected intake of dietary BC per experimental unit between treatments was assumed to be the same. The possible effect of consuming versus not consuming supplemental betacarotene by the experimental groups was thereby evaluated.

2.3. Estrus synchronization, blood sampling & quantification of the somatotropic axis hormones

During the second half of October, estrus was synchronized by using intravaginal sponges containing 45 mg of fluorogestone acetate (Chronogest®, Intervet International B.V., Boxmeer, Holland) left in place for 10 d. 9 d after the insertion of the sponge (day −3; day 0 = estrus), goats received a single i.m. dose of 1 mL of a prostaglandin $F_{2\alpha}$ analogue (0.075 mg $goat^{-1}$ of cloprostenol; Prosolvin-C®, Intervet International B. V., Boxmeer-Holland). Thereafter, on day −2, sponges were removed, and 24 h later (day −1) five goats from each group were randomly selected to perform an intensive blood sampling. Blood samples (10 mL) were collected by jugular venopuncture every 15 min for 6-h, starting 3 h after the morning feeding. The intensive blood sampling included 25 samples per goat, 125 samples per treatment, for a total of 250 serum samples per treatment.

Blood samples were collected into sterile vacuum tubes (Corvac, Kendall Health care, St. Louis, MO) and allowed to clot at room temperature for 30 min. Serum was separated by centrifugation (1500 x g, 15 min), decanted and transferred in duplicate to polypropylene micro tubes (Axygen Scientific, Union City, CA, USA) and stored at −20 °C until hormonal analysis. Peripheral serum GH and IGF-1 were determined in duplicate by radioimmunoassay (RIA). Concentrations of GH were determined in all samples in a single RIA assay (Hoefler and Hallford, 1987); intra-assay CV was 9.4%, and a detection limit of 0.2 ng mL^{-1}. The area under the curve (AUC) for GH was calculated using a trapezoidal summation procedure, while the pulsatility was characterized using the Cluster Pulse Analysis Program considering a 16.2% CV, a 0.95 S.D., and a detection limit of 0.3 ng mL^{-1} (Veldhuis and Johnson, 1986). Because the release of IGF-1 is non-episodic, samples collected at two hour intervals were considered to evaluate serum IGF-1 concentrations by double antibody RIA previously described (Berrie et al., 1995), using primary antisera and purified standard and iodination preparations supplied by the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, USA). An assay of total IGF-1 was conducted after acid-ethanol inactivation of binding proteins and resulted in intra- and inter-assay CV of 12 and 15, respectively, and a detection limit of 0.2 ng mL^{-1}.

2.4. Ultrasonographic scanning of ovarian function: follicular growth & ovulation rate

To evaluate ovarian activity, on day 17 post-estrus, towards the end of the luteal phase, an ultrasonographic scan was performed by a single skilled operator using a 7.5 MHz linear-array transducer for veterinarian use (Toshiba Medical Systems, Ltd, Crawley, UK). The total number of antral follicles (AF) and corpus luteum (OR) observed in each ovary were recorded and the structures were measured according to procedures previously outlined (Dickie et al., 1999). Ovaries were visualized at an image magnification of 1.5×, and number and size of antral follicles (> 5 mm) and corpus luteum, were registered. The total ovarian activity (TOA) was defined as the sum of AF and OR recorded in each animal (Fig. 1).

2.5. Statistical analyses

The ovarian variables AF, OR, TOA and GH-AUC were compared considering a CRD-ANOVA. Due to the non-parametric nature of GH

