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ASPECTOS DE LA FISIOLOGÍA DE LA ADAPTACIÓN DE LOS JUVENILES DE *Litopenaeus vannamei:* UNA PERSPECTIVA INMUNOLÓGICA

T E S I S

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p r e s e n t a :

CRISTINA PASCUAL JIMÉNEZ

Director de Tesis: Dr. Carlos Rosas Vázquez

Comite Tutoral:
Dra. Ana Margarita Trigo de Sousa Roque
Dr. Edgar Zenteno Galindo

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Prefacio

La presente tesis está basada en los siguientes trabajos:

Artículos publicados:

1. Pascual, C., G. Gaxiola, C. Rosas. 2003. Blood metabolites y hemocyanin of the shrimp *Litopenaeus vannamei*: the effect of culture condition and a comparison whit other crustacean species. Marine Biology. 142: 735-745.

2. Pascual, C., L. Arena, G. Cuzon, G. Gaxiola, G. Taboada, M. Valenzuela y C. Rosas. 2004. Effect of a size-based selection programm on blood metabolites and immune response of *Litopenaesu vannamei* juveniles fed with different dietary carbohydrates levels. Aquaculture 230: 405-416.

3. Pascual, C., Zenteno, E., Cuzon, G., Sánchez, A., Gaxiola, G., Taboada, G., Suárez, J., Maldonado, T., y Rosas, C. 2004. Energetic balance and immunological responses of *Litopenaeus vannamei* juveniles to dietary proteins. Aquaculture. 236: 431-450.

4. Pascual, C., Sánchez, A., Zenteno, E., Cuzon, G., Gaxiola, G., Brito, R., Gelabert, R., Hidalgo, E. y Rosas, C. Biochemical, physiological, and immunological changes during starvation in juveniles of *Litopenaeus vannamei*. Aquaculture. Aceptado.

Artículo en colaboración:

5. Arena, L., Cuzon, G., Pascual, C., Gaxiola, G., Suyes C., VanWormhoudt, A., Rosas, C. 2003. Physiological and genetic variations in domesticated and wild populations of *Litopenaeus vannamei* fed with different carbohydrates levels. Journal of Shellfish Research 22-1: 269-279.

Trabajos presentados en congresos:

1. Pascual C., Rosas C., Gaxiola G. y L. Arena. Respuesta fisiológica de los juveniles de *Litopenaeus vannamei* silvestres y cultivados, alimentados con bajos y altos (3 y 44 %). VI Simposium Internacional de Nutrición Acuícola, Cancún, Quintana Roo, 3 al 6 de septiembre de 2002.

2. Pascual, C., Sánchez, A., López, N., Martínez, G., Hidalgo, M., Arévalo, M., Taboada, G., Gaxiola, G., y C. Rosas. Las proteínas dietéticas modulan el estado nutricional y la respuesta inmunitaria de *Litopenaeus vannamei* (Crustacea: Decapoda: Penaeidae). X Congreso Latinoamericano de Ciencias del Mar, San José, Costa Rica del 22 al 26 de septiembre de 2003.

3. Pascual, C. y L. Arena. Efecto de la domesticación sobre el estado de salud del camarón blanco *Litopenaeus vannamei*. V Semana de Biología Molecular y Biotecnología. Benemérita Universidad Autónoma de Puebla. Noviembre de 2003.

4. Pascual, C., Sánchez, A., Gaxiola, C., y Rosas, C. Indicadores de la condición fisiológica del camarón blanco *Litopenaeus vannamei*. 1ª Reunión Regional de Estudiantes de Posgrado en Ciencias Biológicas. Centro de investigaciones Científicas de Yucatán, 3 de Noviembre de 2004.

5. Pascual, C., Sánchez, A., Suárez, J., Cuzon, G., Gaxiola, G. y Rosas, C. Energetic metabolism and immune response of Litopenaeus vannamei juveniles to dietary protein: feeding and starving conditions. VII Simposium Internacional de Nutrición Acuícola, Hermosillo, Sonora, México del 16 al 19 de noviembre de 2004.

Abreviaturas

aa	aminoácidos
AAL	pool de aminoácidos libres
A-CoA	acetil-Coenzima-A
B	branquias
C	intermuda
C	cutícula
CAG	ciclo de los ácidos grados
CG	ciclo del glutamato
CK	ciclo de Krebs
CR	cadena respiratoria
CHO	carbohidratos
FO	penoloxidasa
GDH	glutamato desidrogenasa
GL	glucólisis
H	hemolinfa
HHC	hormona hiperglicemiante
LP	lipoproteínas
LPS	lipopolisacaridos
LT	lectina
M	músculo
Oxi	oxihemocianina
PAM	péptidos antimicrobianos
PD	proteínas dietéticas
ProFO	profenoloxidasa
SN	sistema nervioso
ßG	beta-glucanos
TH	tejido hematopoyético
TPH	tasa de proliferación de hemocitos

Abreviaturas (por sus siglas en inglés)

A	asimilated energy
AG	acylglycerids
AM	adult males
ARB	respiratory burst activated
BRB	respiratory burst basal
BWd	body weight/day
C	inter.-molt stage
CP	crude protein
CP	clotein protein
CHH	hyperglycemic hormone
CHO	carbohydrate
DG	digestive gland
DGC	daily growth coefficient
DGG	digestive gland glycogen
DGT L	digestive gland total lipids
DP	dietary proteins
DPL	dietary protein levels
FCR	feed conversion ratio
FP	females from ponds
FW	from wild populations
GC	granular cells
H	heces
HC	hemocyanin
[HC]	hyaline cells
HCHO	high carbohydrate
HP	high protein
I	ingestion rate
J	juveniles
LCHO	low carbohidrate
LP	low protein
LPSBP	lipopolysaccharide binding protein
OC	osmoregulatory capacity
OP	osmotic pressure
Oxy	oxyhemocyanin
P	production
PO	phenoloxidase
PpA	prophenoloxidase-activing enzyme
ProPO	prophenoloxidase
R	respiratory rate
R_{AHI}	repiraton of procces ingested food
R_{RUT}	respiration of rutine metabolic
ßGBP	beta glucans binding protein
TAG	triacylglycerols
TCA	trichloroacetic acid
TH	total hemocytes
U	urine products

CONTENIDO

Resumen

La presencia y la diseminación de las enfermedades infecciosas representan el punto de mayor riesgo para el cultivo de camarón debido a la falta de un diagnóstico preventivo y de tratamientos eficientes. Los componentes de la hemolinfa han sido ampliamente utilizados para determinar el estado fisiológico de los crustáceos, pero poco se conoce acerca de los patrones de variación a nivel poblacional. En el presente estudio se elaboró una base de datos para algunos componentes de la hemolinfa de juveniles de *Litopenaeus vannamei*. El grupo de datos se construyó con mínimo 60 camarones en intermuda por condición de cultivo (experimental, 90 l, y piloto comercial, 20 t). Los resultados indican que los componentes de la hemolinfa derivados del metabolismo intermediario (proteínas, glucosa, acilglicéridos y lactato), reflejan las rutas metabólicas involucradas en el aprovechamiento y el uso de la energía. La concentración de la oxihemocianina (OxiHc) señala la reserva de proteínas en la hemolinfa y esta relacionada a la ruta gluconeogénica. Se compararon estos datos con los reportados en la literatura para *L. vannamei* y otros crustáceos. Las variaciones en los componentes de la hemolinfa presentaron una relación con la actividad, consumo de oxígeno, amplitud metabólica y el grosor del caparazón. En este sentido, los componentes de la hemolinfa parecen reflejan las adaptaciones fisiológicas de los crustáceos, por ser el resultado de la demanda de energía de los organismos. Desde el punto de vista aplicativo la hemolinfa de los camarones es fácil de obtener y sus componentes brindan importante información biológica, por lo cual, podría utilizarse para monitorear la condición fisiológica de organismos silvestres y cultivados.

El estudio de los componentes de la hemolinfa de los camarones silvestres y cultivados (F_7) provenientes de un programa de selección por tamaño señaló los ajustes fisiológicos asociados al proceso de domesticación, y a las transformaciones de la energía derivada del alimento. El estudio de los juveniles cultivados indica una reducción en la capacidad para aprovechar mayores

niveles de carbohidratos en la dieta (44 % CHO), lo cual afecta el estado nutricional e inmunológico de los camarones. Los juveniles domesticados metabolizan preferentemente a las proteínas, lo cual representan una mayor dependencia de este nutriente después de varias generaciones.

El estudio del balance energético, el estado nutricional y la respuesta inmunitaria en juveniles de *L. vannamei* (F_9) alimentados con 5, 15 y 40 % de proteínas dietéticas (PD) indica el carácter central del catabolismo de las proteínas en los camarones cultivados y seleccionados por tamaño, ya que el crecimiento, las reservas y el estado inmunológico de los juveniles de *L. vannamei* estuvieron asociados al nivel de las proteínas en la dieta. La concentración de los hemocitos circulatorios y la actividad fagocítica de los juveniles varió de manera directa y significativa ($P < 0.05$) con el nivel de proteínas dietéticas. Cuando los organismos fueron alimentados con 5 % de PD lograron mantenerse e incorporar una ligera biomasa durante los 50 días experimentales. No obstante, la energía asimilada fue insuficiente para mantener al sistema inmunitario (cantidad significativamente menor de hemocitos, actividad de fenoloxidasa y estallido respiratorio), y también, para generar reservas (bajos niveles de glucógeno en la glándula digestiva y OxiHc). El estudio realizado con organismos en ayuno prolongado (21 días) permitió señalar que las proteínas dietéticas, aportadas previamente (5 y 40 %), están asociadas con el manejo de las reservas y que esto afecta el estado nutricional e inmunológico de los camarones.

Tomando esto en consideración, se elaboró un modelo conceptual que integra el aprovechamiento del alimento de organismos silvestres y cultivados, las rutas metabólicas y diversos mecanismos fisiológicos involucrados en el mantenimiento de la homeostasis de los juveniles de camarón. El esquema señala el carácter central del metabolismo de proteínas en los juveniles de *L. vannamei*, e incluye las principales vías metabólicas, sus interconexiones y productos, lo cual representa el flujo de la energía química desde el alimento hacia los procesos fisiológicos, la incorporación de biomasa, la síntesis de hemocitos y la generación de reservas.

Abstract

Presence and dissemination of the infectious diseases represent the point of major risk for the culture of shrimp due to the absence of a preventive diagnosis. The components of the hemolymph have been widely used to determine the physiological state of the crustaceans, but little is known about distribution levels of metabolites in a population. In the present study it was elaborated a baseline for some components of the hemolymph of the juvenile of *Litopenaeus vannamei* in intermolt stage. Statistical analyses were done with minimum 60 shrimps cultured indoor (90 l tanks) and outdoor, (20 t ponds). The results indicate that proteins, glucose, acylglycerol and lactate reflect the metabolic routes involved in the use of the energy. The concentration of the oxyhemocyanin (OxyHc) indicates the reserves of proteins in the hemolinfa, which is related to the catabolism of proteins, oxygen demand and the glyconeogenic pathway of the shrimps. The values obtained were compared with blood metabolites reported in the literature for *L. vannamei* maintained under similar conditions, and with other crustaceans. The changes in the components of the hemolinfa presented a relation with the activity, consumption of oxygen, metabolic largeness and the thickness of the shell. Possibly the components of the hemolymph and OxyHc could be reflecting the morphological and physiological adaptations of the crustaceans, for being the result of energetic demands. From the point of view applicative the hemolymph of the shrimps it is easy to obtain and his components offer important biological information, for which, there could be used for determine the physiological condition of wild and cultivated organisms.

In the present investigation the components of the hemolinfa reflected the physiological adjustments associated with the process of domestication, and to the transformations of the energy derived from the food. The study of the juvenile proveniences of a program of selection for size (F_7) indicated a reduction in the aptitude to use of major levels of carbohydrates in the diet (44 % CHO).These

organisms metabolize preferably to the proteins, which they represent a major dependency on this nutrient after several generations.

The growth, the reserves and the immunological state of the juvenile ones of L. vannamei were directly associated at the level of the diet proteins (5, 15 and 40 % DP). The concentration of the circulatory hemocytes and the activity of phagocytosis (respiratory burst) of the juvenile changed in a direct and significant way ($P < 0.05$) at the level of dietetic proteins. When the organisms were fed by 5 % of DP they survived and incorporated a light biomass (50 experimental days). Nevertheless, the assimilated energy was insufficient to support to the immunological system (significantly minor quantity of hemocytes, activity of phenoloxidase and respiratory burst), and also, to generate reserves (low levels of glycogen in the digestive gland and OxyHc). The studies realized with organisms in long fasting (21 days) allowed to demonstrate that the dietetic proteins, previously feed (5 and 40 %) are associated with the use of the reserves, determining the nutritional and immunological state of the shrimps.

Taking this in consideration, there was elaborated a conceptual model that integrates the metabolic routes and diverse physiological mechanisms involved in the maintenance of the homeostasis of the shrimp wild and cultivated (F7 y F9). The scheme indicates the central character of the metabolism of proteins in juveniles of *L. vannamei*. The model includes the principal metabolic routes, his interconnections and products, which represent the flow of the chemical energy from the food towards the physiological processes, the incorporation of biomass, the synthesis of hemocytes and the generation of reserves.

Introducción

El cultivo de camarón es una actividad económicamente importante a nivel mundial que actualmente enfrenta una crisis debido a la aparición y diseminación de enfermedades infecciosas (Hossain et al., 2001; Chamberlain, 2002). La experiencia generada en los sistemas de cultivo señala que los brotes de camarones enfermos generalmente son el resultado de la coinfección de diversos patógenos y, que en muchos de los casos, están asociados con alteraciones ambientales (Lightner y Redman, 1998; Le Moullac y Haffner, 2000a).

El desarrollo de un proceso infeccioso es generado por la ruptura del equilibrio entre patógeno, hospedero y ambiente. Este equilibrio está directamente vinculado a los procesos fisiológicos, que permiten canalizar la energía del alimento hacia los ajustes metabólicos, originados por la interacción dinámica entre el organismo y su ambiente (Síma y Vétvicka, 1990). La aclimatación de los camarones a los sistemas de cultivo implica una constante compensación fisiológica para enfrentar las fluctuaciones ambientales, entre las que sobresalen importantes cambios en los parámetros fisicoquímicos, en la calidad nutricional del alimento y los asociados al manejo de altas densidades (Costa et al., 1998; Rodríguez et al., 2000; Dias, 2000).

Aunque mucho se ha avanzado en el conocimiento de las condiciones óptimas de cultivo y los requerimientos nutricionales del camarón blanco *Litopenaeus vannamei* (Rosas et al., 1995a; Shiau, 1998; Lignot et al., 1999; Palacios, 1999; Sánchez, 2001), la aparición de enfermedades sigue representando una merma en el crecimiento, y continúa generado cuantiosas pérdidas por las altas mortalidades (Bachére et al., 1995a; Rodríguez y LeMoullac, 2000). Con el fin de evitar las infecciones, en muchos casos se adicionan al alimento antibióticos, pero su uso frecuente puede propiciar resistencia por parte de los patógenos, además de bioacumularse en el camarón y generar efectos ambientales colaterales (Sun

et al., 1996; Rengpipat et al., 1998). Adicionalmente, el origen viral de muchas de las infecciones de los camarones limitan la eficiencia de tales tratamientos (Le Moullac et al., 1998b).

Un mayor conocimiento de los procesos inmunológicos asociados a la bioquímica fisiológica permitiría identificar las mediciones que pudieran proporcionar mayor información a cerca del estado fisiológico, el cual a su vez, pudiera ser utilizado para determinar la susceptibilidad de los organismos ante ciertas condiciones de cultivo o alguna enfermedad (Bachére et al., 1995b; Lightner y Redman, 1998; Le Moullac et al., 1998a).

Al respecto, diversas investigaciones han propuesto algunos componentes de la hemolinfa como indicadores de la condición fisiológica de los camarones. Las variaciones de la capacidad osmótica y la concentración plasmática de metabolitos han sido utilizadas para determinar el estado fisiológico en relación con la talla, el estadio de muda y la presencia de contaminantes (Lin y Ting, 1994; Lignot et al., 1997), el oxígeno disuelto (Charmantier et al., 1994), la calidad de los reproductores (Palacios et al., 1999; Pascual, 2000; Racotta y Hernández-Herrera, 2000; Sánchez et al., 2001) y el tipo de alimentación (Rosas et al., 2000; 2001b; 2002).

No obstante, en la mayoría de las investigaciones, la fisiología y la inmunología de los camarones han sido estudiadas por separado, y por ello, la vinculación entre los procesos metabólicos e inmunológicos no ha sido contemplada. Bajo este enfoque, el presente trabajo fue diseñado para abordar de manera integral aspectos metabólicos, energéticos e inmunológicos de los juveniles de *Litopenaeus vannamei* asociados con el proceso de domesticación, la condición nutricional y dos tipos de cultivo (condiciones controladas, 90 l, y escala piloto comercial, 20 t).

Antecedentes

1. Estado actual y perspectivas del cultivo de camarón

El cultivo de camarón ha representado una buena alternativa ante la disminución de la pesca en altamar. En el 2002 de las 100,478 toneladas de camarón producido en México, el 45.63 % fue cultivado, lo cual revela que la camaronicultura en nuestro país ha ido adquiriendo mayor participación en el suministro de este recurso (CONAPESCA, 2002; Caro Ros, 2003). El camarón blanco del Pacífico, *Litopenaeus vannamei*, es la principal especie cultivada en América y la segunda a nivel mundial (Benzie, 2000). No obstante, se considera que para mantener e incrementar su producción, es necesario controlar la presencia de enfermedades (Bachére et al., 1995b; Le Moullac y Haffner, 2000a).

La diseminación de infecciones representa el punto de mayor riesgo durante el cultivo debido a la falta de un diagnóstico preventivo y de tratamientos eficientes (Bachére et al., 2000; Rodríguez y LeMoullac, 2000). Diversas investigaciones señalan que la modificación de los parámetros ambientales, como temperatura y salinidad, junto con un estado nutricional deficiente, pueden incrementar la susceptibilidad de los camarones hacia las infecciones (Lightner, 1999; Lignot et al., 2000; Rodríguez et al., 2000).

La evaluación continua de patógenos específicos en los camarones cultivados, principalmente virus y bacterias, indica que los organismos pueden estar infectados, pero no necesariamente enfermos (Lightner y Redman, 1998). Este tipo de pruebas ha permitido desarrollar cepas de camarones resistentes o libres de patógenos específicos, pero la presencia de un nuevo agente infeccioso o un cambio es su virulencia suele cambiar de manera significativa la susceptibilidad y la supervivencia (Bedier et al., 1998). Por esto, los avances en los programas de domesticación en camarones peneidos, señalan que los criterios de selección de poblaciones cultivadas deben incluir pruebas moleculares para evaluar la

variabilidad genética, y también, indicadores fisiológicos e inmunológicos que permitan conocer la capacidad de autorregulación que los camarones despliegan ante los diversos ambientes de cultivo, incluyendo el aprovechamiento del alimento, las variaciones ambientales o la resistencia a patógenos (Vargas-Albores et al., 1998; Arena et al., 2003).

2. *Adaptaciones fisiológicas*

Dentro del marco de la teoría evolucionista, la adaptación ambiental ocurre de forma extremadamente lenta en una especie, a través de millares de generaciones, y depende de la adquisición de información genética que codifique para un nuevo y adaptado fenotipo (Darwin, 1859; Torres, 1995). Por otro lado, las adaptaciones fisiológicas se refieren a los ajustes funcionales, los cuales favorecen la actividad biológica normal en un ambiente alterado o estresado (Newell, 1976). Hay numerosos casos de adaptaciones fisiológicas que ocurren dentro del tiempo de vida de un individuo y que normalmente requieren de horas o de meses para completarse. Estos procesos son denominados como ambientación o aclimatización cuando se trata de un cambio fisiológico, bioquímico o anatómico de un organismo expuesto a una nueva condición ambiental, la cual es causada por una alteración en su entorno natural. Durante la aclimatación se observa el mismo proceso pero los cambios son inducidos, ya sea en condiciones de laboratorio o de campo (Prosser, 1950).

Durante su etapa juvenil los camarones se desarrollan en estuarios, lo cual significa que están adaptados para compensar súbitos y frecuentes cambios de salinidad, de temperatura y de oxígeno disuelto. Bajo condiciones tan fluctuantes el sostenimiento de la toma de O_2 permite a los organismos mantener la actividad metabólica para compensar otros cambios ambientales. La capacidad oxireguladora de algunos decápodos está relacionada con el incremento de la tasa de ventilación, elevada presión sanguínea y mayor concentración del pigmento respiratorio en la hemolinfa, lo cual permite en su conjunto una mayor

transferencia de O_2 (Hochachka, 1970). Algunas especies de camarones, como *L. setiferus* y *L. schmitti*, cambian la capacidad oxireguladora en función de la salinidad (Rosas et al., 1995b), lo cual revela el efecto sinérgico de algunos de los principales factores fisicoquímicos sobre los mecanismos compensatorios y el mantenimiento de la homeostasis (Newell, 1976).

La capacidad de osmorregulación está directamente relacionada con la distribución de los organismos acuáticos, con su ciclo de vida y con su comportamiento reproductivo. El punto isosmótico de la mayoría de las especies de camarones estudiadas hasta ahora es cercano a 25 UPS, lo cual señala que la gran mayoría de las especies de camarones están adaptadas para aprovechar los recursos en los estuarios, donde los recursos alimenticios y el ambiente son propicios para el crecimiento de estos organismos (Brito et al., 2000).

El estudio del balance iónico de los camarones peneidos ha recibido gran atención pues durante un tiempo se creyó que la salinidad de los estanques de cultivo debería coincidir con el punto isosmótico para disminuir el costo energético asociado a la regulación iónica y osmótica. Sin embargo, el mejor crecimiento en muchas especies se observa a una salinidad distinta a su punto isosmótico (Brito et al., 2000), lo cual ha generado una controversia alrededor del costo energético de los mecanismos osmorreguladores y su relación con el metabolismo. Al parecer, la salinidad óptima para el crecimiento esta más bien asociada al metabolismo de proteínas debido a la participación de los aminoácidos libres en la regulación y mantenimiento del volumen celular (Claybrook, 1983).

Aunque el cambio inicial en la osmolaridad de la célula es generado por el transporte de iones, muchos de los ajustes osmóticos se deben a las variaciones en la concentración de los aminoácidos intracelulares (Dall, 1975). En crustáceos, la principal contribución es realizada por la glicina, alanina, prolina y glutamato (Claybrook, 1983). La enzima relacionada con la regulación del volumen celular

es la glutamato deshidrogenasa (GDH), la cual controla la incorporación o remoción del amonio desde el "pool" de aminoácidos libres al glutamato (Rosas et al., 2002). Bajo condiciones de estrés metabólico como hipoxia (Speed et al., 2001), inanición (Hagerman, 1983; Dall y Smith, 1986) o presencia de contaminantes (Chen y Cheng, 1993a; 1993b) el catabolismo de los aminoácidos libres está directamente relacionado con el abastecimiento de energía, y por lo tanto, con los mecanismos homeostáticos que permiten mantener la constancia relativa del medio interno de los camarones, revelando el carácter central del metabolismo de las proteínas en los procesos de adaptación de los organismos.

3. Sistema inmunitario

El sistema inmunitario participa en el mantenimiento de la integridad biológica de cualquier organismo, ya que permite reconocer y neutralizar a moléculas nocivas propias o extrañas, provenientes del ambiente o de los procesos fisiológicos. Se considera que la inmunidad de los invertebrados es muy simple ya que carece de algunos mecanismos presentes en los vertebrados, como son: inmunoglobulinas y memoria inmunológica. No obstante, los invertebrados actuales constituyen uno de los grupos más antiguos y diversificados en el reino animal, por lo cual, podemos considerar que representan una importante suma de estrategias exitosas contra las infecciones (Dunn, 1986; Karp, 1990).

Los mecanismos de defensa de los invertebrados incluyen ambos componentes; barreras físicas pasivas y una respuesta activa contra organismos invasores. En los crustáceos las barreras físicas pasivas, están representadas por el rígido exoesqueleto y la membrana peritrófica que envuelve el bolo alimenticio para proteger el epitelio del sistema digestivo (Dunn, 1990), mientras que la respuesta activa implica normalmente un rápido cambio en el número de hemocitos, y la síntesis de nuevas proteínas en la hemolinfa (Destoumieux et al., 2000; Johansson et al., 2000). En adición, mecanismos hemostáticos reparan las heridas en el

integumento para limitar la extensión de posibles infecciones por medio del proceso de coagulación (Gillespie y Kanost, 1997).

Los crustáceos poseen un sistema circulatorio abierto. El corazón distribuye la hemolinfa a través de arterias hasta llegar a senos, en donde los diferentes sistemas son bañados por la hemolinfa (Bayne, 1990). Este sistema permite que las proteínas de reconocimiento en la hemolinfa y los hemocitos circulatorios tengan una mayor probabilidad de encontrar a los elementos extraños. Por otro lado, la presencia de hemocitos fijos en tejidos como las branquias y la glándula digestiva de diversos crustáceos significa una elevada capacidad para aislar a los agentes infecciosos en los lugares de mayor contacto con el medio (Smith y Ratcliffe, 1980; van de Braak et al., 2002).

Los hemocitos de los decapados son considerados como la primera línea de defensa ya que participan directamente en procesos de reconocimiento, procesamiento y amplificación de la respuesta inmunitaria (Söderhal, 1982; Söderhäll y Häll, 1984; Johansson y Söderhall, 1988). Investigaciones recientes señalan la vinculación entre los hemocitos y elementos séricos de la respuesta inmunitaria, como las peneidinas y crustinas (Vargas-Albores et al., 2004), las cuales son peptidos con actividad antimicrobiana (Destoumieux et al., 2000; 2001). También se ha detectado la asociación de los hemocitos con peroxinectinas, que presentan función opsonizante y actividad de peroxidasa (Johansson et al., 1995; Johansson, 1999).

La amplificación de la respuesta inmunitaria de los crustáceos está directamente relacionada al sistema profenoloxidasa (proFO) (Hernández-López et al., 1996; Gollas-Galván et al., 1997; 1999) y el proceso de coagulación (Montaño-Pérez et al., 1999), ambos sistemas son multiméricos y su activación requiere de la exocitosis regulada de los hemocitos circulatorios (Söderhäll y Häll, 1984; Johansson et al., 2000).

El sistema profenoloxidasa es uno de los mecanismos mejor entendido de la respuesta inmunitaria de los decápodos y ha sido considerado análogo al sistema del complemento en mamíferos por tratarse de una compleja cascada enzimática. Los componentes del sistema proFO se encuentran compartamentalizados en el interior de los gránulos de los hemocitos (Söderhal, 1982; Söderhall y Smith, 1983), y el sistema es liberado directamente por estimulación con beta-glucanos (ßG) o lipopolisacaridos (LPS) de hongos y bacterias (Söderhäll y Häll, 1984), o a través de proteínas séricas de reconocimiento que se unen a ßG (Vargas-Albores et al., 1996; 1997) o a LPS (Vargas-Albores et al., 1993b). Una vez liberado el contenido granular en el plasma, la proFO es activada por una proteinasa, en fenoloxidasa (FO), la cual es responsable de la oxidación de fenoles a quinonas y que finalmente se convierten en melanina (Ashida y Söderhäll, 1984; Vargas-Albores y Yepiz-Plascencia, 2000).

El sistema proFO, al activarse, genera algunos factores que estimulan a los hemocitos para que eliminen el material extraño por medio de procesos como fagocitosis, formación de nódulos y encapsulamiento (Söderhäll y Häll, 1984; Sung et al., 1998). La amplificación de la respuesta inmunitaria y la comunicación celular asociada al sistema proFO es muy poderosa y debe ser controlada. En crustáceos, la reacción en cadena está sujeta a la regulación de ciertos inhibidores de proteasas, como la α_2-macroglobulina (Hergenhahn et al., 1998; Gollas-Galván et al., 2003) y un inhibidor de tripsina de 155 kDa, llamado pacifastina (Liang et al., 1997). Recientemente otros inhibidores de proteinasas han sido descritos en los hemocitos y podrían estar involucrados en la regulación de la protéolisis (Jiménez-Vega y Vargas-Albores, 2005).

Uno de los mecanismos más importantes de la respuesta inmunitaria realizada por los hemocitos es la fagocitosis. Durante el proceso se origina el fagolisosoma y se liberan sustancias líticas como el peroxido, superóxido y derivados del óxido nítrico, los cuales son biológicamente muy reactivos (Muñoz et al., 2000; Campa-Córdova et al., 2002). Este proceso es conocido como estallido respiratorio y

juega un papel muy importante en la actividad microbicida de los hemocitos (Song y Hsieh, 1994).

Hasta ahora, la numerosa información generada sobre el sistema inmunitario de crustáceos incluye la caracterización de importantes moléculas, y también, la descripción de algunos mecanismos. No obstante, se conoce muy poco de los cambios del sistema inmunitario asociados a importantes procesos biológicos, como el crecimiento, la reproducción o la condición nutricional. También hace falta profundizar en el carácter constitutivo o innato de la respuesta inmunitaria, y la posible existencia de una forma peculiar de respuesta inducida. En *Penaeus japonicus* se ha observado un incremento significativo en la tasa de proliferación de hemocitos en los camarones retados por segunda vez con el mismo antígeno fúngico, a comparación de la respuesta generada por el primer reto (Sequeira et al., 1996; Arala-Chaves y Sequeira, 2000). La memoria inmunológica se caracteriza por una repuesta que se distingue en forma y función de una primera respuesta realizado con el mismo antígeno (Kaatari, 1994), de tal manera que los resultados observados en *P. japonicus* señalan la posible existencia de una respuesta inmunitaria adaptativa en camarones, lo cual podría constituir la base de estrategias de inmuno-protección para enfermedades infecciosas comunes en los sistemas de cultivo.

4. Indicadores de la condición fisiológica

Los límites de tolerancia de una especie, ante una condición ambiental particular, no son fijos ya que están sujetos a la historia de vida de los individuos, revelando así, la profunda asociación entre el grado de compensación homeostático y la condición fisiológica de los organismos (Newell, 1976; Torres, 1995). En este sentido, los marcadores de la condición fisiológica son importantes para profundizar en el entendimiento de los mecanismos compensatorios ante cambios ambientales, deficiencias nutricionales y presencia de patógenos.

Por sus implicaciones en la tolerancia ambiental, los ajustes producidos para el mantenimiento de la presión osmótica han sido utilizados como indicadores del estado fisiológico de los camarones (Lignot et al., 2000), mientras que los niveles de glucosa, colesterol, acilgliceroles, proteínas, hemocianina y lactato han sido utilizados para conocer el estado nutricional (Racotta y Palacios, 1998; Rosas et al., 2001a; 2002). Por su parte la respuesta inmunitaria ha sido evaluado a través de diversas formas entre las cuales destacan las variaciones en la concentración de hemocitos, la actividad fenoloxidasa, la actividad de las proteínas de regulación (macroglobulinas y pacifastina) y la actividad fagocítica evaluada directamente o a partir del estallido respiratorio, entre otras (Le Moullac et al., 1998b; López et al., 2003).

La medición de hemocianina ha sido propuesta como un indicador para evaluar el estado de salud y la condición nutricional de los crustáceos (Spicer y Baden, 2000). La hemocianina, además de ser el pigmento respiratorio, funciona como una proteínas de reserva en la hemolinfa (Hagerman, 1983; Chen y Cheng, 1993b; Van Holde et al., 2001), actúa como osmolito (Hagerman y Weber, 1981), transportador de ecdisona (Van Holde et al., 2001), y posiblemente también participa en la respuesta inmunitaria, debido a sus componentes fungistáticos (Destoumieux et al., 2001), y por su actividad tipo fenoloxidasa (Adachi et al., 2003).

Estudios recientes demuestran la fusión dual de las lipoproteínas de crustáceos. Las lipoproteínas son las principales proteínas involucradas en el transporte de lípidos (Hall et al., 1995; Yepiz-Plascencia et al., 2000b) y también participan en el sistema de defensa como proteínas de reconocimiento de beta-gucanos (ßGBP) (Yepiz-Plascencia et al., 1998) o como proteína de coagulación (CP) (Hall et al., 1999; Yepiz-Plascencia et al., 2002). La multifuncionalidad de las lipoproteínas y la de la hemocianina revelan la intrincada relación que existe entre los procesos digestivos y el sistema de defensa de los organismos.

Los conocimientos adquiridos en inmunología de vertebrados, señalan a las carencias nutricionales como el factor externo más importante de inmunodeficiencias. Una reciente investigación indica que el manejo de ciclos cerrado y la selección artificial con base en el crecimiento conduce a la pérdida de alelos del gen de la ∝-amilasa en poblaciones cultivadas de *Litopenaeus vannamei* (Arena, 2004). Debido a la importante participación de la amilasa en la degradación inicial de los carbohidratos, la reducción de la frecuencia alélica para este gen genera podría afectar el estado fisiológico y el sistema inmunitario al generar un cambio en la capacidad de los camarones para aprovechar los carbohidratos. Realizar las evaluaciones de los componentes de la hemolinfa en poblaciones de *L. vannamei* silvestres y cultivados permitiría señalar por un lado, el alcance del uso de los indicadores fisiológicos y a su vez, iniciar el estudio de los ajustes metabólicos e inmunológicos que las poblaciones presentan como consecuencia del manejo de los ciclos cerrados.

Tomando en cuenta la importancia económica de *L. vannamei* y los retos que enfrenta la industria camaronícola actualmente, es importante profundizar en el conocimiento fisiológico inmunológico de los camarones e identificar cuales indicadores metabólicos y de la respuesta inmunitaria son adecuados para determinar su estado de salud. En este sentido, es necesario abordar la relación entre el aprovechamiento del alimento y la condición inmunológica haciendo hincapié en el metabolismo de proteínas, ya que en el caso de los camarones, el estado fisiológico y el crecimiento depende en gran medida de las proteínas de la dieta (Andrews et al., 1972; Chen, 1998; Rodríguez et al., 2000; Guzmán et al., 2001; Lemos et al., 2001; Rosas et al., 2001b).

Por todo lo anterior el presente estudio pretende abordar las adaptaciones fisiológicas que presentan los juveniles de *Litopenaeus vannamei* debido al tipo de cultivo (experimental, 90 l, y piloto comercial, 20 t), al proceso de domesticación y al estado nutricional, a través de algunos componentes de la hemolinfa.

Hipótesis:

Puesto que la hemolinfa constituye el vehículo de transferencia de metabolitos, energía, transporte de oxígeno y también, la sede principal del sistema inmunitario de los camarones, entonces, variaciones en los niveles de algunos componentes de la hemolinfa pueden reflejar adaptaciones fisiológicas de los juveniles de *Litopenaeus vannamei* asociadas al proceso de domesticación, la condición nutricional y el tipo de cultivo.

Objetivos particulares:

a) Estructurar líneas base de los componentes en la hemolinfa: oxihemocianina, proteína total, glucosa, acilglicéridos y lactato de los juveniles de *L. vannamei* y, comparar estos resultados con lo reportado para *L. vannamei* y para otros crustáceos.

b) Determinar el efecto de las condiciones de cultivo experimental y piloto comercial (90 l y 20 t) sobre las concentraciones de oxihemocianina, proteínas, glucosa, acilglicéridos y lactato de los juveniles de *L. vannamei*.

c) Determinar el efecto del proceso de domesticación y el nivel de los carbohidratos dietéticos (3 y 44 %) sobre el crecimiento, los componentes de la hemolinfa (hemocianina, proteínas, glucosa, colesterol, lactato, y acilglicéridos, hemogramas y actividad de fenoloxidasa) y la capacidad osmótica de los juveniles de *L. vannamei* (silvestres y F_7).

d) Evaluar el efecto del nivel de proteínas dietéticas (5, 15 y 40 %), sobre el balance energético, la respuesta inmunitaria (hemogramas, estallido respiratorio, actividad de fenoloxidasa), el equilibrio osmótico, la oxihemocianina y la reserva de glucógeno en la glándula digestiva de los juveniles cultivados de *L. vannamei* (F_9).

e) Determinar el efecto de un periodo de inanición de 21 días sobre los componentes de la hemolinfa (hemocianina, proteínas, glucosa, colesterol, acilglicéridos, lactato, hemogramas, estallido respiratorio, actividad de profenoloxidasa), el peso, la capacidad osmótica, y las reservas de glucógeno y lípidos de la glándula digestiva en juveniles de *L. vannamei* cultivados (F_9) previamente alimentados con una dieta deficiente y óptima en proteínas (5 y 40 %).

f) Proponer un modelo conceptual sobre el aprovechamiento del alimento y su relación con el manejo de la energía ingerida, la condición nutricional y el sistema inmunitaria de juveniles de *L. vannamei* silvestres y cultivados.

Capítulo 1

Metabolitos sanguíneos y hemocianina del camarón blanco *Litopenaeus vannamei*: el efecto de las condiciones de cultivo y una comparación con otros crustáceos.

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C. Pascual · G. Gaxiola · C. Rosas

Blood metabolites and hemocyanin of the white shrimp, *Litopenaeus vannamei*: the effect of culture conditions and a comparison with other crustacean species

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Abstract Oxyhemocyanin (OxyHc), blood protein, glucose, acylglycerol (AG), and lactate of *Litopenaeus vannamei* juveniles were investigated. Statistical analyses were done on baseline values for each metabolite and on OxyHc from shrimp cultured in outdoor (20-m^2 ponds) and indoor (90-l tanks). The values obtained were compared with blood metabolites reported in the literature for *L. vannamei* maintained under similar conditions. Normal distribution was only found for samples from indoor shrimp. The distribution values for blood metabolites from shrimp cultured in outdoor ponds and indoor tanks were right or left skewed. OxyHc, blood proteins, and glucose levels were higher in shrimp cultured in outdoor ponds than those observed in shrimp maintained in indoor tanks. These differences were attributed to availability of live food in outdoor culture ponds. The OxyHc/protein ratio showed that 60% of blood protein is OxyHc in outdoor-cultured shrimp and was lower than in indoor-cultured shrimp, and this ratio was lower than that found (97%) in indoor-cultured shrimp. The type of food was identified as a dominant factor affecting blood metabolites. AG in outdoor-cultured shrimp was significantly lower than that observed in indoor-maintained shrimp. Blood lactate concentration of shrimp was not affected by the culture conditions. The blood levels for protein, glucose, and lactate reported here are similar to those reported previously for other crustacean species, indicating that these can be used as a reference for evaluating the physiological status of *L. vannamei*. When we compared the OxyHc, blood protein, glucose, and lactate levels of *L. vannamei* juveniles with other crustacean species, we observed that crabs, lobsters (low-activity species), and closed-thelycum shrimp species (mean-activity species) had lower values in comparison to those observed in open-thelycum shrimp species, including *L. vannamei* juveniles. Possibly the blood metabolites and OxyHc could be reflecting the morphological and physiological adaptation of crustaceans, because these metabolites will depend on energetic demands.

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C. Pascual (✉) · G. Gaxiola · C. Rosas
Laboratorio de Biología Marina Experimental,
Fac. de Ciencias UNAM, Apdo. post. 69,
Cd. Del Carmen, Campeche, Mexico
E-mail: crv@hp.fciencias.unam.mx
Fax: + 52-93-83828730

Introduction

The development of shrimp culture and the needs for monitoring the physiological condition of cultured shrimp are constantly increasing. Some blood metabolites and oxyhemocyanin (OxyHc) have been used for monitoring physiological conditions in wild or cultured shrimp species that are exposed to different environmental conditions. Recently Racotta and Palacios (1998) used blood glucose, lactate, acylglycerol (AG), cholesterol, and proteins to evaluate the stress produced by serotonin injection in *Litopenaeus vannamei* juveniles, and reported that glucose and lactate were the best indicators of stress in shrimp. Chen et al. (1994a), testing the combined effect of salinity and ammonia, reported that blood protein and OxyHc were significantly reduced in *Penaeus monodon* juveniles exposed to low salinity and high ammonia-N levels. Similar results were observed in *Penaeus japonicus* exposed to similar ammonia and salinity conditions (Chen et al. 1994b). Palacios (2000) used AG, glucose, cholesterol, and protein to monitor the physiological condition of wild and cultured populations after repeated spawning of adult females of *L. vannamei*. Sánchez et al. (2001) reported variations in protein, lactate, glucose, AG, and cholesterol, as well as in hemocytes, prophenol oxidase activity, and osmotic pressure of *Litopenaeus setiferus* adult males from wild populations and during acclimation at 27°C and 30°C. In that study they observed that laboratory conditions

produced lower values of proteins, AG, and cholesterol than those observed in freshly captured shrimp.

Blood metabolites have also been used to monitor shrimp nutritional conditions. Recent studies showed that blood protein and glucose are highly sensitive to dietary protein and carbohydrate (CHO) contents for *L. setiferus* and *L. vannamei* juveniles exposed at high (35–40‰) and low (15‰) salinities (Rosas et al. 2001a, 2001b). In these studies, blood protein was related to ammonia hemolymph levels, indicating that a protein-rich diet could regulate the rate of ammonia production and in consequence the gill glutamate dehydrogenase activity. From the data in these publications, it was evident that *L. vannamei* and *L. setiferus* can convert protein to glycogen by the gluconeogenic pathway, which permitted shrimp to maintain a minimum level of circulating glucose, independent of dietary CHO.

However, there has been no attempt until now, to characterize the variation patterns at the shrimp population level. There is no information available on baseline levels that could be used as a reference for studying experimental conditions in cultured or wild populations of shrimp. When reporting blood metabolites, most publications present data as means (±standard deviation or standard error), assuming that all metabolites are normally distributed. However, few studies obtain sample sizes that are sufficiently large for testing normal distribution of values, and verification of normal distribution is required. Recently, Spicer and Baden (2000) analyzed OxyHc in three crustacean species, and showed that the OxyHc distribution data changed between species and between populations. Only one of the five frequency distributions obtained for OxyHc was normally distributed when *Nephrops norvegicus*, *Liocarcinus depuratos*, and *Hyas araneaus* were studied. Those results stress the need for caution in the interpretation of results obtained for shrimp and other crustacean species when blood metabolites are studied and are used as a tool for monitoring the physiological condition in wild or cultivated animals.

The white shrimp, *L. vannamei*, is the most important shrimp species cultivated in the Americas. More than 90% of the shrimp cultivated in 1998 on the American continent were *L. vannamei* (Rosemberry 1998). Therefore, a study of the distribution levels of metabolites in a population could be very useful as it will provide reference baseline levels for evaluating environmental conditions. The baseline reference levels could also be used as a diagnostic tool for determining whether shrimp are under stress. For that reason, the work described in this paper was aimed at obtaining information on baseline levels of the blood metabolites of *L. vannamei*, cultivated in outdoor ponds (20 m^2) and indoor experimental tanks (90 l). The baseline levels were determined for the blood metabolites glucose, proteins, CHO, lactate, and AG, as well as for OxyHc . Results were compared with blood metabolite data previously published for *L. vannamei*. Comparisons with other shrimp and crustacean species offered some explanations for possible differences between crustacean species related to behavioral and morphological characteristics.

Materials and methods

Animals

Litopenaeus vannamei juveniles, 0.08 and 0.5 g body weight, were obtained from Pecis Industries (Yucatán, Mexico). Shrimp (n = 2,400) were maintained in three outdoor ponds (20 m^2) with aerated natural seawater, a daily water exchange between 10% and 20%, and a density of 800 shrimp $pond^{-1}$ (40 shrimp m^{-2}). The seawater of the ponds was inoculated with *Tetraselmis chuii* algae (200 l $pond^{-1}$; 800,000 cells ml^{-1}) before introducing the shrimp. Shrimp were fed with a commercial food (35% protein, Api camarón ultra Malta Clayton, Mexico) twice a day at a rate of 20% of live weight. When shrimp reached 2 g wet weight, a group of 400 shrimp (150 shrimp $pond^{-1}$) were transported to the laboratory and stored in 40 plastic tanks (0.22 m^2) at a density of 10 shrimp $tank^{-1}$ (45 shrimp m^{-2}; formally called "indoor tanks"). Tanks were supplied with aerated and filtered (20 μm) natural seawater in a flow-through system. In the other three outdoor ponds used (formally called "outdoor ponds"), shrimp (n = 800 shrimp $pond^{-1}$) were maintained for 110 days between July and October 2000 at temperatures ranging from 27°C to 29°C, dissolved oxygen > 5 mg l^{-1}, pH > 8.0, and a salinity between 32‰ and 36‰. In indoor tanks, shrimp were maintained for 40 days using filtered seawater (20 μm) at a temperature of 28 ± 1°C, dissolved oxygen > 5 mg l^{-1}, pH > 8.0, and a salinity between 32‰ and 36‰. The main difference between the two culture systems was in the food. In outdoor ponds, shrimp had access to natural live food in addition to 35% protein pellets (Api camarón ultra Malta Clayton) given at a rate of 5% body weight day^{-1}. In indoor tanks, shrimp had access only to the food pellets at a rate of 15% body weight day^{-1} (Table 1). Shrimp were fed twice a day (0800 and 2000 hours) in both culture systems. In inside tanks uneaten food particles and feces were removed twice a day. At the end of the experimental time all the shrimp from inside tanks (7 ± 0.5 g) and 300 shrimp (100 $pond^{-1}$) from outside ponds were sampled (10 ± 1 g). At the time of sampling a density of 41 and 36 shrimp m^{-2} was obtained in inside tanks and outside ponds, respectively. In consequence survival of > 90% was obtained in both culture systems.

Sampling and analysis of hemolymph

Twelve hours before sampling, shrimp were not fed, to avoid the interference of food nutrients in the blood metabolites (Rosas et al. 2001b). Before sampling, shrimp were placed in chilled (18°C) and aerated seawater for 5 min, to reduce metabolic activity and decrease the effect of manipulation prior to hemolymph extraction. Previous studies demonstrated that the stress caused by sampling can be significantly reduced in this manner (Rosas et al. 2000a; 2001b; 2002). Only shrimp in the intermolt stage (stage C) were used. Hemolymph extractions (approximately 200–300 μl $shrimp^{-1}$) were individually sampled through a chilled syringe needle inserted at the base of the fifth pereiopod, after the shrimp had been dried with a paper towel. To avoid clotting, blood samples were taken without air, because air accelerates the coagulation process. The blood sample from each shrimp was carefully placed on chilled (4°C) parafilm placed over ice. From that drop, subsamples were taken. The weights (±0.05 g) of individual shrimp were measured, and the molting stage of each was observed using the uropod characteristics described by Drach and Tchernigovtzeff (1967).

For OxyHc measurements, 10 μl of hemolymph was immediately diluted with 990 μl of distilled water in a 10 mm cuvette for UV-wavelength spectroscopy (1.0 ml; 1 cm path length), and the

Table 1 Pelletized food used to feed *Litopenaeus vannamei* juveniles in indoor tanks

	Amount
Krill paste	480 g kg^{-1}
Yeast	340 g kg^{-1}
Pre-cooked corn starch	20 g kg^{-1}
Native wheat starch	73 g kg^{-1}
Cellulose	54 g kg^{-1}
Robimix-b (vitamins)[a]	20 g kg^{-1}
Soybean lecithin	1 g kg^{-1}
Cod oil	2 g kg^{-1}
Na_2HPO_4	4 g kg^{-1}
KH_2PO_4	2 g kg^{-1}
$CaCO_3$	1 g kg^{-1}
$MgCl_2$	3 g kg^{-1}
Carbohydrate	219 g kg^{-1}
Protein	44 g kg^{-1}
Lipids	10 g kg^{-1}
Total energy	12.1 mJ kg^{-1}

[a]Robimix-b from Hoffmann La Roche, no. 1720: retynil palmitate (vitamin A): 8,000,000 UI; cholacalcyferol(vitamin D_3): 196,000 UI; α-tocopherol acetate (vitamin E): 10,000 mg kg^{-1}; vitamin K_3: 800 mg kg^{-1}; ascorbyl phosphate(vitamin C): 15,000 mg kg^{-1}; thiamin (vitamin B_1): 700 mg kg^{-1};riboflavin (vitamin B_2): 2,000 mg kg^{-1}; pyridoxine (vitamin B_6): 1,000 mg kg^{-1}; niacin (vitamin PP): 10,000 mg kg^{-1}; calcium pantothenate: 5,000 mg kg^{-1}; cyanocobalamine(vitamin B_{12}): 50 mg kg^{-1};folic acid: 250 mg kg^{-1}; biotin: 30 mg kg^{-1}; inositol: 30,000 mg kg^{-1} (Hoffmann La Roche, Bâle, Switzerland). Coefficients for energy concentration: 21, 39, 17 kJ for protein, lipid, and carbohydrate, respectively (Cousin 1995)

absorbance was measured at 335 nm. Using an extinction coefficient of $E = 17.26$ calculated on the basis of a functional subunit of 74,000 Da for shrimp, the OxyHc concentration was determined (Chen and Cheng 1993a, 1993b).

Plasma was obtained from the remaining hemolymph, which was diluted immediately with prechilled (8°C) anticoagulant (1:2 hemolymph:anticoagulant). Shrimp salt solution was prepared according to Vargas-Albores et al. (1993): 450 mM NaCL, 10 mM KCL, 10 mM HEPES, pH 7.3, 850 mOsm kg^{-1}. The anticoagulant solution for hemolymph extraction was prepared by adding 10 mM of EDTA-Na_2 to the shrimp salt solution. Hemolymph plus anticoagulant was centrifuged at 800 *g* for 3 min, and the supernatant was separated for plasma determinations.

Commercial kits were used for glucose (GOD-PAD, Merck cat. 740393), lactate (Sigma cat. 735), and AG (GPO-PAP, Merck cat. 14354) determinations. These determinations were made using 20 µl of plasma and 200 µl of enzyme chromogen reagent in microplates. Absorbance was recorded on a microplate reader (BIO-RAD model 550), and concentrations were calculated from a standard solution of substrate. Plasma was further diluted 1:500 for protein determination by the Bradford (1976) technique adapted to a microplate method using commercial chromogen reagent (Sigma cat. 610) and bovine serum albumin as a standard.

Statistical analysis

Descriptive statistical analysis was used to characterize metabolite data in both shrimp groups. Normal distribution of values of blood samples for each group (outdoor and indoor) of raised shrimp was tested using a Chi-squared test ($P < 0.05$). When data fit a normal distribution, results were expressed as a means (± *s*, standard deviation). When data did not fit a normal distribution, results were expressed as a median (± quartile) range. When data had a normal distribution, the outdoor–indoor blood metabolite comparison was done using a Student's *t*-test. When data did not have a normal distribution, the outdoor–indoor blood metabolite comparison was done using a Wilcoxon paired-sample test ($P < 0.05$) (Zar 1974). When no differences were detected between groups, data were pooled and a new statistical parameter was calculated, tested for normality, and a mean distribution curve was calculated. Baseline levels were thus obtained with unpooled or pooled data, and compared with literature data using the quartile range or standard deviation as an interval of confidence of median or mean for each blood variable (Zar 1974). The baseline values for each metabolite were compared with data reported for *L. vannamei* by Racotta and Palacios (1998), Racotta and Hernández-Herrera (2000), Rodríguez et al. (2000), and Rosas et al. (2000b, 2001a, 2001b, 2002).

Results

Baseline values of metabolites

The OxyHc values of outdoor- and indoor-raised *Litopenaeus vannamei* were statistically different, with high values in the shrimp raised outdoors (1.98 ± 0.48 mmol l^{-1}) and low values in indoor-raised shrimp (1.59 ± 0.55 mmol l^{-1}) ($P < 0.05$). The frequency distribution of OxyHc values in outdoor- and indoor-raised shrimp did not show a normal distribution (Table 2).

For proteins, statistical differences were observed between outdoor- and indoor-raised groups, with a non-normal distribution and higher blood protein concentration in outdoor shrimp (median ± quartiles of 224.2 ± 64.19 mg ml^{-1}). Normally distributed values were found for indoor shrimp (mean ± *s* of 102.8 ± 17.4 mg ml^{-1}; Table 2). The OxyHc/protein ratios calculated with mean or median data were 0.6 for outdoor shrimp and 0.97 for indoor shrimp, indicating that in *L. vannamei* 60% and 97% of the proteins in blood were OxyHc for outdoor and indoor shrimp, respectively.