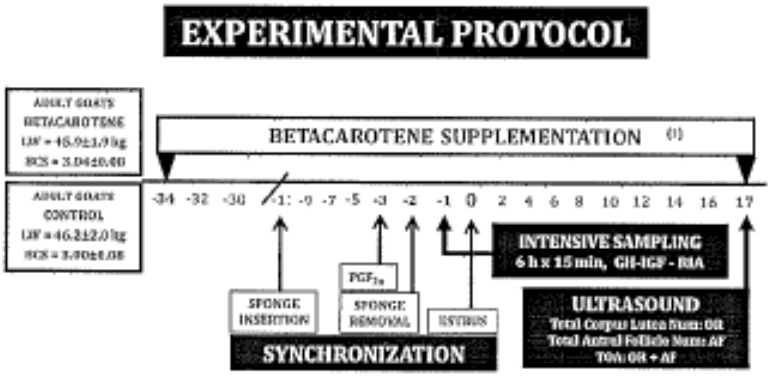

Fig. 1. A schematic representation of the procedure for estrous cycle synchronization, betacarotene supplementation, intensive blood sampling and ultrasound scanning in adult crossbred goats (n = 22), Control (CONT) or supplemented with Betacarotene under natural photoperiodic conditions in northern Mexico (October-November, 28° LN). Note: Once synchronized with the use of intravaginal sponges containing 45 mg of fluorogestone acetate (d–10), goats received a single i.m. dose of 1 mL of a prostaglandin $F_{2\alpha}$ analogue (d −3); on day −2, sponges were removed; and 24 h later (d–1) an intensive blood sampling (every 15 min × 6 h) for GH and IFG-1 serum quantification was performed 36 h prior to the estrus day (d 0). On day 17 post-estrus, an ultrasonographic scan was performed to relate the GH and IGF-1 secretion pattern and ovulation rate (OR), measured as number of corpora lutea present in each ovary; additionally, the antral follicle number (AF) was also recorded to define total ovarian activity (TOA = OR + AF).

(1) Oral supplementation, 50 mg/goat/day

pulse frequency, GH-PULSE was analyzed using the Kruskal–Wallis test. LW and BCS as well as serum GH and IGF-1 concentrations across time were determined by split-plot analysis of variance for repeated measures. The models included treatment in the main plot, which was tested using goat within treatment as the error term. Time and the time x treatment interaction were included in the subplot and were tested by using the residual mean square (Littell et al., 1998). When significant F values were observed, mean separation was conducted using comparisons generated from the least-square mean procedure (PDIFF option) from the PROC GLM. All statistical analyses were computed using the procedures of SAS (SAS Inst. Inc. Cary, NC, USA). Since no treatment by time interactions were observed regarding either GH or IGF-1, the overall average per treatment across time per hormone are described. Pearsons correlations were used to evaluate the association among LW, BCS and the OR. Reported values are defined as least-square mean ± SE; the most conservative SE is presented.

3. Results

Least-square mean for live weight (LW, kg), body condition score (BCS, units), antral follicles (AF, units), corpus luteum number (OR, units), total ovarian activity (TOA = AF + OR) and serum concentrations of growth hormone (GH, ng mL^{-1}) and insulin-like growth factor-1 (IGF-1, ng mL^{-1}) are shown in Table 1. Average LW and BCS at the beginning and the end of the study were 45.35 ± 1.4 kg, 2.97 ± 0.08 units, and 45.05 ± 1.4 kg, 3.27 ± 0.08 units, respectively. Hence, no differences were found, neither at the beginning ($p > 0.05$) nor during the entire ($p > 0.05$) experimental period between groups. Yet, increases in ovulation rate (3.4 vs. 2.8 ± 0.2 units; $p = 0.05$), number of antral follicles (5.0 vs. 3.4 ± 0.6 units; $p = 0.05$) and total ovarian activity (8.4 vs. 6.2 ± 0.6 units; $p = 0.05$) were observed in the BC-supplemented group. Interestingly, the lowest values of serum GH average concentrations (10.0 vs. 14.3 ± 1.0 ng mL^{-1}; $p = 0.01$) and GH-AUC (3670.4 vs. 5235.7 ± 369.8 units; $p = 0.05$) were observed in the BC-supplemented group. However, neither differences for serum IGF-1 concentrations (254.6 ± 28.9 ng mL^{-1} $p > 0.05$) nor for GH-PULSE (1.4 ± 0.5 pulses $6\,h^{-1}$ $p > 0.05$), occurred between treatments (Table 1). In addition, a positive correlation between live weight ($r = 0.42$; $p = 0.04$) and body condition score ($r = 0.47$; $p = 0.02$) with respect to ovulation rate was observed in our study.