Blood glucose and AG values from outdoor- and indoor-raised shrimp did not show a normal distribution, and statistical differences were found between these groups ($P < 0.05$; Table 2). Blood glucose levels in outdoor shrimp were 26% higher (0.38 ± 0.21 mg ml^{-1}) than those of indoor shrimp (0.28 ± 0.17 mg ml^{-1}) ($P < 0.05$). In contrast, AG levels for indoor shrimp (1.34 ± 0.70 mg ml^{-1}) were 23% higher than those of outdoor shrimp (1.03 ± 0.63 mg ml^{-1}) ($P < 0.05$; Table 2).

Blood lactate levels in outdoor- and indoor-raised shrimp were not distributed normally, and differences were not found between groups ($P > 0.05$; Table 2).Therefore, data from both groups were pooled, and blood lactate levels showed a median value of 0.11 ± 0.11 mg ml^{-1}.

Comparisons between reports for *L. vannamei*

OxyHc levels were compared with two other data sets obtained for indoor-raised shrimp, reported by Racotta and Hernández-Herrera (2000) and Rosas et al. (2002). According to the data in Table 2, a range between 1.26

Table 2 *Litopenaeus vannamei*. Descriptive statistics and normality test for hemocyanin (*Hc*), blood metabolites, and digestive gland glycogen (triacylglycerol, *TAG*) of juveniles (*A* outside ponds; *B* inside experimental tanks). Statistical differences at $P<0.05$

	N	Mean	Confident limits, 95%		SD	Mean ± 1 SD range		Median	Quartile range	Quartile		χ^2 test for normality, *P*
			+	−		+	−			Upper	Lower	
[Hc], mmol l^{-1}												
A	70	1.93	2.01	1.85	0.33	2.26	1.60	1.98	0.48	2.17	1.69	0.02
B	83	1.52	1.60	1.44	0.39	1.91	1.13	1.59	0.55	1.81	1.26	0.001
A ≠ B												
Proteins, mg ml^{-1}												
A	60	217.5	229.7	205.4	47.08	264.6	170.4	224.2	64.19	250.9	186.8	0.01
B	211	102.8	105.1	99.4	17.4	127.6	85.4	98.9	19.2	118.1	79.7	0.20
A ≠ B												
Glucose, mg ml^{-1}												
A	213	0.42	0.43	0.39	0.16	0.58	0.26	0.38	0.21	0.50	0.29	0.0001
B	167	0.31	0.33	0.29	0.15	0.46	0.16	0.28	0.17	0.39	0.22	0.001
A ≠ B												
Lactate, mg ml^{-1}												
A	205	0.14	0.15	0.13	0.12	0.26	0.02	0.09	0.10	0.15	0.06	0.0001
B	319	0.15	0.16	0.15	0.11	0.26	0.04	0.12	0.10	0.19	0.18	0.0001
A = B	532	0.15	0.16	0.14	0.12	0.27	0.03	0.11	0.11	0.18	0.07	0.0001
TAG, mg ml^{-1}												
A	60	1.12	1.20	1.00	0.43	1.50	0.69	1.03	0.63	1.39	0.76	0.0001
B	307	1.45	1.54	1.37	0.74	2.9	0.71	1.34	0.70	1.72	1.02	0.001
A ≠ B												

and 1.81 mmol l^{-1} could be considered a baseline level, with a median value of 1.59 mmol l^{-1}. This value was higher than that observed as a baseline level by Racotta and Hernández-Herrera (2000) (1.35 ± 0.04 mmol l^{-1}) and similar to that obtained by Rosas et al. (2002; 1.51 ± 0.08 mmol l^{-1}) (Fig. 1). When shrimp were stressed by high ambient ammonia-N concentration (2.14 mmol l^{-1}) or fed with high dietary CHO levels (61%) lower OxyHc levels were observed (1.12 ± 0.11 and 1.02 ± 0.02 mmol l^{-1}, respectively) (Fig. 1).

Protein blood levels were compared with data obtained from the literature (Fig. 2). For outdoor shrimp a median (± quartile) range value was used as the baseline interval; for indoor shrimp a mean (± *s*) value was used. The outdoor baseline protein level (224.2 mg ml^{-1}) was higher than other blood protein concentrations (Fig. 2). The baseline values for indoor shrimp (102.8 mg ml^{-1}) in the present study were similar to those for indoor shrimp reported by Racotta and Palacios (1998; 100 mg ml^{-1}) and Rodríguez et al. (2000; 127 mg ml^{-1}). Furthermore, the baseline values in the present study were in agreement with those obtained in stressed shrimp (130 mg ml^{-1}; Racotta and Palacios 1998) and for shrimp fed pelletized food with 22% (90 mg ml^{-1}) and 50% protein (100 mg ml^{-1}) (Rodriguez et al. 2000). Under nutritional stress (shrimp fed with only frozen shrimp and squid), indoor-raised shrimp showed a lower blood protein value (52.5 mg ml^{-1}; Rodriguez et al. 2000), than the protein baseline values observed in the present study (Fig. 2).

The glucose level in outdoor shrimp (0.38 mg ml^{-1}) was higher than that of indoor shrimp (0.28 mg ml^{-1}) ($P<0.05$; Table 2), and was similar to levels reported in shrimp fed between 1% and 36% dietary CHO (Rosas et al. 2001a, 2001b). It was higher than the baseline values obtained by Racotta and Palacios (1998; 0.15 mg ml^{-1}), although they determined a higher glucose hemolymph level in stressed shrimp (0.58 mg ml^{-1}) (Fig. 3).

AG values for both outdoor- and indoor-raised shrimp (1.03 and 1.34 mg ml^{-1}, respectively) were higher than those for stressed shrimp obtained by Racotta and Palacios (1998; 0.4 mg ml^{-1}), and of saline-shock-stressed shrimp reported by Rosas et al. (2000a; 0.50 mg ml^{-1}) (Fig. 4).

Lactate baseline levels (0.11 mg ml^{-1}) were higher than those reported by Racotta and Palacios (1998; 0.015 mg ml^{-1}) and lower than those obtained for

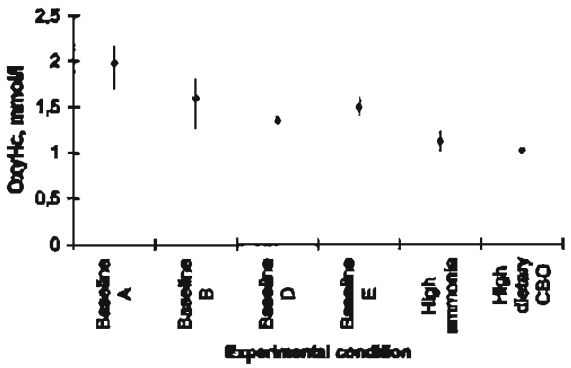


Fig. 1 *Litopenaeus vannamei*. Blood oxyhemocyanin concentration (*OxyHc*, mmol l^{-1}) of juveniles maintained in outdoor ponds (*baseline A*; median ± quartile range) and in indoor tanks (*baseline B*: mean ± *s*). Results are compared with data from Racotta and Hernández-Herrera (2000) (*baseline D* and *high ammonia*: mean ± SE) and with data from Rosas et al. (2002) (*baseline E* and *high dietary CHO*: mean ± SE)

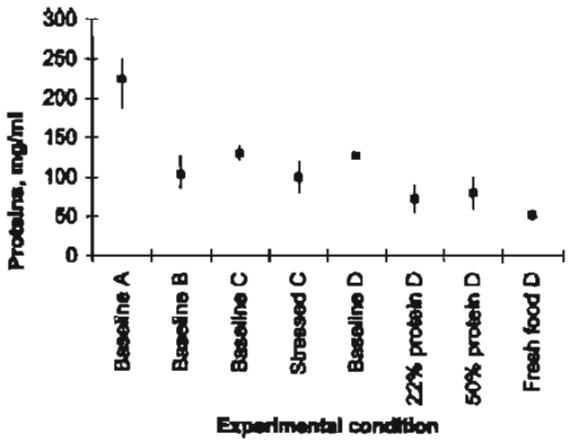


Fig. 2 *Litopenaeus vannamei*. Blood protein concentration (mg ml^{-1}) of juveniles maintained in outdoor ponds (*baseline A*: median ± quartile range) and in indoor tanks (*baseline B*: mean ± *s*). Results are compared with data from Racotta and Palacios (1998) (*baseline C* and *stressed C*: mean ± SE) and with data from Rodríguez et al. (2000) (*baseline D, 22% protein D, 50% protein D*, and *fresh food D*: mean ± SE)

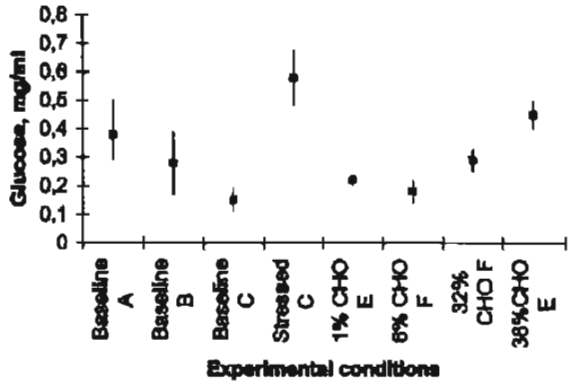


Fig. 3 *Litopenaeus vannamei*. Blood glucose concentrations (mg ml^{-1}) of juveniles maintained in outdoor ponds (*baseline A*: median ± quartile range) and in indoor tanks (*baseline B*: median ± quartile range). Results are compared with data from Racotta and Palacios (1998) (*baseline C* and *stressed C*: mean ± SE) with data from Rosas et al. (2001a) (*1% CHO E* and *32% CHO F*: mean ± SE) and with data from Rosas et al. (2001b) (*6% CHO F* and *36% CHO E*: mean ± SE)

stressed shrimp and reported in the same publication (0.3 mg ml^{-1}). The lactate values reported by Rosas et al. (2000a) from saline-shock-stressed shrimp (0.2 mg ml^{-1}) were close to the upper limits of the quartile interval of the lactate baseline presented in the present study (Fig. 5).

Discussion

Results show that not all blood metabolites and molecules studied in *Litopenaeus vannamei* juveniles have a

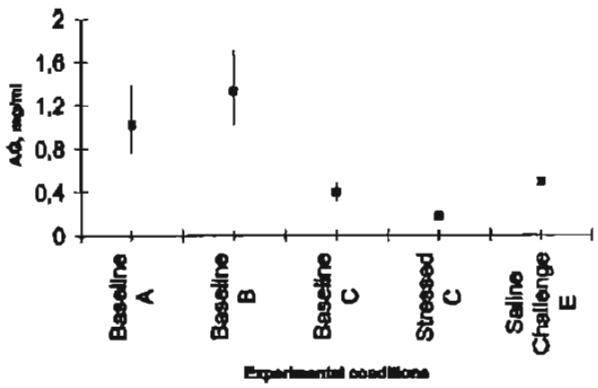


Fig. 4 *Litopenaeus vannamei*. Blood acylglycerol (*AG*, mg ml^{-1}) of juveniles maintained in outdoor ponds (*baseline A*: median ± quartile range) and in indoor tanks (*baseline B*: median ± quartile range). Results are compared with data from Racotta and Palacios (1998) (*baseline C* and *stressed C*: mean ± SE) and with data from Rosas et al.(2000b) (*saline challenge E*: mean ± SE)

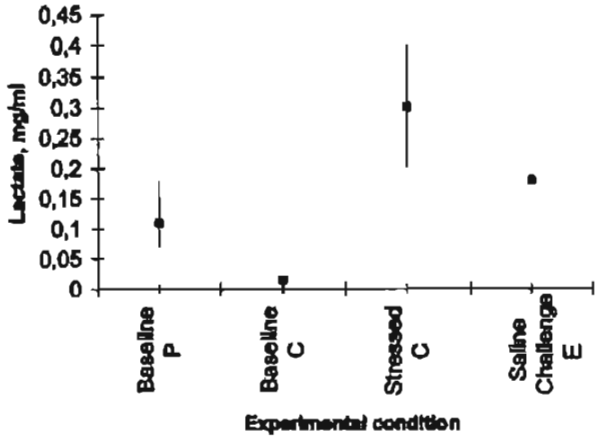


Fig. 5 *Litopenaeus vannamei*. Blood lactate (mg ml^{-1}) of juveniles maintained in outdoor ponds and indoor tanks (*baseline P*: median ± quartile range). Results are compared with data from Racotta and Palacios (1998) (*baseline C* and *stressed C*: mean ± SE) and with data from Rosas et al. (2000b) (*saline challenge E*: mean ± SE)

normal distribution. Only blood protein values from indoor shrimp were normally distributed. Proteins (outdoor shrimp), OxyHc, glucose, AG, and lactate levels of both culture conditions were right or left skewed.

The difference between outdoor- and indoor-raised shrimp in OxyHc, protein, and glucose concentrations reflects different nutritional conditions in outdoor ponds, where shrimp were offered both live and pelletized food, and the laboratory-raised shrimp that were fed only pelletized food. Outdoor-maintained shrimp showed relativity high metabolite values in comparison to those shrimp maintained in filtered seawater under laboratory conditions. In farms, many producers recognize the benefits of promoting primary and secondary production as a source of supplemental food for shrimp, and these benefits have been quantified experimentally. Anderson

et al. (1987) reported that between 53% and 77% of the growth of *L. vannamei*, raised in earthen ponds, was caused by the assimilation of natural pond biota. In another study, it was demonstrated that *L. vannamei* exhibited significantly greater growth rates in eutrophic pond water compared to shrimp grown in clear water (Leber and Pruder 1988). According to Otoshi et al. (2001), shrimp growth can be improved when shrimp are cultivated with pond water containing bacteria, microalgae, and microbial–detrital aggregates that afford protein and vitamins. In addition to direct nutritional benefits, live food enhances digestive enzyme activity, increasing the assimilation of food (Guzmán et al. 2001), which is also reflected in the blood metabolites.

The present results indicate that nutritional information derived from clean-water experiments is limited in applicability, because these conditions differ significantly from pond environments where the effects of pond water, filled with living food and organic material, on shrimp physiology are profound, as was reflected in differences between some blood metabolites between inside- and outside-raised shrimp. However, not all blood metabolites were positively affected in pond-reared shrimp. Blood AG levels were higher in indoor-raised shrimp, indicating the effect of pelletized food on lipid metabolism. Lipid requirements are relatively low in juvenile shrimp (around 11% of total nutrients in the food), but lipids are more important during vitellogenesis (Mourente et al. 1994). The differences observed in the present study could be related to the quantity of food that is given to the shrimp in outdoor ponds (5% of body weight) in comparison to that given under indoor conditions (15% of body weight). These results indicate that live food in an outdoor pond does not offer the same quantity of lipids that can be offered with palletized food under laboratory conditions. In consequence blood protein, OxyHc, and glucose levels in outdoor-raised shrimp could be used as a reference for determining the nutritional status of shrimp and the role of live food in nutrition during shrimp culture. A reduction in blood protein levels could indicate a reduced consumption of live food and, consequently, a higher dependence on pelletized food for shrimp. On the other hand, blood AG could be used as a reference for the quality of the pelletized food used in the laboratory or outside pond, because AG levels are affected directly by the quality and quantity of lipids in food. If AG blood levels are considered together with blood cholesterol levels, a stronger index of the quality of food could be obtained, because shrimp cannot synthesize cholesterol and, in consequence, the cholesterol levels in blood are directly associated with the levels in the ingested food. In conclusion, with the present results two types of baselines could be proposed, one reflecting the nutritional status of shrimp (blood OxyHc, protein, glucose) and the other (blood AG) reflecting the quality of food offered to shrimp, independent of the culture system used.

Another difference between inside tanks and outside ponds is the stress associated with the characteristics of each culture system. An outside pond is a more natural system, with microalgae and living food, and in which the natural light is determined by turbidity of the water. In inside tanks shrimp are in clear water and are subject to the daily manipulations associated with the extraction of uneaten food particles and feces. In spite of this maintenance routine, it is interesting to note that, although inside-reared shrimp were manipulated more, their blood OxyHc, protein, and glucose levels were lower than those observed in shrimp reared in outside ponds. These results could indicate that the manipulation of inside tanks was not important enough to affect the blood components of the shrimp, showing that in such tanks shrimp may be conditioned to the routine manipulations used in this type of clear-water system. In this sense we can conclude that the differences between blood components of shrimp tested in various culture systems are more related to different nutritional conditions (i.e. the food offered) than to the stress associated with the characteristics of the culture system.

Baseline values from shrimp cultured in outdoor ponds or in indoor tanks could be valuable if they are proven to reflect general trends. The metabolite values from our study appear to fulfill this condition when they were compared with literature data for the same shrimp species. OxyHc, protein, glucose, and lactate levels are similar to values reported in the literature, indicating their usefulness as reference indicators for the physiological status of shrimp. Significantly lower OxyHc and blood protein levels were reported by Rosas et al. (2002) and Rodríguez et al. (2000) in nutritionally stressed shrimp (fed with frozen shrimp and squid). Several researchers have proved that OxyHc is a storage protein for *L. vannamei*, *Melicertus kerathurus*, and *Marsopenaeus japonicus* juveniles (Cuzon and Ceccaldi 1971; Cuzon et al. 1980; Rosas et al. 2002). In this sense Rosas et al. (2002) showed that OxyHc synthesized in the digestive gland was higher in shrimps fed with low dietary CHO (5% and 0%) than that observed in animals fed with high dietary CHO (61%), evidencing that diet can modulate the OxyHc level in shrimps. According to Rosas et al. (2001a, 2001b, 2002) high dietary CHO could inhibit protein absorption in the digestive gland, due to the CHO excess stored as glycogen. Although we do not know if the low OxyHc value observed in extreme dietary CHO levels was a consequence of an inhibition of protein absorption and/or the effect of a low dietary protein level, certainly the nutritional status of shrimps maintained at that level of dietary CHO affected OxyHc metabolism, compromising the oxygen-carrying and oxygen-storage capacity of OxyHc. Lower OxyHc was observed in shrimp exposed to high ambient N-ammonia levels, when compared with baseline values. The oxidation of OxyHc to methoxyhemocyanin by nitrite derived from ammonia could be an explanation for the reduction of OxyHc (Racotta and Hernández-Herrera 2000). With this information it is evident that OxyHc values could be used as a tool for monitoring the nutritional status of shrimp and/or the effects of high environmental ammonia levels.

In the study by Rodríguez et al. (2000) a high correlation between protein level and the immunological state of shrimp was suggested, concluding that protein levels could be used as a strong indicator of shrimp health status. That conclusion was based on recent studies that had demonstrated that several hemolymph proteins are involved in the immunological response, including the clotting proteins, serine proteinase, peneacidins, and the prophenoloxidase system (Hall et al. 1999; Montaño-Pérez et al. 1999; Destoumieux et al. 2000; Huang et al. 2000). In *L. vannamei*, proteins are the most important source of energy (Rosas et al. 2002) and molecules for the immune system (Rodríguez et al. 2000). In a previous paper (Rosas et al. 2001a), we observed, in shrimp fed high-quality fish protein, that the blood protein level varied according to dietary protein levels, with low values (200 mg ml^{-1}) in shrimp fed 30% protein and high concentrations (250 mg ml^{-1}) in shrimp fed 50% protein, indicating a close correlation between dietary proteins, blood proteins, and growth rate. In another study, similar blood protein values in control and stressed shrimp were reported by Racotta and Palacios (1998). These values were similar to those found in indoor-shrimp protein levels, indicating that the stress produced by injection with a saline solution did not affect protein metabolism in *L. vannamei*, possibly because of the action of other compensatory mechanisms related to glucose metabolism. With all this information, we can conclude that blood protein levels are affected more by nutritional stress than manipulation stress, suggesting that shrimp blood protein levels could reflect the changes in shrimp health status, including modifications in immunological response. In a recent paper Destoumieux et al. (2001) showed antifungal activity at the C terminus of *L. vannamei* hemocyanin and suggested that a limited proteolysis of OxyHc could be relevant to the shrimp's immune system reactions after microbial infection. If OxyHc represents a high proportion of the total proteins in the blood (58–98%) and it has an antifungal function, OxyHc and other immunological proteins dissolved in blood could represent a high proportion of the total proteins involved in responses of the immune system, evidencing the importance of blood proteins in the determination of the health status of shrimp.

Blood glucose levels are highly sensitive to dietary CHO, and therefore it was not surprising that levels in outdoor-raised shrimp were different from those of indoor-raised animals. The live food, bacteria, and detritus in outdoor ponds contain different quantities and qualities of CHO that affect the blood glucose levels. Similar to protein, glucose hemolymph levels could be used to indicate the nutritional status of pond shrimp, identifying the time when live food in the pond is declining and consequently the dependence on pelletized food is increasing. In *L. vannamei* juveniles maintained in indoor tanks, Rosas et al. (2001a) observed recently that blood glucose levels depended on dietary CHO levels, with values ranging between 0.22 and 0.45 mg ml^{-1} in shrimp fed between 1% and 36% dietary CHO. Those values are within the blood glucose quartile range observed as the baseline glucose levels of indoor shrimp (0.17–0.5 mg ml^{-1}), indicating that the actual glucose baseline is strong enough to absorb the effect of a wide range of dietary CHO. Notwithstanding, the indoor shrimp baseline (0.28 mg ml^{-1}) was higher than the baseline levels observed by Racotta and Palacios (1998; 0.15 mg ml^{-1}). That difference could be related to the type of food. In the present study, indoor shrimp were fed with an experimental diet containing high-quality protein and CHO, which presumably covered all the nutritional requirements of shrimp. Racotta and Palacios (1998) used commercial pellets that certainly were made of lower quality ingredients than those used in our experimental diet. Because the blood glucose level depends on the gluconeogenic pathways and, in consequence, on protein metabolism, the differences observed between these studies could be related to differences in dietary characteristics that permit a high or low gluconeogenesis, depending on the digestibility of nutrients and their assimilation. Blood protein and glucose are better indicators of the nutritional status of shrimp, because both reflect the regulation of other processes that exceed those involved only in diet catabolism. Those results show that the actual glucose baseline level of indoor shrimp could be useful to test commercial foods, because it is highly sensitive to diet quality and, jointly with blood proteins and OxyHc, could help to evaluate the nutritional effects on shrimp.

When shrimp are stressed, blood glucose increases to be used as a rapid source of energy (Claybrook 1983). Under such circumstances (escape metabolism), the blood glucose level could be elevated above the glucose baseline, such as demonstrated in a previous study (Racotta and Palacios 1998).

According to Mourente et al. (1994), AGs are the major energy source and the predominant form of energy storage for shrimp. Taking into account that AG blood values depend on dietary lipid levels, a generalized reference level is difficult to pinpoint, because it will depend on each type of food used in a particular situation. For example, the AG values reported by Racotta and Palacios (1998) were lower than values obtained in the present study, evidencing differences in the type of food used in every experiment. The dependence of blood AG on dietary lipid levels is related to the ability of shrimp to store and synthesize lipids. Mourente and Rodríguez (1991) and Teshima (1998) showed that because of the limitation of space in the digestive gland of *P. kerathurus* and *P. japonicus*, lipids must be processed rapidly and delivered into the hemolymph, where they are stored and transported to the different tissues to be metabolized. Under such circumstances, blood AG levels will be a reflection of dietary lipid concentrations, indicating when shrimp are fed with a food of suboptimum lipid content. The use of lipids as an index of performance in fish as well as the eggs and larvae of shrimp has demonstrated that AG can provide a

reference point if every researcher or farmer has his own baseline, which will depend on the food quality used in each place (Angus 1989; Palacios et al. 1999).

Culture conditions did not affect blood lactate levels. Outdoor- and indoor-raised shrimp had similar values for lactate levels, indicating that in both situations similar stress conditions exist (Table 1). But how much lactate does indicate stress in *L. vannamei* shrimp? Racotta and Palacios (1998) reported values > 30 mg ml^{-1} in stressed *L. vannamei*, in comparison to values of 0.015 mg ml^{-1} obtained in unstressed shrimp. With the present results obtained from blood lactate concentrations of > 500 *L. vannamei* juveniles, we can expect that all *L. vannamei* lactate values 0.18 mg ml^{-1} (upper quartile range value) above the expected normal range could show the organism to be under stress.

OxyHc represents 60–95% of the total protein in the hemolymph of crustaceans (Djangmah 1970). Our results show that, in *L. vannamei* juveniles that were tested at stage C, i.e. during the premolt cycle, between 60% and 97% of blood protein is OxyHc. Blood OxyHc was affected by dietary CHO in *L. vannamei* maintained for 40 days under indoor conditions (Rosas et al. 2002). In the present study, shrimp fed 5% dietary CHO had a greater growth rate and higher OxyHc levels than the values observed in shrimp fed 32%, 38%, or 61% dietary CHO. The OxyHc values obtained in shrimp fed 5% dietary CHO (1.45 mmol l^{-1}) are inside the range of quartiles proposed as a OxyHc reference values for *L. vannamei* in the present study.

To understand the relationship between blood metabolites and morphological and activity adaptations, we made a comparison between the OxyHc, protein, glucose, AG, and lactate blood levels of the present study and the data published previously for different crustacean species. We considered only data obtained from wild populations or for crustaceans maintained under optimum environmental conditions (Figs. 6, 7, 8, 9, 10). Lobster and crab (low activity and heavy carcass) had OxyHc values lower (between 0.2 and 0.6 mmol l^{-1}; Senkbeil and Wriston 1981; Hagerman 1983; Spicer and Baden 2000) than those observed in closed-thelycum shrimp species (burrowing and nocturnal shrimp; OxyHc values between 0.7 and 1.1 mmol l^{-1}; Hagerman and Weber 1981) (Chen and Cheng 1993a, 1993b, 1995), or those in more active shrimp species (*L. vannamei*; diurnal shrimp; OxyHc values between 1.6 and 2 mmol l^{-1}; present paper). From these results, we can assume that the OxyHc levels may be related to crustacean activity that is a consequence of morphological and physiological adaptations. It has previously been observed (Sánchez et al. 1991) that the ash content of different crustacean exoskeletons is positively related to the oxygen consumption rate, with lower oxygen consumption rates in heavy exoskeleton-weight crabs (burrowing crabs, *Calapa sulcata* and *Hepathus ephelitucus*), intermediate values in intermediate exoskeleton-thickness species (swimming crabs, *Callinectes sapidus* and *Portunus spinicarpus*), and high oxygen consumption

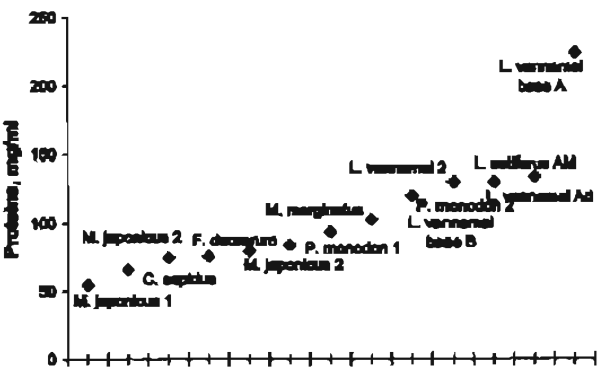
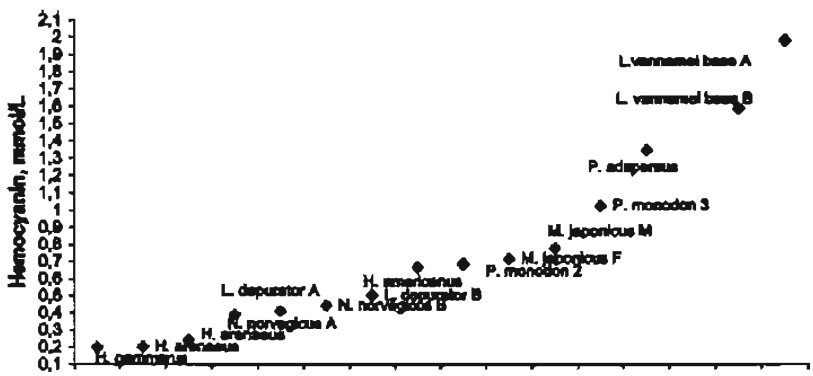


Fig. 6 Mean oxyhemocyanin level (Oxy*Hc*, mmol l^{-1}) of different decapod crustacean species: *Homarus gammarus* (Hagerman 1983); *Homarus arenaeus*, *Nephrops norvegicus*, and *Liocarcinus depurator* (Spicer and Baden 2000); *Homarus americanus* (Senkbeil and Weber 1981); *Penaeus monodon* (Cheng et al. 1994a); *Marsupenaeus japonicus* and *P. monodon* (Chen and Cheng 1993b); *Palaemon adspersus* (Hagerman and Weber 1981); and *Litopenaeus vannamei* baseline A and B (present paper)

Fig. 7 Mean blood protein level (mg ml^{-1}) of different decapod crustacean species: *Marsopenaeus japonicus* 1 (Chen et al. 1994b); *Callinectes sapidus* (Lynch and Webb 1973); *M. japonicus* 2 (Cuzon et al. 1980); *Farfantepenaeus duorarum* (Chen and Cheng 1993a); *M. japonicus* 3 (Vazquez-Boucard et al. 1985); *Penaeus monodon* 1 and 2, *Metapenaeus marginatus* (Chen and Cheng 1993a), *Litopenaeus vannamei* adults (*Ad*) and adult males (*AM*) (Racotta and Palacios 1998); and *L. vannamei* baseline A and B (present paper)



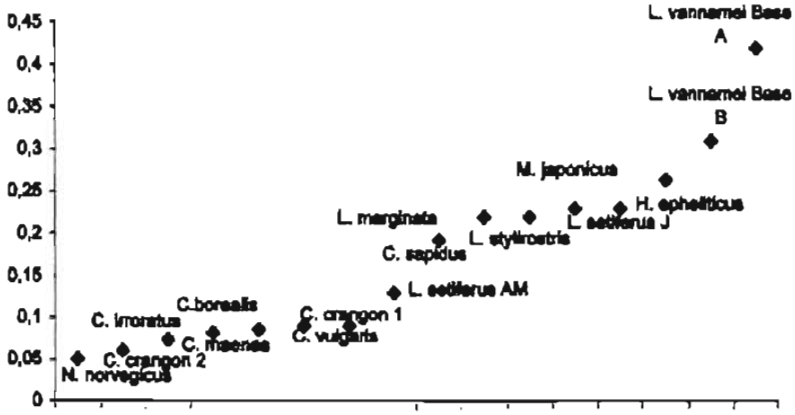


Fig. 8 Mean blood glucose level (mg ml^{-1}) of different decapod crustacean species: *Nephrops norvegicus* (Schmitt and Uglow 1997); *Crangon crangon* 1 (Poolsanguan and Uglow 1974); *C. crangon* 2 (Spazzgaren and Heafner 1987); *Cancer borealis* (Djangmah 1970); *Carcinus maenas* and *Cancer irroratus* (Telford 1968); *Crangon vulgaris* (*Callinectes sapidus*, *Hepathus ephelliticus* and *Libinia marginata*, Dean and Vernberg 1965); *Marsopenaeus japonicus* (Cuzon et al. 1980); *Litopenaeus stylirostris* (Rosas et al. 2000a); *L. setiferus* juveniles (*J*) (Rosas et al. 2001b); *L. setiferus* adult males (*AM*) (Sánchez et al. 2001); and *L. vannamei* baseline A and B (present paper)

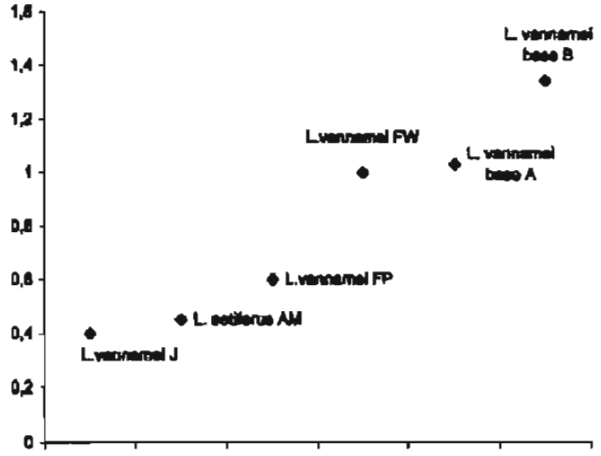


Fig. 9 Mean acylglycerol (*AG*, mg ml^{-1}) levels of different shrimp species; *Litopenaeus vannamei* juveniles (*J*) (Racotta and Palacios 1998); *L. vannamei* females from ponds (*FP*) (Palacios et al. 1999); *L. vannamei* from wild populations (*FW*) (Palacios et al. 1999); *L. setiferus* adult males (*AM*) (Sánchez et al. 2001); and *L. vannamei* baseline A and B (present paper)

rates in light-carcass species (shrimp, *Farfantepenaeus aztecus*).

It is tempting to assume that the more active species have a high OxyHc in response to higher oxygen metabolic demands. This adaptation, together with their hydrodynamic shape, would allow shrimp to migrate long distances in a short time (Williams 1984). In the less active species, the lower OxyHc concentration could be a reflection of lower metabolic oxygen demands associated with the low activity related to their burrowing behavior and slow locomotive activity, and associated with low energy expenditure.

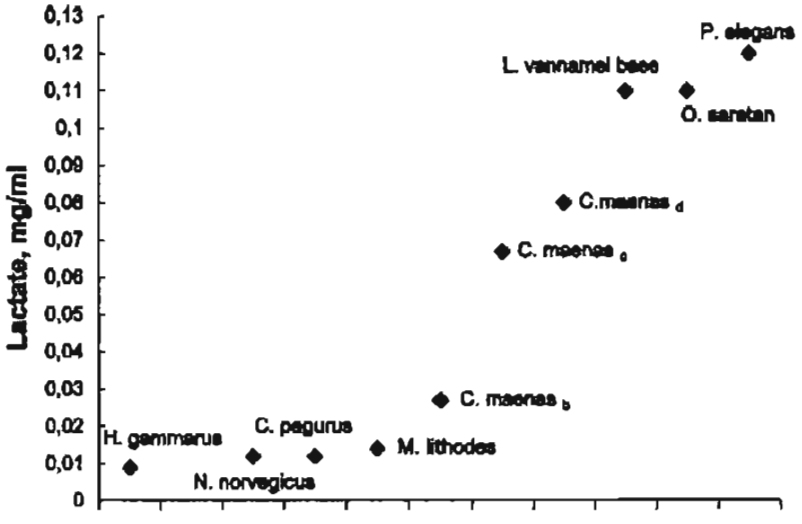


Fig. 10 Mean lactate levels (mg ml^{-1}) of different shrimp species; *Homarus gammarus*, *Nephrops norvegicus*, *Cancer pagurus*, *Maia lithodes*, and *Carcinus maenas*$_b$ (Phillips et al. 1977); *C. maenas*$_c$ (Johnson and Uglow 1985); *C. maenas*$_d$, *Ocypode saratan*, and *Palaemon elegans* (Morris and Taylor 1988); and *Litopenaeus vannamei* baseline (present paper)

When we compared the blood protein, glucose, and lactate levels of *L. vannamei* juveniles with other crustacean species, we observed that crabs, lobsters, and closed-thelycum shrimp species had lower values than those observed in open-thelycum shrimp species, including *L. vannamei* juveniles (Figs. 7, 8, 10). It was demonstrated that *L. vannamei* are well adapted to live without dietary CHO (Rosas et al. 2001a, 2002), and base their metabolism almost entirely on proteins. This result was related to the adaptation of shrimp to respond to protein and carbohydrate variations in the wild. Donaldson (1976) demonstrated that proteins are the most abundant molecules in benthic ecosystems, ranging between 46% and 72% in comparison to 1–2.5% CHO measured in the same area; this suggests that shrimp are adapted to synthesize CHO from dietary proteins by adjusting their digestive and metabolic processes. According to Vinagre and Da Silva (1992), muscle lactate might be an important source of carbon chains for gluconeogenesis or for producing metabolic energy under specific catabolic conditions when the catabolism of the free amino acid pool is not favored, such as in high-salinity-acclimated shrimp (Vinagre and Da Silva 1992; Rosas et al. 2001b). Rosas et al. (2001b, 2002) observed that OxyHc concentration is closely related to protein and CHO metabolism, because, in shrimp, OxyHc can be used to store proteins.

Although AG baseline levels have limited application, because they depend on the diet used to feed shrimp, we have now provided sufficient AG values, from both outside ponds and inside tanks, to be used for reference purposes. The present results indicate that all other baseline values could be used for practical applications. However, this would entail determining the blood metabolite levels in shrimp at the various culture facilities and in wild populations, to evaluate the range of variability. A wider spectrum of tropical crustacean species should be studied to define whether the differences observed between species are related to environmental characteristics (temperature–salinity–dissolved oxygen, or combinations thereof) or whether they are related to morphological and physiological adaptations, independent of environmental conditions.

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Capítulo 2

Efecto de un programa de selección por tamaño sobre los metabolitos de la hemolinfa y la respuesta inmunitaria de los juveniles de *Litopenaeus vannamei* alimentados con diferentes niveles de carbohidratos.

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Effect of a size-based selection program on blood metabolites and immune response of *Litopenaeus vannamei* juveniles fed different dietary carbohydrate levels

Cristina Pascual[a], Leticia Arena[a], Gerard Cuzon[b], Gabriela Gaxiola[a], Gabriel Taboada[a], Manuel Valenzuela[a], Carlos Rosas[a,*]

[a] *Laboratorio de Ecología y Biología Marina Experimental, Facultad de Ciencias, UNAM, Calle 26, No 1 Playa Norte Cd. del Carmen, Campeche, Mexico*
[b] *Centre Océanologique du Pacifique, BP 7004, Taravoo, Tahiti, French Polynesia*

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Abstract

Blood metabolites in wild and seventh-generation cultivated shrimp were measured to determine how size-based selection could alter the nutritional and immunological conditions of *Litopenaeus vannamei*. Wild *L. vannamei* juveniles and a sample of seventh-generation cultured shrimp were acclimated under identical conditions. During 55 days, shrimp were fed a high (HCHO: 44%) or a low (LCHO: 3%) carbohydrate diet for 55 days. Wild shrimp showed a direct relation between dietary CHO and lactate, protein and hemocyte levels indicating that dietary CHO was used for protein synthesis via transamination pathways. In seventh-generation cultured shrimp these parameters were inversely proportional to dietary CHO level, indicating the capacity to synthesize protein from dietary CHO was repressed in cultured shrimp. Farmed shrimp showed a limited capacity to respond to LCHO diets demonstrating high protein dependence in their metabolism and immune response. These results demonstrate that during size-based breeding programs other metabolic process than CHO catabolism can be selected. The incapacity of shrimp to use dietary CHO could limit

* Corresponding author. Tel./fax: +52-938-3828730.
E-mail address: crv@hp.fciencias.unam.mx (C. Rosas).

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protein reduction of diets and limit the efforts of the shrimp industry to be ecologically and environmentally profitable.

Keywords: Blood metabolites; *Litopenaeus vannamei*; Artificial selection; Immune response; Dietary carbohydrates levels

1. Introduction

Proteins are involved in recognizing foreign glucans through the lipopolysaccharide binding protein (LPSBP) and β-glucan binding protein (BGBP) (Vargas-Albores and Yepiz-Plascencia, 2000). A clotting protein is involved in engulfing foreign invading organisms and prevents blood loss upon wounding (Hall et al., 1999; Montaño-Pérez et al., 1999). Defense reactions in shrimp are often accompanied by melanization, where ProPO activating system leads to the synthesis of melanin (Sritunyalucksana and Söderhall, 2000) and is regulated by a number of proteins. Antimicrobial peptides are produced against Gram-positive bacteria (Destoumieux et al., 2000) and hemocyanin (Hc) is a multifunctional protein with both nutritional and immunological roles (Chen and Cheng, 1995; Destoumieux et al., 2001; Rosas et al., 2002) and a precursor of ProPO-like enzyme (Adachi et al., 2003). Recent studies showed that blood protein and glucose of *L. setiferus* and *L. vannamei* juveniles are highly sensitive to dietary protein and carbohydrate (CHO) contents (Rosas et al., 2001a,b). Blood protein was related to ammonia hemolymph level indicating that a protein-rich diet could regulate the rate of ammonia production and, in consequence, the gill glutamate dehydrogenase activity. From these data, it is evident that *L. vannamei* and *L. setiferus* can convert protein to glycogen by the gluconeogenic pathway, which allows the shrimp to maintain a minimum circulating glucose independently from dietary CHO. In this sense, Rosas et al. (2002) showed that protein metabolism is the modulator of the general metabolism in cultured shrimp, since, through protein metabolism, shrimp can synthesize their own carbohydrates (CHO), regulate their osmotic pressure and glycogen synthesis, or store protein as hemocyanin. Previous results demonstrated that blood glucose, triacylglycerols, cholesterol, and lactate together with blood protein, osmotic pressure, oxy hemocyanin (OxyHc), hemocytes and ProPO are good indicators of nutritional and immunological health in wild (Sánchez et al., 2001; Pascual et al., 2003a) and cultivated shrimp (Rosas et al., 2001a).

The white shrimp *L. vannamei* is the major aquaculture species in the American Pacific region. Diseases in this species have received considerable attention as bacteria and viruses have affected *L. vannamei* farms with losses of hundreds of millions of dollars. In response, shrimp farmers established selective breeding programs for *L. vannamei* to reduce the risks for infections associated with uncontrolled populations movements (Wyban et al., 1993; Bedier et al., 1998). Accordingly, as a part of a project to determine the effect of artificial selection on shrimp physiology, we measured blood metabolites in wild and seventh-generation cultivated shrimp to determine how the size-based selection can alter the nutritional and immunological conditions of *L. vannamei*. We used dietary CHO as a tool

to determine whether other metabolic responses aside from CHO and protein metabolism (Arena et al., 2003) could be changed as a consequence of selection, including part of the immune system (hemocytes, ProPO, and Hc).

2. Material and methods

2.1. Origin of shrimp

L. vannamei ($n=200$; 0.8 ± 0.1 g wet weight) were collected from Huizache and Caimanero Lagoon on the Pacific Coast of Mexico and were transported by plane in plastic bags with cool seawater (30‰; 20 °C) to the Experimental Marine Biology Laboratory of UNAM in Cd. del Carmen, Campeche, Mexico. Shrimp were acclimated to laboratory conditions for 2 weeks before being used in any experimental study. Shrimp were placed in a circular external pond (20 m^2) with aerated (O_2>5.0 mg/l) natural seawater (32‰; 29 ± 2 °C). During acclimation, shrimp were fed twice a day with a commercial shrimp diet with 45% protein (Api Aba camarón ultra, Malta Clayton, SA, Mexico). At the same time, a sample of seventh-generation cultured shrimp ($n=200$; 0.03 ± 0.03 g live weight) from a farm located in Sisal, Yucatán, Mexico, was transported to the laboratory in cool seawater (35‰, 24 °C), and acclimated under identical conditions to the wild shrimp.

After acclimation, a sample of each population was randomly chosen and distributed into 90-l experimental plastic tanks. Shrimp were reared for 55 to 58 days in a flow-through sea water system (salinity 32‰) at a density of 10 shrimp per tank. Shrimp were fed three times a day (0800, 1400, and 2000), uneaten food particles were removed twice a day (0730 and 1700) and water quality variables were maintained, i.e., temperature 28 ± 1 °C, dissolved oxygen >5.0 mg/l, and pH >8.0. In both locations, the photoperiod was set at 12 h light/12 h dark. At the end of the experiment, shrimp live wet weight was measured.

2.2. Diets

L. vannamei juveniles were fed formulated diets with two levels (3% and 44%) of carbohydrate (CHO). Experimental diets were prepared by thoroughly mixing dry ingredients with oil and then adding water until a stiff dough resulted. The dough was passed through a mincer with a 2-mm die, and the resulting spaghetti-like strings were air dried at 60 °C. After drying, the strings were broken up and sieved to appropriate pellet size and stored at −4 °C. Six tanks were randomly assigned to each CHO level (Table 1).

2.3. Physiological, nutritional, and immunological evaluations

After day 55, 35 shrimp per treatment were sampled for nutritional (hemolymph, lactate, cholesterol, triacylglycerol, protein, and hemocyanin, and digestive gland glycogen), immunological (ProPO oxidase activity in degranulated of hemocytes and blood cells), and physiological (osmotic pressure) evaluations. Blood metabolite measurements were made on fasted (12 h) shrimp. Before sampling, shrimp were placed in pre-

Table 1
Composition of the experimental diets

Ingredients	LCHO	HCHO
Squid meal	15	15
Casein	40	5
Cod liver oil	5	5
Starch	0	40
Lecithin	3	3
Cholesterol	0.6	0.6
Vitamins[a]	1.7	1.7
Minerals[b]	0.8	0.8
Na_2HPO_4	1	1
KH_2PO_4	1	1
Cellulose	4	4
Filler	22.4	22.4
Protein (%)	44	14
Carbohydrates (CHO%)	0	40
Lipids (%)	8.6	8.6

HP = High protein, LP = low protein. g/100 of diet.
Coefficient for energy concentration: 23/35/15 kJ for protein, lipid and carbohydrate, respectively (Rosas et al., 2001b).
[a] From Ralston Purina, without Vitamin C. Vitamin C from Stay C, La Roche.
[b] From Ralston Purina.

chilled (18 °C) and aerated seawater for 5 min to reduce the effect of manipulation before hemolymph extraction. Only shrimp in inter-molt stage (C stage) were used. Hemolymph (approximately 200–300 μl per shrimp) was individually sampled through a pre-chilled syringe needle inserted at the base of the fifth pereiopod after the shrimp had been dried with a paper towel. The individual weight (± 0.05 g) was recorded and molting stage was observed using uropod characteristics (Drach and Tchernigovtzeff, 1967).

For OxyHc measurements, 10 μl hemolymph were immediately diluted with 990 μl distilled water in a 10-mm cuvette and absorbance was measured at 335 nm. OxyHc concentration was calculated using an extinction coefficient of $\varepsilon = 17.26$ calculated on the basis of the 74,000-Da functional subunit (Chen and Cheng, 1993).

Commercial kits were used for lactate (Sigma-cat. 735), triacylglycerol (TAG; GPO-PAP, Merck, cat. 14354), and cholesterol (CHOD-PAP, Merck, cat. 14349). Determinations were adapted to a microplate using 20 μl of plasma obtained at 8000 × g centrifugation and 200 μl of enzyme chromogen reagent. Absorbance was recorded in a microplate reader (BIO-RAD model 550) and concentrations were calculated from a standard substrate solution. Plasma was further diluted 1:300 for protein (HP) determination by the Bradford (1976) technique adapted to a microplate method using commercial chromogen reagent (Sigma, cat. 610) and bovine serum albumin as standard.

2.4. Immune response

As ProPO system can be activated by endotoxins, all glassware was washed with E-Toxa-Clean® and all solutions were prepared using pyrogen-free water. Shrimp salt

solution was prepared according to Vargas-Albores et al. (1993); 450 mM NaCL, 10 mM KCL, 10 mM HEPES, pH 7.3, 850 mosM kg^{-1}. An anticoagulant solution for hemolymph extraction was prepared by adding 10 mM of EDTA-Na_2 to the shrimp salt solution. A sodium cacodylate buffer (Cac, 10 mM cacodylate, 10 mM $CaCL_2$, pH 7.0) was used to determine phenoloxidase activity.

Prophenol oxidase (ProPO) activity was measured in a sample of 50 μl which was incubated for 3 min at 25 °C with 50 μl of trypsin type IX (0.1 mg/ml). Then 50 μl of L-DOPA (3 mg/ml in Cac buffer) was added and incubated for 10 min. The absorbance at 490 nm was measured (Hernández-López et al., 1996).

Hemocytes were counted using a sample of 150 μl of hemolymph mixed with an Alsever solution (113 mM glucose, 27.2 mM sodium citrate, 2.8 mM citric acid, 71.9 mM NaCl) and 10% formaldehyde (v/v). This sample was stored frozen until analyzed. Hemocyte counting was done with a microscope and a Malassez chamber. Cellular characterization was based on size, shape, and granular content of the cells (Le Moullac et al., 1997).

2.5. Statistical analysis

Differences between populations and dietary CHO levels were assessed using a two-way ANOVA. A probability level of 0.05 was used to assess significance in all measured parameters.

Table 2
Two-way ANOVA summary of the effect of origin of the population and dietary CHO on blood metabolites of *L. vannamei* juveniles maintained for 55 days in experimental conditions

	df effect	MS effect	*df* error	MS error	*F*	*P*-level
ProPO						
Origin	1	0.180	102	0.018	10.056	**0.002**
CHO level	1	0.354	102	0.018	19.808	**0.000**
Origin vs. CHO level	1	0.137	102	0.018	7.661	**0.007**
Hemocytes, Total cells						
Origin	1	464,879,648	137	247,890,832	1.875	0.173
CHO level	1	53,063,372	137	247,890,832	0.214	0.644
Origin vs. CHO level	1	1,635,434,112	137	247,890,832	6.597	**0.011**
Hialine cells						
Origin	1	229,230,208.000	137	83,546,064	2.744	0.100
CHO level	1	37,727,860.000	137	83,546,064	0.452	0.503
Origin vs. CHO level	1	1,943,812,736.000	137	83,546,064	23.266	**0.000**
Granular cells						
Origin	1	41,225,616.000	137	55,062,672	0.749	0.388
CHO level	1	179,277,936.000	137	55,062,672	3.274	**0.046**
Origin vs. CHO level	1	13,309,299.000	137	55,062,672	0.242	0.624

N=35 data per experimental condition.

3. Results

3.1. Blood metabolites

A significantly higher lactate value (0.46 mg/ml) was observed in wild shrimp fed high dietary CHO (HCHO) as compared to the other treatments (mean value of 0.22 mg/ml) (Table 2; $P<0.001$). A significant interaction of dietary CHO and population origin was observed in lactate levels (Table 1, $P<0.05$). Origin of the population affected blood cholesterol values of *L. vannamei* juveniles with high values in wild (mean value of 0.32 mg/ml), and low in seventh-generation cultivated shrimp (mean value of 0.24 mg/ml) (Fig. 1; $P<0.001$). Blood triacylglycerols (TAG) were affected by dietary CHO levels yielding lower values in wild shrimp fed low dietary CHO (LCHO) ($P<0.001$). An inverse relation between TAG values and dietary CHO in each population produced a significant interaction ($P<0.001$, Table 1). Higher blood protein levels were observed in wild shrimp and in shrimp fed HCHO (Fig. 1, $P<0.05$). In contrast, the highest blood protein level in seventh-generation cultured

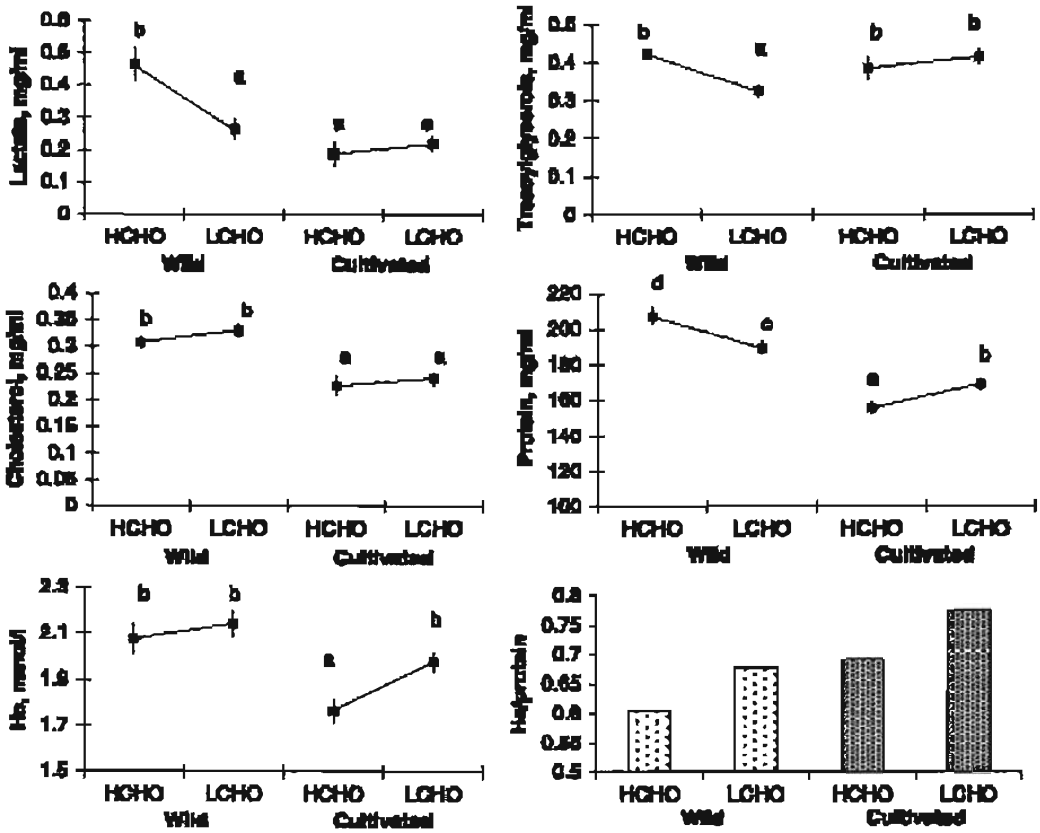

Fig. 1. Blood metabolites oxy hemocyanin (OxyHc) and OxyHc/protein ratio of wild and seventh-generation cultivated *L. vannamei* fed high (40%) and low (0%) dietary CHO. Mean ± S.E. Different letters mean statistical differences ($P<0.05$).

shrimp was obtained in those fed LCHO. A significant interaction of dietary CHO and population was also observed (Table 2, $P<0.05$). Different OxyHc levels were observed according to the origin of the population, with high levels in wild and low levels in seventh-generation cultivated shrimp, a significant interaction was also observed (Fig. 1, Table 2, $P<0.05$). The proportion of OxyHc of total blood protein varied between 60% and 77% with low values in wild shrimp fed HCHO and high values in seventh-generation cultured shrimp fed LCHO (Fig. 1).