Table 1
Least-square means for live weight (LW, kg), body condition score (BCS, units), antral follicles (AF, units), total corpus luteum (OR, units) and total ovarian activity (TOA, AF + OR, units) at ultrasound evaluation as well as serum growth hormone concentrations (GH, ng mL^{-1}), GH area under the curve (GH-AUC, arbitrary units), GH pulsatility (GH-PULSE, units every 6 h) and insulin-like growth factor (IGF-1, ng mL^{-1}) in adult crossbred goats (n = 22), Control (CONT) or supplemented with Betacarotene (BC) under natural photoperiodic conditions (October-November, 28° LN).

	Treatments			
	BC	CONT	p-value	SE*
LW, (kg)	45.8	46.3	0.80	1.46
BCS, (units)	3.25	3.30	0.80	0.08
AF, (units)	5.0	3.4	0.05	0.6
OR, (units)	3.4	2.8	0.05	0.2
TOA (AF + OR)	8.4	6.2	0.05	0.6
GH, (ng mL^{-1})	10.0	14.3	0.01	1.0
GH-AUC, (units)	3670.4	5235.7	0.01	369.8
GH-PULSE (units)	1.6	1.2	0.62	0.6
IGF-1, (ng mL^{-1})	261.1	248.2	0.75	028.9

* SE, most conservative standard error is presented.

4. Discussion

Current results support our working hypothesis in that BC-supplementation improved ovarian activity in adult goats with increases in both ovulation rate and antral follicle number, which paralleled decreases in GH serum concentration, yet without differences in serum IGF-1 between treatments. Interestingly, since BC-supplementation continued up to d-17 post-estrus, the observed effect of such dietary supplementation upon antral follicle number can be defined as an "acute effect" of BC administration on the growth of the AF population, suggesting a physiologic ovarian scenario prone to an increased follicular steroidogenesis. Our findings suggest a potential positve role of BC supplementation during the aromatization process of the antral follicles, a steroidogentic scenario previously observed (Salem et al., 2015). Moreover, an intrincate association has been proposed between serum antioxidants and endogenous hormones, supporting the hypothesis that concentrations of serum vitamins affect steroidogenesis even after adjustment for oxidative stress (Mumford et al., 2016). Undoubtedly, the precise site of action engaged by BC-supplementation throughout the HPG axis cannot be established without further research.

In addition, changes in metabolic status are strongly related to fluctuations in both live weight and body condition score (Meza-Herrera et al., 2007, 2008; Scaramuzzi et al., 2011). Yet no differences ($P > 0.05$) occurred regarding LW or BCS between treatments. Thus, other endocrinological or metabolic pathways should be involved in the different ovarian outcome observed. Fluctuations in blood concentrations of metabolic hormones are important signals that inform the nutritional status of mammals (Meza-Herrera et al., 2011, 2014). A possible explanation is that the response to supplemental feeding alters the glucose-insulin system (Scaramuzzi et al., 2011), leptin or IGF-I (Gamez-Vazquez et al., 2008; Guerra-García et al., 2009), and probably other reproductive and metabolic hormones (Meza-Herrera et al., 2004; Scaramuzzi et al., 2011) as well as genomic cues (Meza-Herrera et al., 2010a,b).

In ruminants, different studies have shown a direct relationship between serum BC concentration, ovarian function and reproductive performance. In beef cattle, BC supplementation affected the size of corpora lutea and the level of progesterone secretion (Haliloglu et al., 2002). Also, in adult goats, short-term BC supplementation positively affected ovarian follicular development and ovulation rate under short-day photoperiods (Arellano-Rodriguez et al., 2007), as well as ovarian and luteal function and progesterone secretion (Arellano-Rodriguez et al., 2009). Also, a positive relationship between serum BC concentrations and ovarian activity during the first follicular wave was reported in bovine animals (Kawashima et al., 2009), while a positive relationship among BC supplementation, serum retinol concentration, serum gammaglutamyl transpeptidase concentration and luteal activity occurred in dairy cattle (Kawashima et al., 2010).