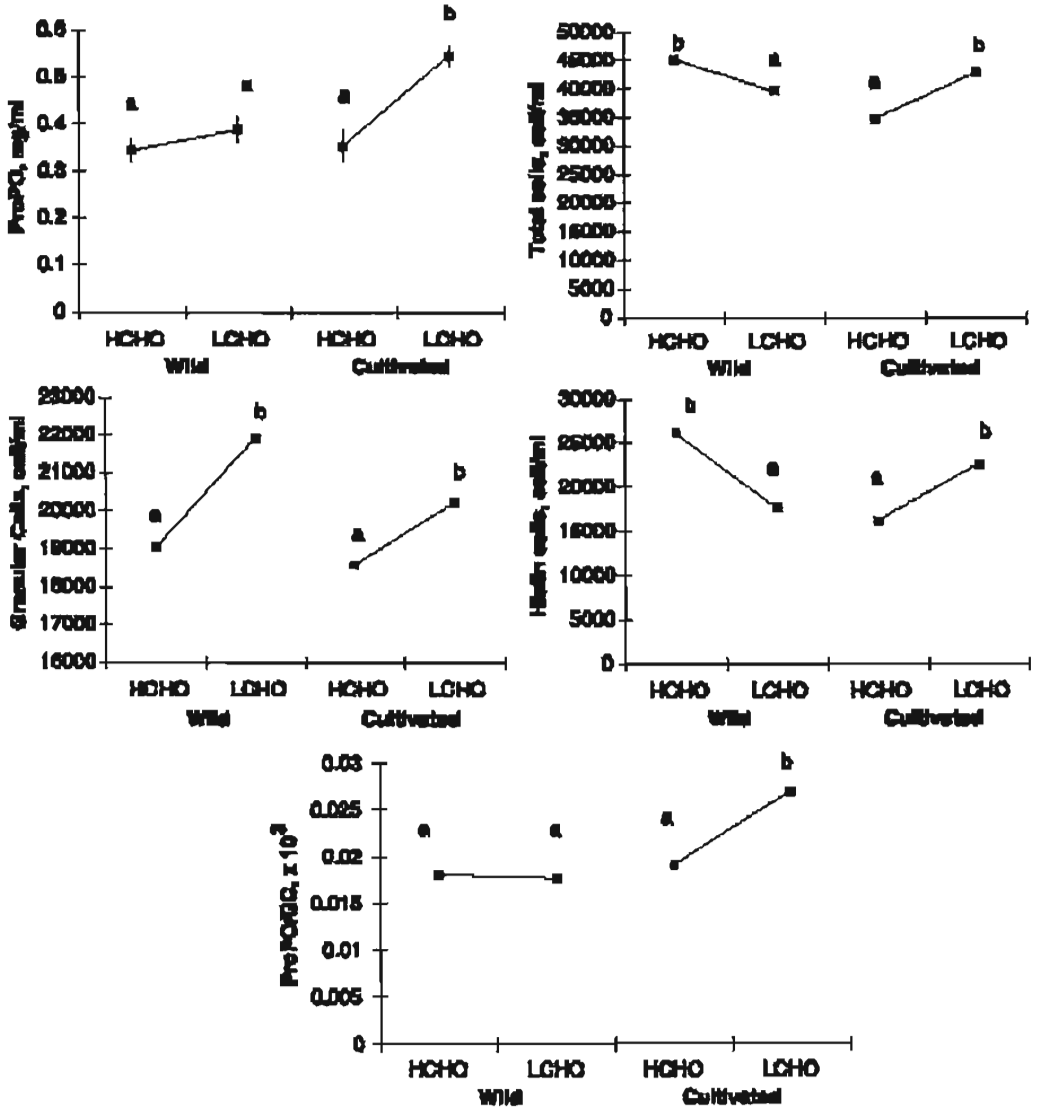

Fig. 2. Immune response (prophenol oxidase activity [ProPO]) hemocytes concentration and ProPO/granular cells (ProPO/GC) ratio of wild and seventh-generation cultivated *L. vannamei* fed high (40%) and low (0%) dietary CHO. Mean ± S.E. Different letters mean statistical differences ($P<0.05$).

3.2. Immune response

Population type, dietary CHO, and their interaction affected ProPO activity of shrimp (Fig. 2, Table 3; $P<0.05$). A high ProPO level was observed in seventh-generation cultured shrimp fed LCHO (0.55 OD_{490}), which was 52% higher than that obtained for the other treatments (mean value of 0.36 OD_{490}; Fig. 2). Total cells and hyalin cells presented a significant interaction between type of diet and population origin. High cell concentration was observed in both wild shrimp fed HCHO and seventh-generation cultured shrimp fed LCHO (Fig. 2). A significant effect of type of diet was observed on granular cells (large granular + granular cells) of both populations, with low values in shrimp fed HCHO and high cell concentrations in shrimp fed LCHO (Fig. 2, Table 3, $P<0.05$). A higher value of ProPO/granular cell ratio was observed in seventh-generation cultured shrimp (0.027 OD_{490} /cell $ml^{-1} \times 10^3$) as compared to that obtained for the other treatments (0.018 OD_{490}/cell $ml^{-1} \times 10^3$) (Fig. 2).

Table 3
Two-way ANOVA summary of the effect of origin of the population and dietary CHO on blood metabolites of *L. vannamei* juveniles maintained for 55 days in experimental conditions

	df effect	MS effect	*df* error	MS Error	*F*	*P*-level
Lactate						
Origin	1	0.664	105	0.039	17.081	**0.000**
CHO level	1	0.185	105	0.039	4.746	**0.032**
Origin vs. CHO level	1	0.340	105	0.039	8.747	**0.004**
Cholesterol						
Origin	1	0.190	105	0.004	47.064	**0.000**
CHO level	1	0.009	105	0.004	2.118	0.149
Origin vs. CHO level	1	0.000	105	0.004	0.088	0.767
Triacylglicerols						
Origin	1	0.017	105	0.009	1.890	0.172
CHO level	1	0.025	105	0.009	3.233	**0.045**
Origin vs. CHO level	1	0.103	105	0.009	11.509	**0.001**
Proteins						
Origin	1	33,425.945	105	420.591	79.474	**0.000**
CHO level	1	104.029	105	420.591	0.247	0.620
Origin vs. CHO level	1	6302.306	105	420.591	14.984	**0.000**
OxyHc						
Origin	1	0.930	119	0.180	5.173	**0.002**
CHO level	1	0.161	119	0.180	0.894	0.470
Origin vs. CHO level	1	0.350	119	0.180	1.949	**0.035**
CO						
Origin	1	346.029	109	3554.926	0.097	0.756
CHO level	1	89,327.969	109	3554.926	25.128	**0.000**
Origin vs. CHO level	1	183,285.875	109	3554.926	51.558	**0.000**

$N=35$ data per experimental condition.

4. Discussion

Blood metabolites and immune response of the wild shrimp were essentially different from those of seventh-generation cultured shrimp, demonstrating that during size-based breeding programs genes affect blood lactate, TAG, and protein, as well as ProPO and total and hyalin hemocytes. Statistical significant interaction between population type and type of diet demonstrated that wild and farmed populations responded differently to the same type of diet.

When CHO levels are changed in the diet, in isocaloric and isolipidic diets, protein must also be changed (Table 1). In the present study, diets were formulated to contain 3% CHO (LCHO; 66.5% protein) and 44% CHO (HCHO; 30% protein) and were isocaloric (18 kJ/gDE). Shrimp were exposed therefore to variation in both CHO and protein concentrations. We observed that blood lactate, TAG, and protein were higher in wild shrimp fed HCHO than in wild shrimp fed LCHO. However, in seventh-generation cultured shrimp, lactate and TAG were not affected by dietary CHO whereas a high blood protein level was observed in shrimp fed LCHO. Lactate is the end product of glycolysis after a reduction of pyruvate by NADH (Hochachka, 1970) and, in this sense (Huggins, 1966), showed that a high percentage of glucose metabolized during glycolysis in Crustacea (50%) is directed to form amino acids through the transamination pathway. High levels of blood lactate can regulate glycogen synthesis via crustacean hyperglycemic hormone (CHH): a reduction of CHH in blood is observed when a high lactate level is present, activating the glycogen synthetase in muscle and promoting glycogen synthesis (Santos and Keller, 1993). This mechanism was observed in *Carcinus maenas* (Santos and Keller, 1993) and *Chasmagnatus granulata* (Da Silva and Kucharski, 1992; Vinagre and Da Silva, 1992). High lactate levels observed in wild shrimp could indicate that high glycolysis activity was induced by a more active CHO digestion, promoting a more appropriate regulation of CHO metabolism in all pathways. The increment in blood protein observed in wild shrimp fed HCHO demonstrates that high protein level in blood could be related with amino acids synthesis obtained from the transamination pathway, and its posterior storing as hemocyanin or other peptides. Huggins (1966), using marked glucose, observed that pyruvate tends to be transaminated or reduced rather than oxidized via the tricarboxylic acid cycle. In other study, Boulton and Huggins (1970) showed that the presence of glutamate-pyruvate and glutamate-oxaloacetate aminotransferases confirmed that crab tissue extracts catalysed a direct interconversion of glutamic acid + pyruvic acid or oxaloacetic acid to give α-ketoglutamic acid + alanine or aspartic acid and vice versa, demonstrating that Crustacea can synthesize amino acids from CHO through glycolysis pathway. If wild shrimp can digest more dietary CHO than farmed shrimp, produce more amino acids and store it in hemocyanin, wild shrimp could improve its immune system and its general health status just for the important role that hemocyanin has as a multifunctional protein that participates in the defense mechanism and nutritional status of shrimp.

In seventh-generation cultivated shrimp, lactate was not affected by CHO dietary levels indicating that dietary CHO levels did not affect glycolysis. According to Arena et al. (2003), the limited capacity for starch digestion is related to a repression of amylase alleles that, at the same time, limit dietary starch digestion and CHO metabolism in general. The

present results suggest that, in addition to lactate, metabolism was affected by artificial selection, limiting protein synthesis (through transamination pathway) when shrimp are fed HCHO diets.

If this limitation is extended to proteins of the immune system, a higher sensitivity to pathogens (Xu et al., 2001) could be inducted by the segregation or selection of genes when size-based breeding programs based on high protein diets are applied in shrimp. Using the relation between ProPO/granular cells $\times 10^3$ as an index of immune system condition in *L. setiferus* (Pascual et al., 2003a,b) this ratio in wild shrimp in the present study was independent of diet composition. In contrast the index in seventh-generation cultured shrimp was affected by the type of diet yielding high values with LCHO (high protein level) and low ones with HCHO, suggesting that ProPO/granular cell levels depended on dietary CHO in the farmed population. Shrimp immune system has a strong protein base. If transamination and, in consequence, protein synthesis are limited, use of high dietary protein levels in a selection program could compromise the immune system and could be limited by a size breeding program. That means that during a size-based program with a regular diet of ~ 20% starch, although shrimp could grow more during the first years of selection, during this time they could lose metabolic capabilities to process CHO, affecting protein anabolism with consequences on protein requirements and immune system.

Acknowledgements

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Capítulo 3

Balance energético y respuesta inmunitaria de los juveniles de *Litopenaeus vannamei* a las proteínas de la dieta.

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Litopenaeus vannamei juveniles energetic balance and immunological response to dietary protein

Cristina Pascual[a], Edgar Zenteno[b], Gerard Cuzon[c], Ariadna Sánchez[a], Gabriela Gaxiola[a], Gabriel Taboada[a], Jaime Suárez[a], Teresita Maldonado[d], Carlos Rosas[a,*]

[a] *Laboratorio de Ecología y Biología Marina Experimental, Facultad de Ciencias, UNAM, Calle 8 A No 248, Vista Alegre Norte, C.P. 97130 Mérida, Yucatán, Mexico*
[b] *Departamento de Bioquímica, Facultad de Medicina, UNAM, Circuito exterior Cd. Universitaria, 04510 D.F., Mexico*
[c] *Centre Océanologique du Pacifique, PO 7004, Taravao, Tahiti, French Polynesia*
[d] *Facultad de Ciencias Químicas y Biológicas, Universidad Autónoma de Campeche, Mexico*

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Abstract

The present study was designed to evaluate the effect of dietary protein level on survival, assimilation efficiency and immunological condition of *Litopenaeus vannamei* juveniles using two protein levels in a range of optimal reported levels of 15% and 40% (equivalent to 15 and 40 g DP/kg body weight/day [g DP/kg BWd]) and one extremely low (5% equivalent to 5 g DP/kg BWd). In order to reach this goal, effects of dietary protein level on energetic balance were recorded by ingestion rate (I), respiratory rate (R) and biomass production of *L. vannamei* during its growing process (P). Energy lost from feces (H) and urine products (U) was calculated as $(H+U)=I-R+P$ and assimilated energy (As) as $R+P$. At the end of the growth experiment, shrimp immune response, oxyhemocyanin (OxyHc), osmotic pressure and digestive gland glycogen were measured. Dietary protein level enhanced ingestion rate in shrimp fed 5 g DP/kg BWd compared to shrimp fed 40 g DP/kg BWd. However, daily growth coefficient (DGC, %)of *L. vannamei* juveniles was high in shrimp fed 40 g DP/kg BWd in comparison to shrimp fed 5 g DP/kg BWd. An inverse relation between wastes ($H+U$) and dietary protein level was observed indicating that shrimp loose 81% of ingested energy when fed 5 g DP/kg BWd and only 5.6% when fed 40 g DP/kg BWd. A higher assimilation and production efficiency (P/As) was obtained when shrimp were fed 40 g DP/kg BWd, than obtained in shrimp fed 15 or 5 g DP/kg BWd and an inverse relation between R and respiratory efficiency (R/As) in relation to dietary protein levels was also observed. An increase in OxyHc was

observed with increasing dietary protein levels indicating that shrimp accumulated protein as hemocyanin. A reduction of hemocytes occurred when shrimp were fed sub-optimal dietary protein levels indicating that zymogens contained in hemocytes, i.e., prophenoloxidase (ProPO) system, peneidins and their activities (phagocytosis, coagulation), were also reduced. A reduction on respiratory burst was observed indicating that sub-optimal dietary protein level affected the number of cells and the phagocytosis capacity of cells. Notwithstanding, the compensatory mechanism used by *L. vannamei* to respond nutritional stress, sub-optimal dietary protein level (5 and 15 g DP/kg BWd) induced not only a severe reduction in growth rate and assimilation efficiency but also in immune capacities.

Keywords: Protein; Production; Oxygen consumption; Assimilation; Immune response; *Litopenaeus vannamei*

1. Introduction

Several studies have evaluated optimal dietary protein level for growth and feed conversion in *Litopenaeus vannamei*. Colvin and Brand (1977) found a better feed conversion for juveniles when shrimp were fed 25% crude protein when compared with shrimp with 30%, 35% or 40% dietary protein levels. Smith et al. (1984) indicated that maximum growth is obtained when 0.4 g shrimp are fed a diet exceeding 36% protein. Teichert-Coddington and Arrue (1988) proposed a range between 25% and 35% crude protein as optimum for *L. vannamei* maintained in outdoor ponds. Arantakananda and Lawrence (1993) concluded that maximum growth can be obtained with a dietary protein level of 15% with an optimal energy to protein ratio of 120 kJ g^{-1} protein with shrimp fed ad libitum. Cousin (1995) evaluated growth of *L. vannamei* juveniles and recommended a dietary protein level of 30% to obtain a maximum growth rate. More recently, Kureshy and Davis (2002) showed protein requirement for maximum growth with *L. vannamei* juveniles to be higher than 32%; they noticed 48% dietary protein level yielding a better feed efficiency.

Relative requirement in terms of percent dietary protein was largely discussed before (Lawrence et al., 1998; Aquacop and Cuzon, 1989) and optimum proposed for different species; in *Litopenaerus stylirostris*, on the contrary, an optimum protein level is hardly evidenced because growth continue up to 58% CP (Cuzon and Aquacop, 1998). Absolute requirement in protein has been proposed as the minimum or maximum amount of protein needed per animal per day (Cousin, 1995; Guillaume, 1977). In terms of absolute requirement, juvenile *L. vannamei* had maximum growth rate with 43 g DP/kg body weight/day (43 g DP/kg BWd) with an optimum above 32% CP (Kureshy and Davis, 2002). Cousin (1995) reported 33 g DP/kg BWd with an optimum of 30–35% CP and a protein/energy ratio of 20 mg/kJ DE.

Such expression of the requirement takes into account the ingestion rate that can be modulated by shrimp according to the energy density of the diet (Cuzon and Aquacop, 1998). White shrimp, *L. vannamei*, is an example where optimum protein level can be met by an elevation of intake; and then the question is raised about a possible increase in feed conversion ratio (FCR) when the protein level is reduced. This was proposed for *L.*

vannamei (Kureshy and Davis, 2002). Dietary protein consideration can not be dissociated from energy level. Fuel derived from lipid is not considered an adequate source to spare protein (Cousin, 1995; Cuzon et al., 2001). On the other hand, carbohydrates, such as native starch, are recommended up to 30% of the diet. Although a limitation could be found concerning its digestibility for an inbred strain (Rosas et al., 2001a), in a more recent study, a higher starch inclusion (40% CHO) was observed in *L. vannamei* wild specimen (Arena et al., 2003). Moreover, in case of protein-dense diet, shrimp possess an ability to use other metabolic pathways and derive glucose from glucogenic amino acids (alanine, glycine, valine, etc.) (Rosas et al., 2002). Energy/protein ratio of formulated feeds is certainly more useful than relative protein requirement which has been largely expressed by researchers, farmers and feed manufacturers; moreover, less consideration was put on how dietary protein level could affect physiological status of farmed shrimp.

Previous studies (Rosas et al., 2000, 2001a,b; Sánchez et al., 2001) showed a relationship between nutritional requirements, blood metabolites and immune system capacities for *L. vannamei* and *L. setiferus*. Taking into account such studies and data on shrimp protein metabolism from our laboratory, a scheme was elaborated to represent physiological and biochemical events that occur in shrimp fed high or low dietary starch (Rosas et al., 2002). Results demonstrated that shrimp are well adapted to live without dietary starch, channeling energy to growth from protein, although protein metabolism produced a substantial loss of energy through ammonia excretion. Shrimp fed low dietary starch levels had more hemocyanin and protein than observed in shrimp fed high dietary starch, thus confirming a capacity for *L. vannamei* to use dietary protein as an amino acid source to store protein in hemolymph as hemocyanin (Rosas et al., 2002).

The shrimp immune system has a solid protein base and hemocyanin plays an important role in its function. Recent studies have demonstrated that in addition to its multifunctional role (oxygen transporter, storage protein, carotenoids carrier, osmolite, ecdysone transporter) hemocyanin has a fungistatic (Destoumieux et al., 2001) and prophenol oxydase-like function (Adachi et al., 2003). Proteins are also involved in recognizing foreign glucans through lipopolysaccharide binding protein (LPSBP) and β glucan binding protein (BGBP) (Vargas-Albores and Yepiz-Plascencia, 2000). A clotting protein (with the change of fibrinogens to fibrin) is involved in engulfing foreign invading organisms and prevents blood loss upon wounding (Hall et al., 1999; Montaño-Pérez et al., 1999). Defense reactions in shrimp are often accompanied by melanization. Prophenoloxidase (ProPO)-activating system, mediated by hemocytes, is a zymogen of phenoloxidase (PO) enzyme that catalyzes both *o*-hydroxylation of monophenols and oxidation of phenols to quinones leading to synthesis of melanin (Sritunyalucksana and Söderhall, 2000). Conversion of ProPO to PO occurs through a serine protease called prophenoloxidase-activating enzyme (ppA) regulated by another protein, α-2 macroglobulin, a trypsin inhibitor (Perazzolo and Barracco, 1997). The innate immune response of shrimp also relies upon a production, in hemocytes, of antimicrobial peptides called peneidins that are active against a large range of pathogens essentially directed against Gram-positive bacteria via a strain-specific inhibition mechanism (Destoumieux et al., 2000).

According to Lucas (1993), energetics can be defined as the quantification of the exchange and transformations of energy and matter between living organisms and their environment. When this concept is applied to cultured shrimp in nutritional studies,

energetic helps to understand how nutrients modulate the physiological mechanisms related with the transformation of food energy into biomass through the basic equation $I=H+U+R+P$, where I is the ingested energy, H is the energy lost in feces, U is the energy lost in nitrogen metabolism, R is the energy invested in respiratory metabolism and P is the energy invested in production of biomass or gametes. In a practical sense, this equation can provide data to know how much energy of the food is necessary for maximum growth rate and how the different proportions of nutrients in a diet are used as a source of metabolic energy for growth or lost as waste products. Protein requirements according to Kureshy and Davis (2002) can be defined as the level of protein required for maintaining body functions associated with protein metabolism with all other nutrients having been provided in adequate amounts. In this context, protein requirement should consider how much protein is needed to optimally maintain physiological status of shrimp including immune system.

According with many researchers the optimal protein level ranges between 15% and 36%, although more than 32% appears as a dietary protein level that could satisfy shrimp physiological condition (Kureshy and Davis, 2002). In this context, the present study was designed to evaluate the effect of dietary protein level on survival, assimilation efficiency and immunological condition of *L. vannamei* juveniles. Two protein levels in the range of optimal reported levels (15% and 40% equivalent to 15 and 40 g DP/kg BWd) and one extremely low (5% equivalent to 5 g DP/kg BWd) level were used. Immunological conditions were defined as the capacity of shrimp immune system to respond to an experimental condition without stress.

2. Material and methods

2.1. Animals

A group of 150 shrimp (1.59±0.07 g wet weight) were used in this experiment. *L. vannamei* were obtained from Pecis Industries, in Yucatan, Mexico. Shrimp were reared for 50 days in 90-l tanks (10 shrimp/tank) and fed different protein levels: 5%, 15% and 40%. Five tanks were randomly assigned to each treatment. Photoperiod was 12h/12h, water temperature ranged from 28±1 °C, dissolved oxygen was >5.0 mg/l, salinity was 35‰ and pH was between 7.9 and 8.2.

Shrimp were fed at 10% body weight. Feed amount was divided in three rations a day (0800, 1400 and 2000 h). Taking into account that ratio change with changes in body weight, food ratio was adjusted every 10 days. A sample of 10 shrimp were taken from one tank of each treatment. At the end of the trial, all experimental tanks were sampled one time at least. Uneaten food particles and feces were removed once a day by siphoning.

2.2. Preparation of diets

The experimental diets were prepared by thoroughly mixing dry ingredients with oil and then adding water until a stiff dough resulted (Table 1). This was then passed through a meat mincer with a die, and the resulting spaghetti-like strings were air dried at 60 °C for

Table 1
Composition of the experimental diets (g kg^{-1})

Ingredients	Dietary protein, g DP/kg BWd		
	5	15	40
Anchovy fish meal[1]	56	168	450
Squid meal[2]	12.5	37.5	100
Soy bean meal	22.5	67.5	180
Native wheat flour[3]	609.1	495.4	190
Soy bean lecithin[3]	20	20	20
Cod liver oil[3]	43	32	22
Cholesterol	2	2	2
Vitamins mix[a]	17	17	17
Rovimix Stay-C[b]	0.286	0.286	0.286
Minerals[c]	8	8	8
Filler[4]	199.6	142.3	0
Alginate Na	10	10	10
Crude protein (%)	5.6	15	40
Crude lipid (%)	10	10	10
CBH (%)	61	49.5	19
Ash %	23	17	9
Total energy (kJ g^{-1})[*]	16	16	16

[a] Vitamin premix without vitamin C, provided by Agribrands de México.
[b] Ascorbyl phosphate (Stay-C—35%; Roche).
[c] Mineral premix provided by Agribrand de México.
[*] Estimated from Cuzon and Guillaume (1987) with the following coefficients: 21.3, 17.6 and 39.5 kJ g^{-1} for protein, carbohydrates and lipids, respectively.
[1] CORPESCA SUPERPRIME (Chilenean Fish meal, steam dried).
[2] Squid meal (*Loligo* sp., from Gulf of México).
[3] Droguería Cosmopolita, México.
[4] Cellulose.

24 h. After drying, the material was broken up and sieved to a convenient pellet size and stored at −4 °C.

2.3. Growth and survival

Growth rate was evaluated as the difference between wet weight at the start and end of the experiment, calculated as daily growth coefficient (DGC, %) (Bureau et al., 2000):

$$\mathrm{DGC} = 100 \times [(\text{final weight})^{1/3} - (\text{initial weight})^{1/3}]/\text{time (days)}$$

Survival was calculated as the difference between the number of live animals at the start and end of the experiment.

2.4. Energetic balance

Ingested feed was individually measured directly into respirometric chambers of 12 shrimp per diet. That value was obtained from the differences between the given food

and the remained feed obtained at the end of the oxygen consumption measurements. The feed lost into the experimental chamber was calculated as the difference between the remaining feed and the feed obtained from a control chamber without shrimp. To do that, the water of the experimental chambers were filtered using a pump. A pre-weighed Whatman filter no. 20 was used to retain the unconsumed feed that was dried at 60 °C until constant weight. Ingested energy was calculated using the energy content of the feed (15.8 J g^{-1}) that was measured with an adiabatic calorimeter Parr® pump, previously calibrated with benzoic acid. Total energy of the feces was obtained from feces collected directly in experimental growth tanks after fed at 0800 h during 5 days. To do that, shrimp were fed and the remained feed collected by siphon 2 h after. All the water was gently changed without changing the volume of the tank. Once the tank was cleaned, feces were collected by siphoning and filtering the water on a Whatman filter no. 20 coupled to a pump. All feces were pooled by diet and dried at 60 °C until analysis. Feces energy was obtained from three samples per diet burned in the calorimeter pump. Total feces energy was expressed as joules per gram of dry weight feces (J g^{-1} dw).

Energetic balance was estimated using the following equation (Lucas, 1993):

$$I = H + U + R + P$$

where I is the ingested gross energy, H is the energy lost in feces, U the energy lost in nitrogen products, R indicates respiration ($R=R_{rout}+R_{AHI}$) and P is energy invested in biomass production. In the present paper, H and U were not measured but were estimated as

$$(H + U) = I - \mathrm{As}.$$

Assimilated energy (As) was calculated as $R+P$. All values were expressed as J day^{-1} g^{-1} dw. Oxygen consumption was measured in 12 shrimp from each diet. Oxygen consumption was determined individually by a continuous flow respirometer in a closed system (Rosas et al., 2002). Oxygen consumption was calculated as

$$VO_2 = O_{2o} - O_{2ex} \times \mathrm{Fr},$$

where VO_2 is oxygen consumption (mg O_2 h^{-1} $animal^{-1}$), O_{2o} indicates oxygen concentration at entrance to the chamber (mg l^{-1}), O_{2ex} is oxygen concentration at exit (mg l^{-1}) and Fr is the flow rate (ml h^{-1}). Oxygen concentration was measured using a digital oxymeter (YSI 50B digital, USA) with a polarographic sensor (±0.01 mg l^{-1}), previously calibrated with oxygen-saturated seawater at 28 °C. Afterwards, shrimp were fed feed pellet fragments of 0.06±0.002 g distributed in each respirometric chambers. Same amount of feed was placed in a control chamber without organisms to estimate oxygen lost by feed decomposition. Oxygen consumption of fed shrimp was measured every hour for a 5-h period, between 0800 and 1300. Once the experiment was concluded, shrimp were weighed. Routine metabolism (R_{rout}) was estimated from VO_2 (mg g^{-1} h^{-1}) of unfed shrimp considering the time during the day in which shrimp do not feed. Apparent heat increase (R_{AHI}; J g^{-1} h^{-1}) was estimated from the difference

between VO_2 of unfed shrimp and maximum value attained after feeding and considering the time needed for peak oxygen consumption after feeding and the number of rations fed to shrimp per day (n=3) during growth experiment. A 14.3 J mg^{-1} conversion factor of oxygen consumption was used to transform unfed and fed VO_2 to J g^{-1} dry weight (dw) (Lucas, 1993). The AHI coefficient was calculated as $R_{AHI}/I \times 100$. Respiration (R) was calculated as $R_{rout} + R_{AHI}$ and expressed as J day^{-1} g^{-1} dw taking into consideration that shrimp were fed three times a day during the growth trial.

Energy produced (P) was calculated using the actual growth rate of the shrimp obtained during experimental time (50 days). The value of 23.96 ± 0.72 J g^{-1} dw was used to transform the growth data into production units (P; J g^{-1} dw day^{-1}). This value was obtained from analyzing energy content applied to abdominal muscle of 10 shrimp by means of a calorimeter (Parr®), previously calibrated with benzoic acid.

Assimilated, respiratory and production gross efficiencies were calculated as $As/I \times 100$, $R/I \times 100$ and $P/I \times 100$, respectively. Respiratory (R) and production net efficiencies (PE) were calculated as $R/As \times 100$ and $P/As \times 100$, respectively.

2.5. Blood constituents, osmotic pressure and immunological evaluations

Blood measurements were made in living fasted (12 h) shrimp at end of experiment. Before sampling, shrimp were placed in pre-chilled (18 °C) and aerated seawater for 5 min to reduce the effect of manipulation before hemolymph extraction. Only shrimp in intermolt stage (C stage) were used. Hemolymph (approximately 300–500 μl per shrimp) was individually sampled through a pre-chilled syringe needle inserted at the base of the fifth pereiopod after the shrimp had been swiped off with a paper towel. The individual weight (±0.05 g) was recorded and molting stages were determined using uropod characteristics (Drach and Tchernigovtzeff, 1967).

Osmotic pressure of the hemolymph and water was measured in a micro-osmometer with 20 μl of sample per titration (3 MO-PLUS; Advanced Instruments, USA). The osmotic capacity (OC) was defined as the difference between osmotic pressure of hemolymph and external medium (Lignot et al., 1999).

For hemocyanin (OxyHc) measurements, 10 μl hemolymph was immediately diluted with 990 μl distilled water in a 10-mm cuvette, and absorbance was measured at 335 nm. Hemocyanin concentration was calculated using an extinction coefficient of ε=17.26 calculated on the basis of the 74000 Da functional subunit (Chen and Cheng, 1993).

Glycogen in the digestive gland was extracted in the presence of sulfuric acid and phenol (Dubois et al., 1965). The digestive gland was first homogenized in trichloroacetic acid (TCA, 5%) for 2 min at $3340 \times g$. After centrifugation ($7000 \times g$), the supernatant was quantified, this procedure was done twice. Two hundred microliters of supernatant was pipetted into a tube and mixed with 5 vol of 95% ethanol. Tubes were placed in an oven at 37–40 °C for 3 h. After precipitation, the tubes were centrifuged at $7000 \times g$ for 15 min. Glycogen pellet was dissolved by adding 0.5 ml of boiling water. Then, 1 milliliter of concentrated sulfuric acid and phenol (5%) was added and mixed. Tube contents were transferred to microplate and read at 490 nm in a microplate reader (Bio-Rad 550).

2.6. Immune condition

Because the ProPO system can be activated by endotoxins, all glassware was washed with E-Toxa-Clean® and all solutions were prepared using pyrogen-free water. Shrimp saline solution was prepared according to Vargas-Albores et al. (1993): 450 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3, 850 mOsm kg^{-1} The anticoagulant solution for hemolymph extraction was prepared by adding 10 mM of EDTA–Na_2 to the shrimp saline solution. A sodium cacodylate buffer (Cac, 10 mM cacodylate, 10 mM $CaCl_2$, pH 7.0) was used to determine phenoloxidase activity.

2.7. Hemocytes

A sample of 150 μl of hemolymph was mixed with an Alsever solution (113 mM glucose, 27.2 mM sodium citrate, 2.8 mM citric acid, 71.9 mM NaCl) with 10% formaldehyde (v/v). This sample was stored frozen until analysis (8 °C). The hemocytes counting was done with a Malassez chamber. Cellular characterization was done taking into account size, form and granular content of the cells (Pascual et al., 2003).

2.8. Prophenoloxidase activity

A sample of 50 μl of hemolymph was incubated for 3 min at 25 °C with 50 μl of trypsin type IX (0.1 mg/ml). Then, 50 μl of L-DOPA (3 mg/ml in Cac buffer) was added and incubated 10 min. The absorbance at 490 nm was measured (Hernández-López et al., 1996). ProPO activity was expressed as ProPO granular cell $(GC)^{-1} \times 1000$.

2.9. Respiratory burst

Intracellular production of the superoxide anion in hemocytes was quantified using the NBT (Nitroblue Tetrazolium) reduction to formazan (Song and Hsieh, 1994). Fifty microliters of hemolymph diluted in Alsever solution (1:1) was placed in a microplate and centrifuged at $800 \times g$ per 10 min. Plasma was removed and hemocytes were washed with 100 μl of Hank's solution. Next, 100 μl of Zymosan (0.1% in Hank's solution) was added and incubated for 2 h at room temperature. The zymosan was then removed and hemocytes were washed three times with 100 μl of Hank's solution and staining for 30 min with NBT solution (0.3%) at room temperature. After stained solution was removed, fixed hemocytes were washed three times with 100 μl of methanol (70%) and dried for 5 min. Formazan was dissolved with 120 μl of KOH and 140 μl of DMSO, and optical density was read at 630nm using a microplate reader (Bio-Rad Mod. 550).

2.10. Hemagglutinating activity (HU)

Human blood was used to make hemagglutinating activity measurements. Blood used was obtained from Medicine Faculty of the National University of México. Blood cells were suspended in a 2% saline solution (v/v). Shrimp plasma was obtained at $800 \times g$ centrifugation of blood diluted 1:3 with SIC–EDTA. Afterwards, plasma was serially

diluted 11 times (1:2) with 50 µl of saline solution in type Falcon plates wells in U form. Finally, 50 µl of human erythrocytes suspension was added and plates were incubated for 2 h at room temperature (26±2 °C). A column of the plate with erythrocytes and saline solution was used as a control. Hemagglutination title was recorded as the inverse of the last dilution hemagglutinating activity was observed. Results were expressed as specific hemagglutinating activity (HU/mg protein).

2.11. Statistical analysis

ANOVA was applied to results of growth rate, survival, energetic balance and immunological parameters. An arcsin transformation was used before processing percentage data (Zar, 1974).

3. Results

3.1. Energetic balance

Ingestion rate increased significantly according with a reduction in dietary protein levels (Table 2). Ingestion rate of shrimp fed 5 g DP/kg BWd was 231% and 168 % higher than that observed in shrimp fed 15 and 40 g DP/kg BWd, respectively ($P<0.05$; Table 2). At the end of the experiment, ingestion rate was equivalent to 6%, 4% and 3% of body weight for shrimp fed 5, 15 and 40 g DP/kg BWd, respectively. Survival was similar between treatments with values ranging between 84% and 94% ($P>0.05$).

Daily growth coefficient (%) resulted significantly higher in shrimp fed 40 DP/kg BWd (1.96±0.02%) than those observed in shrimp fed 15 DP/kg BWd (1.39±0.01%) and 5 DP/

Table 2
Energy balance of *L. vannamei* juveniles fed different dietary protein levels

	Protein, g DP/kg BWd		
	5	15	40
Ingestion rate, (I) day^{-1} g^{-1} dw	3298.20±362^{c}	1954.00±254^{b}	1427.00±157^{a}
Respiration (R)			
R_{ICA} J day^{-1} g^{-1} dw	6.86±0.16^{a}	49.76±11.55^{c}	60.06±2.52^{b}
R_{RUT} J day^{-1} g^{-1} dw	438.44±65.77^{c}	249.68±19.90^{b}	171.60±20.60^{a}
R=R_{RUT}+R_{AHL}, J day^{-1} g^{-1} dw	445.30±65.93^{c}	299.44±11.10^{b}	231.66±11.56^{a}
Production (P), J day^{-1} g^{-1} dw	155.29±5.95^{a}	664.58±6.14^{b}	1115.97±14.16^{c}
Energy lost (H+U)=I(R+P), J day^{-1} g^{-1} dw	2698.00	991.00	81.00
Assimilation=(P+R), J day^{-1} g^{-1} dw	600.59	964.02	134.63
Assimilation efficiencies As/I, %	18.00±0.001^{b}	49.00±0.002^{b}	94.00±0.003^{a}
Feces energy content J g^{-1} dw	6243±187^{a}	6999±210^{b}	11809±354^{c}
(H+U)/I, %	81.00	50.70	5.60
R/I, %	13.50	28.80	16.30
P/I, %	4.70	34.00	78.20
R/As, %	74.14	31.06	17.19
P/As, %	25.86	68.94	82.81

Means with superscript letters in same row are statistical different.

kg BWd (0.5±0.02%) ($P<0.05$) (Fig. 1). Consequently, produced energy (P) was higher in shrimp fed 40 DP/kg BWd (1.1 kJ day^{-1} g^{-1} dw) than those observed in shrimp fed 15 and 5 DP/kg BWd (0.7 and 0.1 kJ day^{-1} g^{-1} dw, respectively) (Table 2).

Oxygen consumption was measured in fasted and fed shrimp (Fig. 2). A higher fasting oxygen consumption was obtained in shrimp fed 5% protein (1.47 mg O_2 h^{-1} g^{-1} dw) in comparison to that observed in shrimp fed 15 and 40% protein (mean value of 0.99 mg O_2 h^{-1} g^{-1} dw) ($P<0.05$). Consequently, R_{RUT} of shrimp fed 5 DP/kg BWd was 1.49 and 1.92 times higher than those observed in shrimp fed 15 and 40 DP/kg BWd, respectively (Table 2).

Once shrimp were fed, oxygen consumption increased rapidly reaching a peak 1, 2 and 1 h after feeding for 5, 15 and 40 DP/kg BWd level, respectively. A high post-prandial oxygen consumption was recorded in shrimp fed 15% protein. That value was 1.3 and 1.56 times higher than those observed in shrimp fed 5 and 40 DP/kg BWd, respectively ($P<0.05$). It is interesting to note that oxygen consumption of shrimp fed 5 DP/kg BWd returned to pre-feeding values just 1 h after reaching a peak while shrimp fed 15 and 40 DP/kg BWd needed 2 and 4 h to return to post-prandial oxygen consumption values, respectively. In fact, shrimp fed 40 DP/kg BWd maintained high and constant post-prandial oxygen consumption for 4 h (Fig. 2). Apparent heat increment (R_{AHI}) of shrimp fed 5 DP/kg BWd was 86% lower than that obtained in shrimp fed 15 DP/kg BWd and 88% lower than that obtained in shrimp fed 40 DP/kg BWd (Table 2). A high total R ($R_{RUT}+R_{AHI}$) was obtained in shrimp fed 5 DP/kg BWd with 0.4 kJ day^{-1} g^{-1} dw. This value was 1.49 and 1.92 times higher than those observed in shrimp fed 15 and 40 DP/kg BWd, respectively (Table 2). A high assimilated energy ($P+R$) was recorded in shrimp fed 40 DP/kg BWd (1.3 kJ day^{-1} g^{-1} dw). This value was 1.40 and 2.24 times higher than those observed in shrimp fed 15 and 5 DP/kg BWd (Table 2). A significant increment on assimilation efficiency (As/I, %) was recorded according to an increase in dietary protein level with values of 18%, 49% and 94% in shrimp fed 5, 15

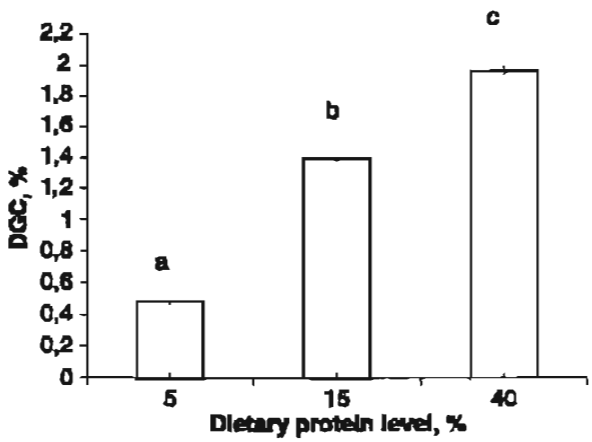

Fig. 1. Daily growth coefficient (%) of *L. vannamei* juveniles fed different protein levels during 50 days. Mean±S.E. (S.E. as a bar). Different letters denote statistical differences at $P<0.05$.

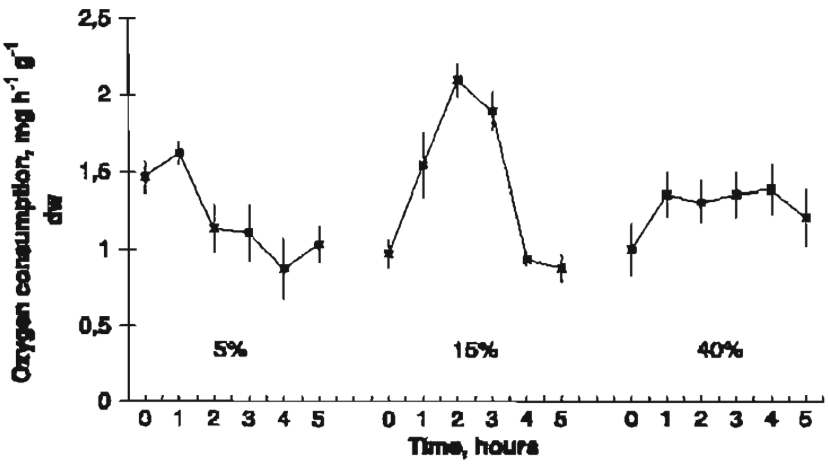

Fig. 2. Mean oxygen consumption (mg O_2 h^{-1} g^{-1} dw) of *L. vannamei* related to feeding time and dietary protein level (%) (mean±S.E.).

and 40 DP/kg BWd, respectively ($P<0.05$). In contrast, energy loss as waste products calculated as $(H+U)=I-(R+P)$ showed a reduction according to an increment in dietary protein with high values in shrimp fed 5 DP/kg BWd and low in shrimp fed 40 DP/kg BWd. Consequently $(H+U)$ was 81%, 51% and 6% of the ingested gross energy in shrimp fed 5, 15 and 40 DP/kg BWd (Table 2).

The total energy content in feces increased according to dietary protein levels with low values in shrimp fed 5 DP/kg BWd (6.2 kJ g^{-1} dw) and high values in shrimp fed 40 DP/kg BWd (11.8 kJ g^{-1} dw). An intermediate value (Table 2) was recorded in shrimp fed 15 DP/kg BWd (7 kJ g^{-1} dw) ($P<0.05$).

3.2. Blood constituents, osmotic pressure and digestive gland glycogen

An increase in hemocyanin (Fig. 3) and digestive gland glycogen (Fig. 4) was observed according with an increase in dietary protein level ($P<0.05$). Osmotic pressure was no

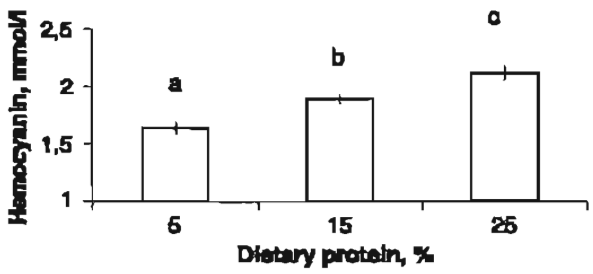

Fig. 3. Effect of dietary protein level (%) on oxyhemocyanin of *L. vannamei* fed during 50 days. Mean±S.E. Different letters denote statistical difference between treatments.

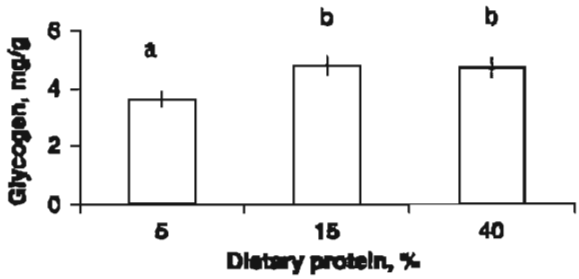

Fig. 4. Effect of dietary protein level (%) on digestive gland glycogen of *L. vannamei* fed during 50 days. Mean ±S.E. Different letters denote statistical difference between treatments.

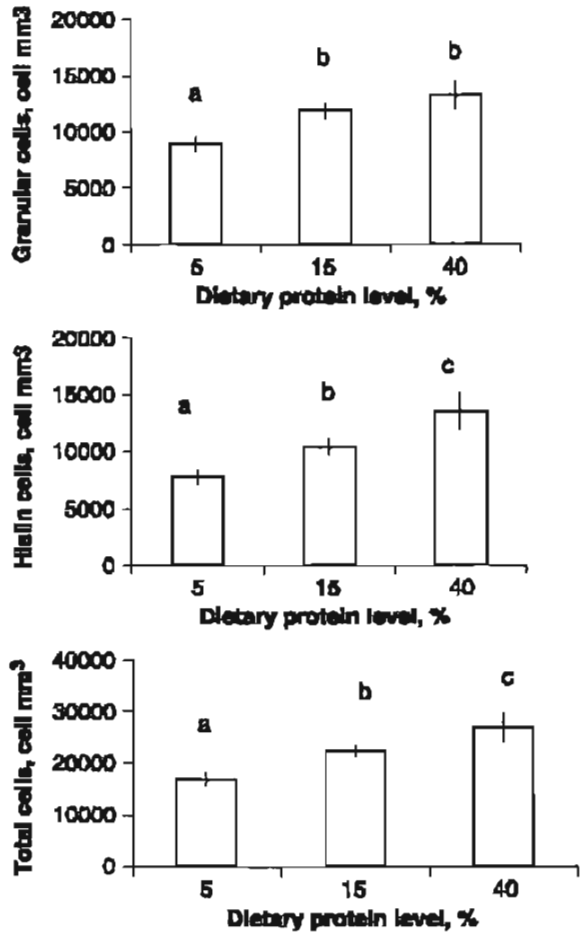

Fig. 5. Effect of dietary protein level (%) on total and differentiated hemocytes of *L. vannamei* fed during 50 days. Mean ±S.E. Different letters denote statistical difference between treatments.

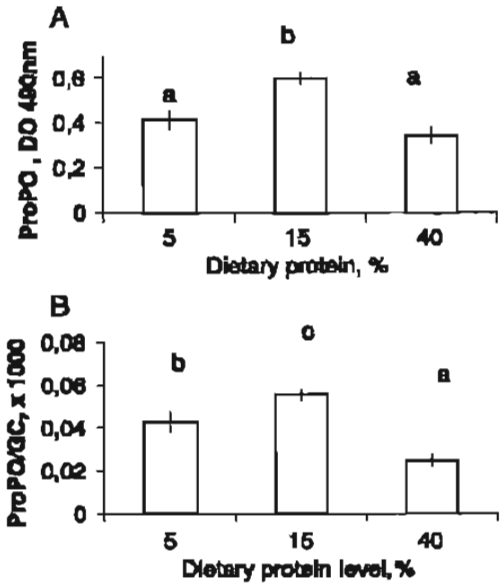

Fig. 6. Effect of dietary protein level (%) on prophenoloxidase (ProPO) in hemocytes degranulated (A) and ProPO/granular cells ratio (ProPO/GC) (B) of *L. vannamei* fed during 50 days. Mean±S.E. Different letters denote statistical difference between treatments.

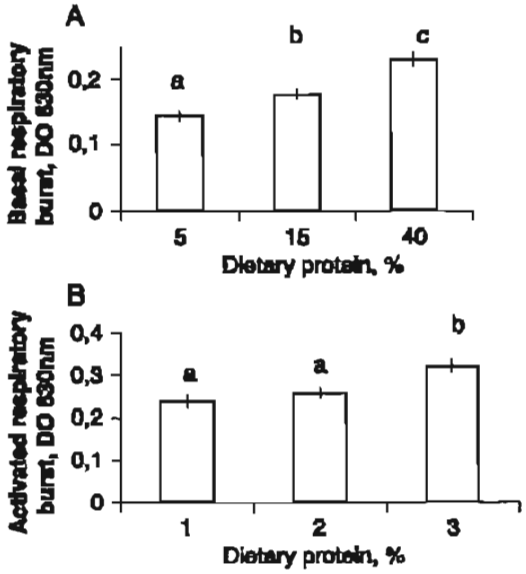

Fig. 7. Effect of dietary protein level (%) on basal (A) and activated respiratory burst (B) of *L. vannamei* fed during 50 days. Mean±S.E. Different letters denote statistical difference between treatments.

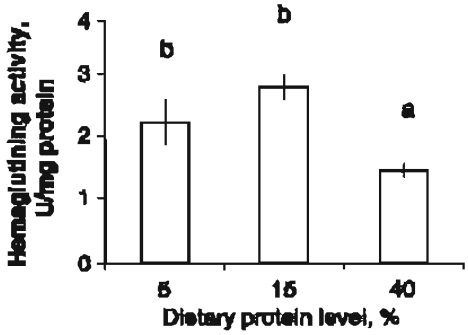

Fig. 8. Effect of dietary protein level (%) hemagglutinating activity of *L. vannamei* fed during 50 days. Mean ± S.E. Different letters denote statistical difference between treatments.

affected by dietary protein level, for that reason, a mean value of 877 mOsm/kg can be calculated for all shrimp used in the present study.

3.3. Immune condition

Hemocytes concentration were affected by dietary protein level with high values in shrimp fed 40 DP/kg BWd and low in shrimp fed 5 DP/kg BWd. Intermediate values were observed in shrimp fed 15 DP/kg BWd ($P<0.05$) (Fig. 5).

ProPO activity in degranulated of hemocytes was affected by dietary protein level with low values in shrimp fed 5 and 40 DP/kg BWd and high values in shrimp fed 15 DP/kg BWd. When ProPO was expressed per granular cell, a lower value was obtained in shrimp fed 40 DP/kg BWd ($P<0.05$) (Fig. 6a and b).

Basal and activated respiratory burst were affected by dietary protein levels with low values in shrimp fed 5 DP/kg BWd and high values in shrimp fed 40 DP/kg BWd levels (Fig. 7a and b). Activated respiratory burst of shrimp fed 5 and 15 DP/kg BWd did not show significant differences ($P>0.05$) with a mean value of 0.25 $DO_{630\ nm}$ (Fig 7b).

A reduction of hemagglutinating activity (Fig. 8) was observed in accordance with an increase in dietary protein levels with high values in shrimp fed 5 and 15 DP/kg BWd (mean value of 2.58 U/mg protein) and low values in shrimp fed 40 DP/kg BWd (1.51 U/mg protein).

4. Discussion

As expected, dietary protein level affected growth rate of *L. vannamei* juveniles promoting a high daily growth coefficient (%) in shrimp fed 40 DP/kg BWd. Although a low DGC, in shrimp fed 15 DP/kg BWd was expected, shrimp fed 5 DP/kg BWd showed a small DGC and a high survival value (94%). This result is probably due to the fact that 5 DP/kg BWd dietary protein level is above the maintenance requirement reported by Kureshy and Davis (2002) (1.8–3.8 g DP/kg BWd). In this same sense, it

is not surprising that shrimp fed 40 DP/kg BWd showed maximal DGC%, because that protein level is close to the one reported as the protein requirement for maximum growth for *L. vannamei* juveniles (43.4 g DP/kg BWd) (Kureshy and Davis, 2002).