In addition, in peri-puberal female goats, long-term BC supplementation positively affected the release pattern over time of the metabolic hormone triiodothyronine (Meza-Herrera et al., 2014). BC supplementation also promoted increases in serum estradiol concentrations and estrus percentage in sheep (Salem et al., 2015). Increases in litter size and number of piglets born alive were observed in BC-supplemented sows (i.m.), suggesting that this scenario was most likely BC-specific and independent from the BC role as a vitamin A precursor (Krammer and Aurich, 2010), an important finding that also supports our results. In the ovary, nutrition stimulates follicular growth associated with both systemic and intra-follicular alterations in the insulin-glucose and IGF-1-leptin systems (Scaramuzzi et al., 2011). Interestingly, no significant associations between intake of BC from food, supplements or both upon IGF-1 or IGFBP-3 concentrations were observed in humans (Tran et al., 2006).

Finally, and considering a translational perspective, since GH promotes cell proliferation and angiogenesis while inhibiting apoptosis, the GH/IGF-1 system has been associated in mammals with the development and/or progression of several types of cancers (Kopchick et al., 2014). Moreover, elevated GH levels have been related to signs of premature aging and reduction of lifespan. Although such premature deaths have been diagnosed as multifactorial in origin, tissue-specific pathological organ damage has been constantly present, with an augmented incidence of tumors (Bartke et al., 2013; Kopchick et al., 2014). Acting through autocrine-paracrine-endocrine routes, increased GH levels have been related to oncogenesis; thus, an attempt to reduce the GH/IGF-1 action under some pathological scenarios could theoretically provide protection from certain types of cancers (Pollack et al., 2001; Kopchick et al., 2014). These findings, merged with our results, open interesting possibilities in the search of possible translational applications considering the GH-lowering effect of BC-supplementation observed in our study; further research considering this approach poses an interesting assignment.

To conclude, our data document that betacarotene supplementation generated an increase in ovarian activity and ovulation rate considering the adult goat as a model. Notably, this physiologic scenario involved a decrease in serum GH, yet without effect on IGF-1 levels. Our study unveils for the first time a potential role of BC as a somatotrophe-modulating molecule. The precise site of BC action throughout the HPG axis in females awaits to be established, this being an important line of investigation for the animal industry and with potential clinical translational implications.

Conflict of interest

The authors declare that there are no conflicts of interest that would prejudice the impartiality of this scientific work.

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5. CONCLUSIONES GENERALES

La suplementación con Betacaroteno incrementa la actividad esteroidogénica y consecuentemente el desarrollo folicular y la tasa ovulatoria en cabras promoviendo así una función ovárica óptima. Tales efectos del betacaroteno se observaron a pesar de una reducción en la liberación pulsátil de LH. Mediante el presente proyecto de tesis se pudo demostrar que la suplementación con betacaroteno genera aumentos séricos en metabolitos sanguíneos tales como las proteínas totales, glucosa y colesterol; e incrementa la secreción pulsátil de progesterona, además de que se pudo revelar que la suplementación de betacaroteno disminuye la GH sérica, pero sin efecto sobre los niveles de IGF-1. Estos hallazgos pueden ser de gran importancia tanto desde el punto biológico como económico, así como también pueden generar importantes aplicaciones prácticas en otras industrias animales.

Debido a nuestro conocimiento fragmentario, se necesitan más estudios para comprender mejor las acciones fisiológicas precisas del betacaroteno en diferentes resultados reproductivos y escenarios fisiológicos, otros estudios destinados a dilucidar los efectos gonadotrópicos o metabólicos específicos de betacaroteno a nivel ovárico contribuirían a una mejor comprensión de la activación del eje hipotalámico-hipofisario-gonadal en la cabra. Esta es una importante línea de investigación para la industria animal y con posibles implicaciones traslacionales de relevancia clínica.

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