Ingestion rate was increased according with a reduction in dietary protein level suggesting that shrimp are adapted to compensate those deficiencies through an increment in food ingestion. That results indicate that shrimp ingestion rate is modulated by dietary protein content and consequently by amino acids as a source of energy and building blocks for protein synthesis. In a recent study, Rosas et al. (2002) showed that shrimp are well adapted to use protein as a source of energy demonstrating that *L. vannamei* have the capacity to synthesize dietary carbohydrates through the gluconeogenic pathway. Although in the present study, ingested amino acids could not be recorded, high ingestion rate of shrimp fed 5 g DP/kg BWd was not enough to compensate for dietary deficiencies of such a diet. One explanation could be based on the energy invested in assimilation and metabolism of the ingested food. At low dietary protein levels, an increment in shrimp ingestion rate could provoke an unnecessarily energy expenditure and at the same time a reduction of amino acid absorption related to faster transit of the ingested feed affecting nutrient assimilation. A higher energy loss from waste products ($H+U$) was obtained in shrimp according to reduction in dietary protein levels indicating that, effectively, a higher proportion of ingested energy was lost when shrimp were fed low (5 DP/kg BWd: 81% of intake I) compared to high dietary protein (15 DP/kg BWd: 51% of intake I; 40 DP/kg BWd: 6% of intake I).

Results of this study suggest a higher assimilation and production efficiency (P/As) obtained when shrimp are fed 40 DP/kg BWd than fed 15 or 5 DP/kg BWd. At the same time, an inverse relation between R and respiratory efficiency (R/As) in relation with dietary protein levels was also observed. According to Lucas (1993), R is the sum of energy used for catabolism and anabolism of biological molecules and represents the energy used for basal metabolic rate and routine metabolic rate (R_{RUT}) and to process ingested food (R_{AHI}). Present results showed that R decreased according to an increment in dietary protein levels, with high values in shrimp fed 5 DP/kg BWd and low values in shrimp fed 40 DP/kg BWd, indicating that shrimp fed 40 DP/kg BWd were metabolically more efficient because those shrimp used less energy to maintain routine metabolic rate despite an increment in energy invested in R_{AHI}. Consequently, energy available for growth was higher in shrimp fed 40% protein than that obtained in shrimp fed 15 and 5 DP/kg BWd.

R_{AHI}, as part of R, also known as specific dynamic action (SDA) has been associated with a calorigenic effect of food. This is a measurement of metabolic activity of post-absorptive processes following food ingestion (Beamish and Trippel, 1990). R_{AHI} in crustaceans depends on the quality, quantity and energetic component balance of food (Hewitt and Irving, 1990; Du-Preez et al., 1992; Rosas et al., 1996). According to present results, R_{AHI} is possibly a response to dietary protein level in *L. vannamei* with a higher value in shrimp fed 40% protein than that observed in shrimp fed 5 or 15 DP/kg BWd. Notwithstanding that increment, R_{AHI} value obtained in shrimp fed 40 DP/kg BWd was only 4.5% of assimilated energy indicating a highly positive cost–benefit balance for shrimp. Studying four shrimp species (*L. setiferus*, *L. schmitti*, *F. duorarum* and *F. notialis*) Rosas et al. (1997) observed that R_{AHI} increased according to dietary protein

level. In that study, an increment on post-prandial nitrogen excretion was also observed in relation to an increment in dietary protein levels indicating that digestion and assimilation of protein could be the main contribution to R_{AHI}, such as was observed in *L. vannamei* juveniles in the present study.

A reduction in R_{RUT} was observed according to an increment in dietary protein levels. In R_{RUT}, the energy used for activity is included (Lucas, 1993). All experimental feeds were designed with similar gross energy content. Therefore, differences in dietary protein affected routine metabolism, indicating that food energy content may not be the limiting factor of routine metabolism as intake of essential amino acids. Kureshy and Davis (2002) postulated that for *L. vannamei* juveniles fed 16% protein, the poor growth observed is probably due to a large quantity of feed that must be consumed to meet daily amino acids requirements. Taking into consideration the observation that the increased of R_{RUT} observed in relation to a decrease in dietary protein level can be explained as a result of an increase in shrimp activity in an attempt to search and ingest more food.

An increase in OxyHc was observed with an increase in dietary protein indicating that shrimp accumulated protein as hemocyanin. *L. vannamei* are well adapted to obtain energy through protein metabolism although some energy can be lost through ammonia excretion (Rosas et al., 2002). That adaptation is related both with its limited capacity to store and process carbohydrates, and with the wide capacity to use protein for glucose synthesis. As evidenced for *L. vannamei*, gluconeogenic capacity was observed through glycogen increase in digestive gland in relation to dietary protein level. Several studies have shown that shrimp and crustaceans in general are well adapted to synthesize glucose through gluconeogenesis, a process that is induced by amino acid metabolism (Huggins, 1966; Vinagre and Da Silva, 1992; Cuzon et al., 2001; Rosas et al., 2001a). From that study and results obtained at present, it is clear that a reduction in dietary protein level will affect essential amino acid availability, glycogen synthesis and growth rate. Glycogen is a source of *N*-acetylglucosamine for chitin synthesis and amino acids are building blocks for muscle growth (Carey, 1965; Stevenson, 1985).

OxyHc is related to immune function along with its role as a nutrient and protein source. OxyHc has a fungistatic role (Destoumieux et al., 2001; Adachi et al., 2003) or can be converted into a ProPO-like enzyme. A reduction in immune function could be expected if shrimp were fed a sub-optimal protein level, as in the present study, in conjunction with nutritional stress. A reduction in hemocytes was found when shrimp were fed sub-optimal dietary protein levels. Zymogens contained in hemocytes (ProPO system, peneidins, etc.) could also be reduced along with its activities, such as phagocytosis and coagulation (Bachere, 2000; Johansson et al., 2000). A reduction in respiratory burst was observed indicating that sub-optimal dietary protein level affected cell numbers and their phagocytosis capacity. When cells were stimulated, the respiratory burst of shrimp fed 15 DP/kg BWd was similar to that observed in shrimp fed 5 DP/kg BWd, loosing up to 80% of its cellular phagocytic capacity.

Although a reduction in GCs was observed with a decrease in dietary protein level, at the same time a was change also observed in ProPO GC^{-1} concentration. Depending on dietary protein level, ProPO and GC showed different behavior. Shrimp fed 40 DP/kg BWd had an elevated concentration of hemocytes indicating that optimal dietary protein

levels promote blood cell synthesis. In contrast, shrimp fed sub-optimal protein had more ProPO per cell showing that shrimp could be enhancing the immunological content of the hemocytes as a response of cell deficit induced by imbalanced dietary protein. A similar response was observed in *L. setiferus* adults (Pascual et al., 2003). In that study, shrimp exposed at an extreme temperature, responded by increasing ProPO per GC when a reduction in hemocytes was observed.

In a similar manner, hemagglutinating activity increased according to dietary protein level, showing that a compensatory mechanism related with lectin activity could be also operating. Several hypothesis can be addressed. Hemocyte lysates have shown high lectin specific activity in lobsters, shrimp and crabs and hemocytes are proposed as site of synthesis for serum lectins (Cassels et al., 1986). If lectins are synthesized in hemocytes and these are less concentrated in shrimp fed sub-optimal protein concentrations then one can explain the hemagglutinating activity increment in the same form that the increment of ProPO per cell was explained: Shrimp could synthesize more lectins, promoting a higher hemagglutinating activity as a result of a reduction in hemocytes concentration. Another hypothesis could come from the fact that lectins could change in activity according to nutritional status of shrimp. In this hypothesis, lectins could become less active in optimal dietary protein levels to highly active an sub-optimal dietary protein level, following the same strategy of compensation observed in ProPO behavior. A mechanism of isoenzymes that are activated by plasma protein could be a possible mechanisms to activate enzymes. A third hypothesis could be related to the role of lectins as glycoprotein transporter (Zenteno et al., unpublished data). In shrimp fed optimal dietary protein level, lectins could be involved in both hemagglutinating and transportation role, taking into account that with this diet, plasma proteins are elevated and part of the lectins are sequestered to be used as transported. In contrast, in shrimp fed sub-optimal dietary protein levels where plasma protein are reduced, less lectins could be sequestered as transported permitting the rest of lectins serve as hemagglutinating molecule and showing an apparently higher activity.

Notwithstanding the compensatory mechanism used by *L. vannamei* to respond to nutritional stress, results suggest that sub-optimal dietary protein level produced not only a reduction in growth rate through an increment in respiratory metabolism but also a reduction in immune capacities via a reduction of OxyHc, hemocytes concentration and phagocytosis capacity. In this context, it seems that the high protein requirement for maximum growth reported by Kureshy and Davis (2002) is related to physiological adaptations of *L. vannamei* to use protein as a main nutrient (Rosas et al., 2002). Actually, the shrimp industry is trying to reduce the dietary protein content in an attempt to reduce production costs. There are many evidence that demonstrate that the protein requirement of *L. vannamei* (one of the most worldwide cultivated shrimp species) is higher than 35% PC. The main reason is that this shrimp species is well adapted to use protein as a source of energy and molecules for growth and for its immune system (among other physiological functions). A reduction in dietary protein levels without other considerations in shrimp culture (i.e., living food management, water exchange, shrimp density, etc.) could reduce the shrimp production on farms via a reduction on biomass production as a result of lower immune condition and, consequently, in the health of shrimp.

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Capítulo 4

Cambios bioquímicos, fisiológicos e inmunológicos en los juveniles de *Litopenaeus vannamei* durante un periodo de inanición de 21 días.

Biochemical, physiological, and immunological changes during starvation in juveniles of *Litopenaeus vannamei*.

Cristina Pascual[1], Ariadna Sánchez [1], Edgar Zenteno [2], Gerard Cuzon[3], Gaxiola Gabriela[1], Roberto Brito[4], Rolando Gelabert[4], Enrique Hidalgo[1], and Carlos Rosas[1].

1. Unidad Multidisciplinaria de Docencia e Investigación, Facultad de Ciencias, UNAM, Puerto de Abrigo s/n, Sisal, Mpo. Hunucmá, Yucatán, México.
2. Departamento de Bioquímica, Facultad de Medicina, Ciudad Universitaria, México 04510, D.F.
3. Centre Oceanologique du Pacifique, PO 7004, Taravao, Tahiti, French, Polynesia
4. Facultad de Ciencias Pesqueras, Universidad Autónoma del Carmen, Cd. del Carmen, Campeche, México.

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Abstract

In an attempt to know how the protein level modulates catabolism and its effects on the immune response, we studied juvenile *L. vannamei* that had been starved for varying period after being conditioned on diet containing either maintenance or optimal dietary protein levels (DPL). The effect of dietary protein level on nutritional reserves management of shrimp and its relation with immune condition was also addressed. Juvenile shrimp were fed for 21 days on diets containing 5 and 40% dietary protein. Hemolymph metabolites (glucose, cholesterol, protein, acylglycerols, and lactate), hemocyanin, osmoregulatory capacity, digestive

gland glycogen and lipids, and immune conditions (hemocytes characterization, phenoloxidase activity, respiratory burst: basal and activated) were evaluated and considered as initial condition. After that time, shrimp were starved for 21 days. During starved time every 7 days nutritional, physiological and immunological condition were evaluated. A reduction in all physiological and immunological indicators was observed with starvation. The protein level of the conditioning diet had a significant effect on this response; generally, the effect was smaller with shrimp previously fed 40 % dietary protein. In this sense the present results demonstrate that shrimp are well adapted to tolerate food deprivation for some time but that this tolerance is closely related to its previous nutritional condition. In the case of shrimp fed 40% DPL, wet weight, nutritional and immune condition was significantly affected after 14 days of starvation. In shrimp previously fed 5% DP, tolerance to starving condition was limited to only a few days (7 days) as a result of low reserves of circulatory and mussel proteins. All these results demonstrate that dietary protein levels can governor the immune condition of shrimp through the management reserves metabolism, indicating that a shrimp with a good nutritional condition can tolerate until 14 days without modify the evaluated immune responses. In this sense it can be conclude that protein metabolism have a central role for shrimp.

Keywords: *Litopenaeus vannamei*, starvation, physiological condition, immunological condition, blood metabolites.

1. Introduction

In the last 20 years world shrimp production has been increasing mainly in the area dedicated to juvenile growth. The increment of stocking densities and environmental changes inside and outside farms has been affected the production negatively mainly due at sickness problems (Bédier et al., 1998; Bachere, 2000). In this sense, health indicators together with the knowledge of the immune system are considered as the key to understand and eventually to avoid

shrimp's health problems (Bachére et al., 1995b; Lightner, 1999; Rodríguez y LeMoullac, 2000).

In shrimp, nutritional and immunological conditions are closely related (Bachere et al., 1995; Yepiz-Plascencia et al., 2000a; Adachi et al., 2003). The biochemical characterization of molecules with an immunological role has demonstrated that many of these molecules are proteins associated with hemocytes (Chen y Cheng, 1993a; Le Moullac y Haffner, 2000b; Destoumieux et al., 2001). Several studies indicate that dietary protein is the regulatory nutrient because shrimp energy balance, physiological and immune conditions depend mainly on proteins (Rosas et al., 2002; López et al., 2003). Recent research demonstrated that shrimp fed with 40% dietary protein level were energetically more efficient than those fed with 15 or 5% dietary protein levels. A lower respiratory metabolism and, in consequence, a higher retained energy was observed in shrimp fed high dietary protein levels than with low levels (Pascual et al., 2004a). At the same time, it was observed that shrimp fed 40% dietary protein levels showed a better immune condition than shrimp fed lower dietary protein levels. These results confirm the high protein requirement reported before, i.e., more than 32% dietary protein level (Kureshy y Davis, 2002), agree with the previously reported ability of shrimp to use dietary protein as the main source of energy (Teshima, 1998; Rosas et al., 2002). It has been demonstrated that shrimp can synthesize carbohydrates (CHO) through the glycogenic pathway, evidencing that protein metabolism is the main source for glycogen and glucose synthesis in *L. stylirostris*, *L. setiferus*, *L. vannamei*, and other crustacean species (Da Silva y Kucharski, 1992; Rosas et al., 2000; 2001a).

According to Barclay et al., (1983) and Dall and Smith (1986), muscle protein is the main protein reserve during starvation periods. Hemocyanin has been proposed as a reserve protein molecule for shrimp. Cuzon and Ceccaldi (1971) observed in *Penaeus keraturus* a qualitative and quantitative reduction of blood protein after four weeks of privation of food. Electrophoresis results showed a reduction in the number of hemocyanin bands and its thickness during starvation periods, ascribed

hemocyanin a role as protein reserve. In a previous research with *L. vannamei*, we observed that hemocyanin changes with the nutritional status of shrimp showing high or low values according to direct changes in dietary protein levels (Rosas et al., 2001b; 2002).

According to Kureshy and Davis (2002) and Pascual et al. (2004a), 5% of dietary protein level is close to the protein maintenance requirement for *L. vannamei*, whereas a 40% dietary protein level (DPL) is close to the optimal level. Although at this time there are no research reports that demonstrate that dietary protein could modulate the immune condition during periods of fasting or starvation; it is possible that shrimp previously fed a diet with optimal protein content will show a better immune condition than obtained in shrimp previously fed sub optimal dietary protein levels. In order to known the role of dietary protein on reserves metabolism and its consequences in immune condition of shrimp, the aim of this study was to determine the effect of starvation condition on *L. vannamei* shrimp previously conditioned at low (5% DPL) and optimal (40% DPL) dietary protein levels. The effect of both dietary protein levels on hemolymph metabolites, digestive gland glycogen, and immune condition of starved shrimp during 21 days are presented.

2. Materials and methods

2.1 Animals and experimental conditions

A group of 80 shrimp (8.85 $\pm$ 0.42 g wet weight) was used in this experiment. *Litopenaeus vannamei* were obtained from Pecis Industries S.C., in Yucatan, Mexico. Shrimp were reared for 21 d in 300-l tanks (1m^2; 13 shrimp for tank) and fed different protein levels: 5 and 40%. Three tanks were randomly assigned to each treatment. Photoperiod was 12h:12h, water temperature was 28 $\pm$ 1°C; dissolved oxygen, >5.0 mg/l; salinity, 33 ‰; the pH ranged from 7.9 to 8.2. Exuvia in tanks were registered daily. The amount of feed was divided in three rations a day (0800, 1400, and 2000 h). Uneaten feed particles and feces were removed once a day by siphoning. Shrimp were fed at 10% body weight during 21 days conditioning

period. After that time, wet weight, some hemolymph components and digestive gland reserves (glycogen and total lipids), were evaluated in shrimp fasted by 12 h. At this time fifteen shrimp per treatment were sacrificed and considered as initial levels (Five shrimp per tank/treatment). The remaining of the shrimp was not fed for another 21 days period. During this time 7- 8 shrimp per treatment were sacrificed every 7 days in the same way (2-3 shrimp per tank/treatment). To minimize cannibalism that could compromise the starvation experiment, tanks were reviewed during the day and dying or dead shrimp were removed immediately.

Table 1 Composition of the experimental diet (g kg-1).

Ingredients	Dietary protein	
	5 %	40 %
Anchovy fish meal[1]	56	450
Squid meal[2]	12.5	100
Soy bean meal	22.5	180
Native wheat flour[3]	609.1	192.8
Soy bean lecithin[3]	20	20
Cod liver oild[3]	43	22
Cholesterol	2	20
Vitamins mix[a]	17	17
Rovimix Stay-C[b]	0.286	0.286
Minerals[c]	8	8
Filler [4]	199.6	0
Alginate Na	10	10
Crude protein (%)	5.6	40
Crude lipids (%)	10	10
CBH (%)	61	19
Ash %	23	9
Total energy (kJ g^{-1}) *	16	16

[a] Vitamin premix without vitamin C, provided by Agribands de México.
[b] Ascorbyl phosphate (Stay-C-35% Roche).
[c] Mineral premix provided by Agribands de México.
[1] CORPESCA SUPERPRIME (Chilenean Fish meal, steam dried).
[2] Squid meal (*Loligo* sp. From Gulf of México)
[3] Droguería Cosmopolita, México,[4] Cellullose.
Estimated from Cuzon and Guilleau (1987) whit the following coefficients: 21.3, 17.6 and 39.5 kJ g^{-1} for protein, carbohydrates and lipids, respectly.

2.2 Preparation of diets

The experimental diets were prepared by thoroughly mixing dry ingredients with oil and then adding water until a dough stiff resulted (Table 1). This was then passed through a meat mincer with a die, and the resulting spaghetti-like strings were air-dried at 60°C for 24 h. After drying, the material was broken up and sieved to a convenient pellet size and stored at -4 °C.

2.3. Hemolymph metabolites, osmotic pressure and molt.

Measurements of blood constituents were made from samples taken from conditioning period and every 7 days during starvation period. Before sampling, shrimp were placed in pre-chilled (23°C) and aerated seawater for 5 min to reduce the effect of manipulation before hemolymph extraction. Hemolymph (approximately 300-500 µl per shrimp) was individually sampled through a pre-chilled syringe needle inserted at the base of the fifth pereiopod. Hemolymph was diluted immediately with pre chilled (8°C) anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA-Na_2, pH 7.3, 850 mOsm kg^{-1}; 1:2) according to Vargas-Albores et al.(1993a). Hemolymph plus anticoagulant was centrifuged at 800 *g* for 3 min at 4°C, and the supernatant was separated for plasma determinations. The cellular pellet from each blood sample was re-suspended in 300 µl of sodium cacodylate buffer (Cac, 10 mM cacodylate, 10 mM $CaCl_2$, pH 7.0) and newly centrifuged at 600O *g* for 3 min to be used as ProPO source (Hernández-López et al., 1996).

Commercial kits were used for lactate (Sigma-cat. 735), glucose (GH) (Bayer Sera Pak Plus B01 4509-01), acylglycerol (Bayer Sera Pak Plus B01 455101), and cholesterol (Bayer Sera Pak Plus B01 4507-01) assessments. Determinations were adapted to a micro plate using 10 µl of plasma and 200 µl of enzyme chromogen reagent. Absorbance was recorded in a micro-plate reader (Biorad 550) and concentrations were calculated from a standard substrate solution. Plasma was further diluted 1:101 for protein determination by the Bradford (1976) technique

adapted to a micro plate method using commercial chromogen reagent (Biorad, Cat. 500-0006) with bovine serum albumin as the standard.

Osmotic pressure of the hemolymph and water was measured in a micro-osmometer with 20 µl sample per titration (3 MO-PLUS; Advanced Instruments, Inc. USA). The osmotic capacity (OC) was defined as the difference between osmotic pressure of hemolymph and the external medium (Lignot et al., 1999). The individual weight (± 0.05 g) was recorded, and molting stages were determined using uropod characteristics (Drach y Tchernigovtzeff, 1967). Only shrimp in inter-molt stage (C stage) were used for statistical analyses.

2.4. Oxyhemocyanin

For hemocyanin (OxyHc) measurements, 10 µl hemolymph was immediately diluted with 990 µl distilled water in a 10-mm cuvette, and absorbance was measured at 335 nm. Hemocyanin concentration was calculated using an extinction coefficient of ε = 17.26 calculated on the basis of the 74000 Da functional subunit (Chen y Cheng, 1993a).

2.5. Glycogen in digestive gland

Glycogen in the digestive gland (GD) was extracted in trichoroacetic acid (TCA) and determined through the reaction with sulfuric acid and phenol (Dubois et al., 1965). The digestive gland was dissected and a section was weighed (20-30 µg) and homogenized in trichloroacetic acid (TCA, 5%) for 6 min at 8,000 *g* (Micro Centrifuge Eppendorf 5415). One hundred µl of supernatant was pipetted into a tube and mixed with 5 volumes of 95% ethanol. Tubes were placed in an oven at 37-40°C for 3 h. After precipitation, the tubes were centrifuged at 3,340 *g* for 15 min. The supernatant was discarded leaving the glycogen as a pellet; by adding 1 ml concentrated sulfuric acid, and 200 µl phenol (5%) glycogen was dissolved. From the mix, 200 µl was transferred to a microplate and read at 490 nm in an ELISA reader (Biorad 550). Total weight of the digestive gland was also recorded.

2.6 Total lipids in digestive gland

Total lipids in the DG were evaluated according to Folch et al. (1957); 1 g of tissue was homogenized with a Teflon tip in 20 ml of a chloroform/methanol (2:1) solution at 500 rpm for 2 min. The whole mixture was agitated during 15-20 min in an orbital shaker at room temperature. The homogenate was filtered through a funnel with a folded filter paper to recover the liquid phase. Samples were washed with methanol- H2O solution and placed into the separation funnel until obtaining two layers. The chloroform-lipids fold was recovered and placed under a desiccation hood at 60°C with an air current to complete evaporation. The total lipid quantity was expressed as mg g-1 of tissue.

2.6 Immune condition

Since the ProPO system can be activated by endotoxins, all glassware was washed with E-Toxa-Clean® and all solutions were prepared using pyrogen-free water.

2.7 Phenoloxidase activity.

A sample of 40 µl of re-suspended sample containing degranulated hemocytes was incubated for 3 min at 25 °C with 40 µl trypsin type IX (0.1 mg ml^{-1}). Then, 40 µl L-DOPA (3 mg/ml) was added and incubated for 10 min (Hernández-López et al., 1996). Absorbance was measured at 490 nm in an ELISA reader (Biorad 550).

2.8 Hemocytes

A sample of 35 µl hemolymph was mixed with Alsever solution (113 mM glucose, 27.2 mM sodium citrate, 2.8 mM citric acid, 71.9 mM NaCl) and 10% formaldehyde (v/v). This sample was stored in a refrigerator until analysis (8°C). Hemocytes were counted with a Malassez chamber. Cellular characterization was done taking into account size, shape, and granular content of the cells (Pascual et al., 2003).

2.9 Respiratory burst

Intracellular production of the super oxide anion in hemocytes was quantified using the NBT (Nitroblue Tetrazolium) reduction to formazan (Song y Hsieh, 1994). Fifty microliters of hemolymph diluted in Alsever solution (1:1) was placed in a well of microplate (for triplicate) and centrifuged at 800 X *g* for 10 min. Plasma was removed and hemocytes washed with 100 µL Hank´s solution. Next, 100 µL Zymosan (0.1% in Hank´s solution) was added and incubated for 2 h at room temperature. The zymosan was then removed and hemocytes washed three times with 100 µl Hank´s solution and stained for 30 min with NBT solution (0.3%) at room temperature. After fixed hemocytes were washed three times with 100 µl methanol (70%) and dried for 5 min. Formazan was dissolved with 120 µl KOH and 140 µl DMSO, and absorbance was read at 630 nm using a microplate reader (Biorad 550).

2.10 Statistical analyses

A two way ANOVA test was applied to growth rate, blood and digestive gland results, as well as to the immunological parameters. A probability level of 0.05 was used to assess significance in all measured parameters (Zar, 1974).

3. Results

3.1. Shrimp and digestive gland weight

At the start of starvation period, there was a significant difference between the wet weight of shrimp in both treatment, with a higher weight of those fed 40% dietary protein level (DPL) (9.8 $\pm$ 0.44 g) than of those fed 5% DPL (7.9 $\pm$ 0.4 g) ($P < 0.05$). The reduction of wet weight with starvation was faster in shrimp previously fed 5% DPL than in shrimp fed 40% DPL. The mean wet weight of shrimp fed 40% DPL did not show statistical differences until day 21 of starvation whereas wet weight of shrimp previously fed 5% DPL showed significant differences at day 7 of starvation (Fig. 1). A reduction during the starvation in digestive gland weight was observed with both treatments (Fig. 1). During the conditioning period survival was

94 and 96%, 5 and 40% DPL, respectively. During the 21 days of the starvation period only one death was registred in the 40% DPL treatment and was observed at the last day of the experiment.

3.1. Hemolymph metabolites and osmotic capacity

Dietary protein level affected hemolymph protein concentration of shrimp during the conditioning period, with high values in shrimp fed 40% DPL and low values in shrimp fed 5% DPL ($P < 0.05$; Fig. 2). As a consequence a higher protein hemolymph concentration was also observed during the starvation period in shrimp fed 40% DPL, as compared to shrimp previously fed 5% DPL. A decrease in hemolymph protein concentration was observed during the starvation period with low values at day 14 in shrimp fed with either diet. At day 21 of the starvation period an increment in hemolymph protein concentration was observed in shrimp fed previously with 5 and 40% DPL, respectively (Fig. 2).

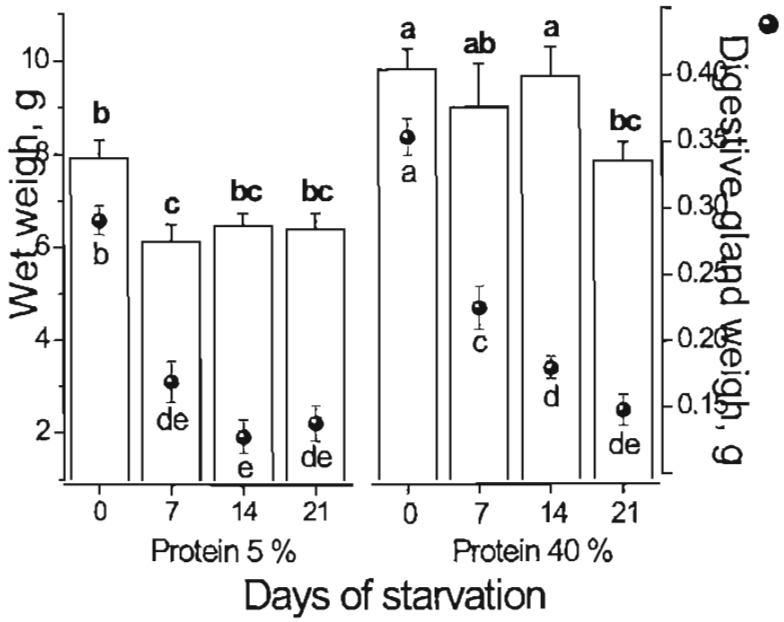

Fig. 1 Wet weight and digestive gland weight of *L. vannamei* juveniles maintained in starvation condition for 21 days and previously fed with 5 or 40% protein diet for three weeks. Mean±S.E. Different letters show statistical differences at $P<0.05$.

A 28% higher oxyhemocyanin (OxyHc) level was observed in shrimp fed 40% DPL as compared to shrimp fed 5% DPL ($P < 0.05$; Fig. 2). During the starving period, OxyHc diminished with time with the lowest value at day 21. At the end of the experiment, a 71 and 63% reduction in OxyHc levels were observed in shrimp previously fed 5 or 40% DPL, respectively ($P < 0.05$).

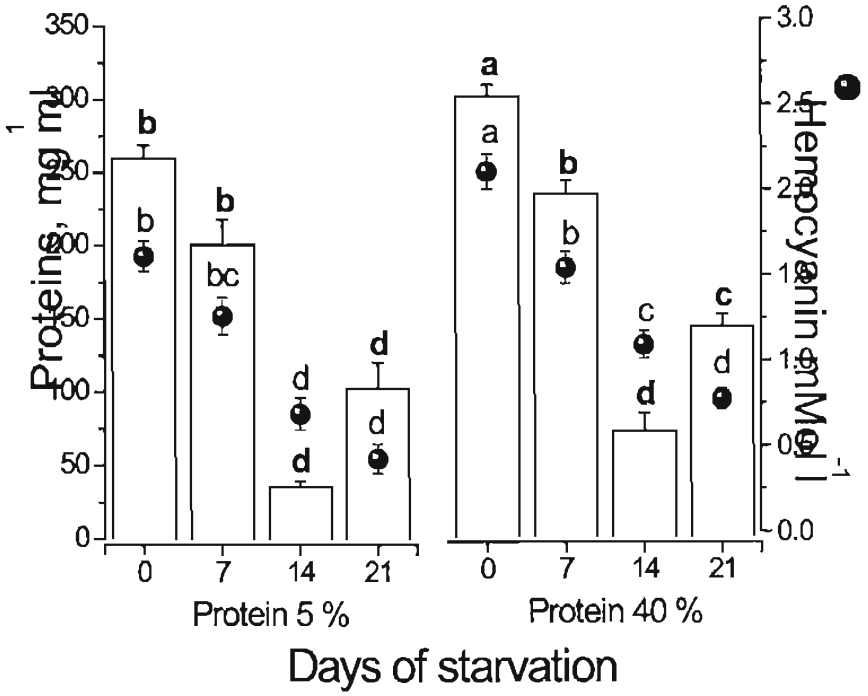

Fig. 2 Blood protein and hemocyanin concentrations of *L. vannamei* juveniles maintained in starvation condition for 21 days and fed previously with 5 or 40 % protein diet for three weeks. Mean±S.E. Different letters show statistical differences at $P<0.05$.

Hemolymph glucose was not affected by the conditioning diets. A mean value of 0.51 mg ml^{-1} was obtained from shrimp after 21 days fed either 5 or 40% DPL (Fig. 3a). In contrast, during the starvation period, glucose levels decreased differentially depending on the conditioning diet with a faster decrease in shrimp fed 5% DPL than in shrimp fed 40% DPL (Fig. 3a). In shrimp previously fed 5% DPL, hemolymph glucose was reduced by 57% at day 7 of starving whereas, in shrimp fed 40% DPL, glucose concentration was similar to that obtained before the

starvation period. At the end of the experiment, shrimp from both treatments showed similar hemolymph glucose levels (mean value of 22.5 mg ml^{-1}) but 55% lower than before the starvation period (Fig. 3a).

Lactate concentration in shrimp form both treatments did not show statistical differences between treatments and starvation time. However shrimp previously fed 5% DPL showed a tendency to lower levels in comparison to that observed in shrimp previously fed 40% DPL.

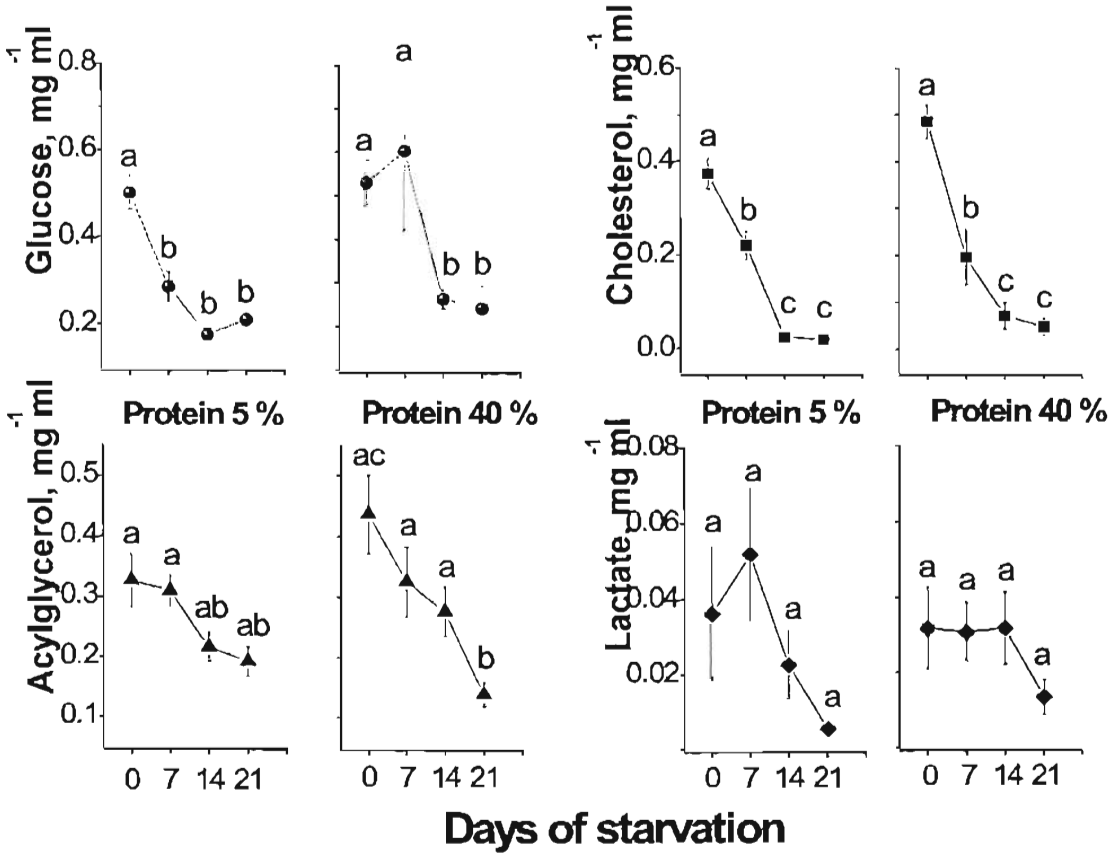

Fig. 3 Glucose, cholesterol, acylglycerol, and lactate concentrations in blood of juveniles of *L. vannamei* maintained in starvation condition for 21 days and fed previously with 5 or 40% protein diet for three weeks. Mean±S.E. Different letters show statistical differences at $P<0.05$.

At the end of the conditioning period (day 0 of starvation period) acylglycerol concentration in the hemolymph (AG) was not affected by the experimental diets (Fig. 3c). During starvation period a mean reduction in AG of 53% was observed in shrimp from both treatments ($P < 0.05$). A logarithmic reduction in hemolymph cholesterol concentration was observed over the starvation period with low values at day 14 and 21 with both treatments (Fig. 3b; $P < 0.05$).

Osmotic pressure (OP) and osmoregulatory capacity (OC) were not significantly affected during starvation of shrimp fed 5% PDL (Fig. 4). In contrast, shrimp fed 40% DPL showed a strong variation in OC with low values at 7 and 21 starving days and high values at the beginning and at day 14 of the starving period ($P < 0.05$; Fig. 4).

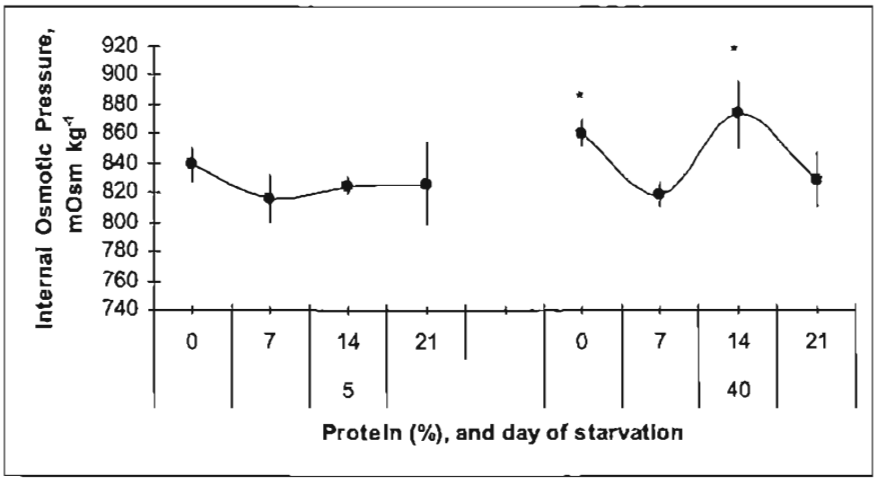

Fig. 4 Osmotic Pressure of *L. vannamei* juveniles maintained in starvation condition for 21 days and fed previously with 5 or 40 % protein diet for three weeks. Mean±S.E. Asterisk shows statistical differences at $P<0.05$.

3.2. *Digestive gland glycogen and total lipids*

Digestive gland glycogen (DGG), was not affected by the dietary protein level during both starving and feeding periods. A significant increase on 21 starving day was observed in both treatments which resulted 58 and 80% higher levels than observed in shrimp fed 5 and 40% DPL, respectively ($P < 0.05$; Fig. 5). In contrast, a

reduction in digestive gland total lipids (DGTL) was observed with both treatments since day 7 to the end of the starvation period ($P < 0.05$; Fig. 5).

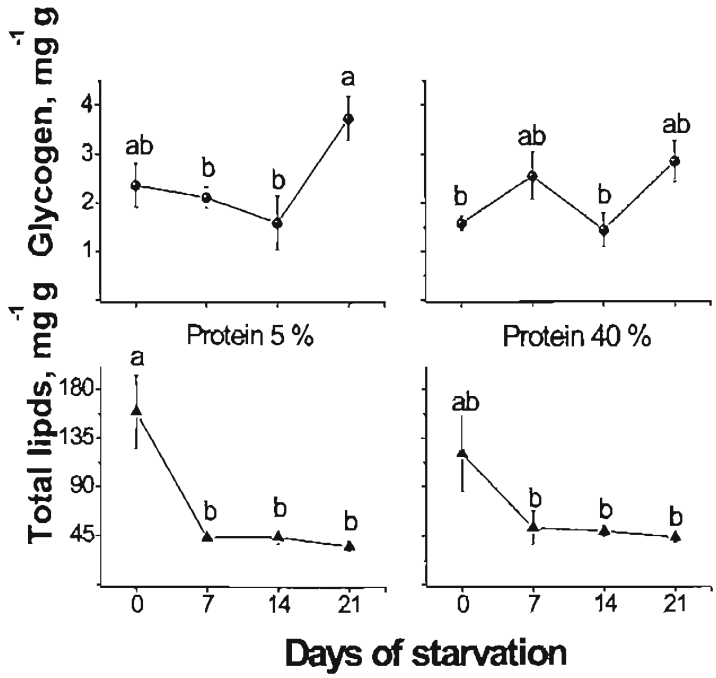

Fig. 5 Glycogen and total lipids in the digestive gland of *L. vannamei* juveniles maintained in starvation condition for 21 days and fed previously with 5 or 40 % protein diet for three weeks. Mean±S.E. Different letters show statistical differences at $P<0.05$.

3.3. *Immune condition*

Total hemocytes (TH) were not affected by the conditioning diet before the starvation period. A mean value of 26,204 cells mm^{-3} was obtained at time 0 for shrimp from both treatments (Fig. 6a). A reduction in TH with starvation was observed in shrimp previously fed 5% DPL. At day 21, TH (8,550 cells mm^{-3}) were 48% lower than at the beginning of the starvation period ($P < 0.05$, Fig. 6a). In shrimp fed 40% DPL, TH were maintained constant between days 0 to 7 of the starvation period with a mean value of 27,900 cells mm^{-3}. A reduction in TH was observed at day 14, reaching the lowest value at day 21 (12,400 cells mm^{-3}). This value resulted 55% lower than that observed in shrimp before the starvation period ($P < 0.05$; Fig. 6a).

Dietary protein content did not affect granular cell (GC) concentration (Fig. 6b). A reduction in GC with starvation was observed in shrimp previously fed 5% DPL whereas in shrimp previously fed 40% DPL, they remained almost constant during the first 7 days of starvation. After that time, a decrease in GC was observed with the lowest value recorded at day 21 ($P < 0.05$; Fig. 6b). The lowest values were 33 and 55% of those observed before the starvation period in shrimp fed 5 and 40% DPL, respectively.

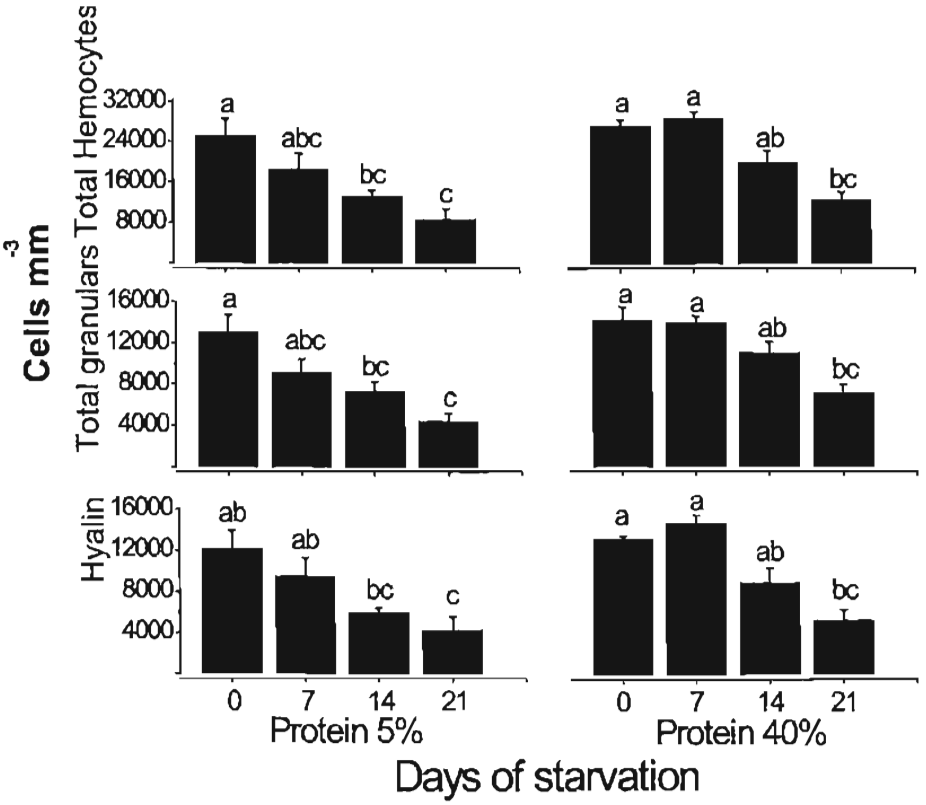

Fig. 6 Total hemocytes, granular and hyaline cells of *L. vannamei* juveniles maintained in starvation condition for 21 days and fed previously with 5 or 40 % protein diet for three weeks. Mean±S.E. Different letters show statistical differences at $P<0.05$.

Hyaline cells (HC) followed a similar trend as TH with a decrease in concentration directly related with the starvation time in shrimp fed 5% DPL whereas, in shrimp fed previously 40% DPL, their concentration was maintained constant for the first 7 days of the starvation period (Fig. 6c).

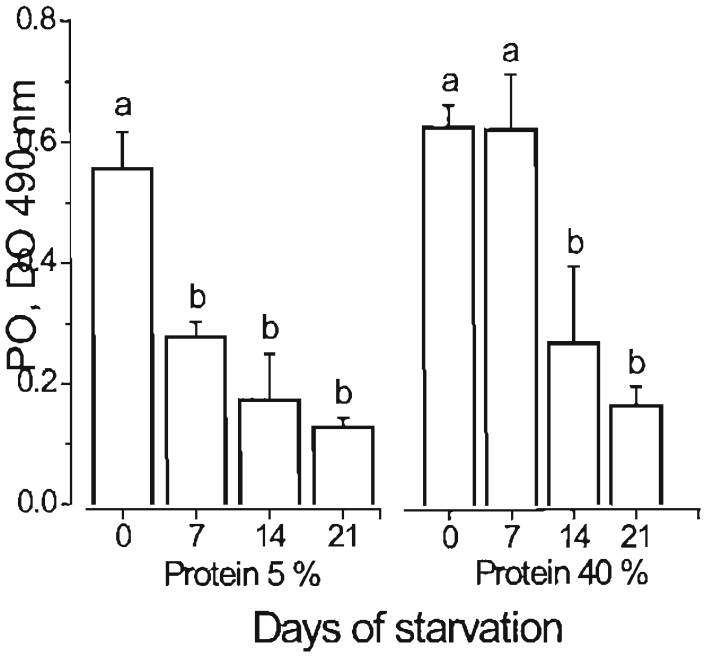

Fig. 7 Phenoloxidase activity in degranulated hemocytes of *L. vannamei* juveniles maintained in starvation condition for 21 days and fed previously with 5 or 40 % protein diet for three weeks. Mean±S.E. Different letters show statistical differences at $P<0.05$.

At the star of the starvation period, similar phenoloxydase (PO) activity was observed in shrimp of both treatments (Fig. 7). However, in shrimp fed 5% DPL a decrease in PO activity was found during the starvation period. The low value registered at day 21 was 22% above that before the starvation period ($P < 0.05$, Fig 7). In contrast, in shrimp fed 40% DPL PO activity was maintained without changes until day 7 after that a decrease was found (Fig. 7). The low value observed at day 21 corresponded to 26% of that before the starvation period. Respiratory burst (basal: BRB, and activated ARB) showed a similar behavior of PO activity; similar values at the beginning of the starvation period and different values during starvation period. Basal and activated respiratory burst in shrimp fed 5% DPL decreased with the starvation time, whereas in shrimp fed 40% DPL, they remained stable during the three weeks of starvation (Fig. 8).

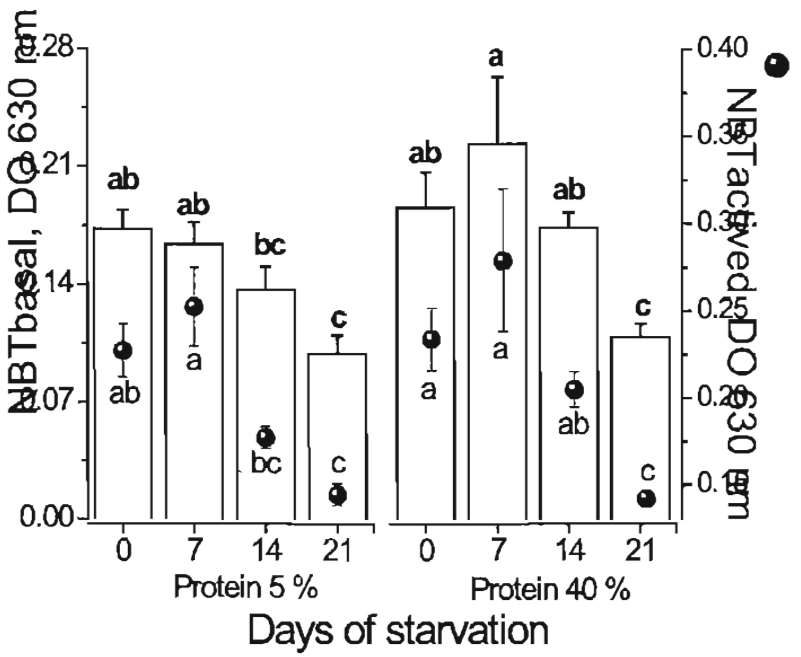

Fig. 8 Respiratory burst (basal and activated) in hemocytes of *L. vannamei* juveniles maintained in starvation condition for 21 days and fed previously with 5 or 40 % protein diet for three weeks. Mean±S.E. Different letters show statistical differences at $P<0.05$.

4. Discussion

After the first 21 days of the conditioning diets, wet and digestive gland weight, protein hemolymph and oxyhemocyanin concentration were significantly higher in shrimp fed 40% DPL than in shrimp fed 5% DPL. These results are agree with other works that show that 40% DPL is near the optimal level and 5% DPL is above the minimum dietary protein level required by *L. vannamei* (Kureshy y Davis, 2002; Pascual et al., 2004c).

None exuvia was recovered during starvation period in both treatments indicating that shrimp can compensate food deprivation using energy that normally is used to exuviation. Stuck et al.(1996) and Comoglio et al. (2004) observed the inhibition of molting in response to starvation in *L. vannamei* shrimp. They concluded that

shrimp are adapted to tolerate starvation, saving energy from exuvia, including the energy challenged to mobilize reserves, chitin digestion, and exuviations.

The influence of starvation on protein, fat, and carbohydrate metabolism has been investigated previously in shrimp and other crustacean species. In those studies, all authors concluded that protein is the main metabolic substrate during starvation conditions. Dall and Smith (1986), showed that the free amino acid pool (FAP's) in *Penaeus esculentus* shrimp is the core of metabolic activity during food deprivation. Vinagre and Da Silva (1992) showed that starved *Chamagnathus granulate* crab previously fed with a high protein diet were able to maintain a higher glucose concentration and glycogen in the digestive gland (DGG) than crabs previously fed with a carbohydrate-rich diet evidencing that protein accumulation promoted a more effective gluconeogenic pathway than carbohydrates.

The reduction of nutritional reserves during starvation in either blood or tissues has been observed in several crustacean species and has been used as an indicator of how nutrients are metabolized. According to the present results, the way in which reserves were used depended on the dietary protein level previously used as shrimp food. According to Rosas et al. (2002), protein levels modulate the use of energy in shrimp because proteins are the main nutritional reserve. However, shrimp have a limited but effective lipid and CHO metabolism that is used according to specific energetic and/or physiological, and/or structural demands that are closely linked with the way in which shrimp use their own protein reserves (Charmantier et al., 1994; Teshima, 1997).

The results obtained in the present study demonstrate that the dietary protein level can modulate the way in which lipids are used as energy source, when the food supply is limited or absent. In shrimp, previously fed 5% DPL, together with protein, lipids were used during the first 7 days of starvation as a source of energy, while in shrimp previously fed 40% DPL, lipid reserves were used in the same period for

glycogen and glucose synthesis producing an increase in digestive gland glycogen and blood glucose. That results could means that shrimp are tolerate food privation, in first instance, thought lipids catabolism.

Similar results were obtained in juvenile *L. vannamei* fed 35% DPL by Comoglio et al.(2004). A high rate of lipid metabolism between days 3 and 9 of starvation indicated that ß-oxidation of lipid reserves occurs first, permitting shrimp the use of lipids as energetic substrates followed by proteins. In the crab *Chasmagnahtus granulate*, carbohydrate and lipid reserves of the DG decreased to near depletion after 8 weeks of food deprivation indicating that newly lipid reserves are exhausted before protein (Vinagre and Da Silva, 1992). In another study, lipid composition of tissues in *Litopenaeus vannamei* exposed to a non-terminal food deprivation (17 days) revealed that only muscular polar lipids were consumed; whereas, all lipid reserves of the digestive gland were utilized to near depletion, confirming that digestive gland lipids are the most important pool of fatty acid reserves during the first days of starving period (Pérez-Velazquez et al., 2001).

In the present study, a lowest DG weight was observed during the first and second week of starvation when glycogen concentration in the DG changed in shrimp fed with either diet protein level. Shrimp previously fed 5% DPL showed a constant reduction of DG glycogen but a significant increase in the last week of starvation; whereas, in shrimp previously fed 40% DPL, glycogen presented two peaks in the second and last weeks of the starvation period. These results could be revealing the synthesis of glycogen from different metabolic pathways: during first 7 days and in shrimp fed 40% DPL glycogen could be obtained from lipids and hemolymph proteins catabolism. At the end of the starvation period glycogen could be obtained from protein when muscle proteins were transferred to blood to be processed in digestive gland via gluconeogenesys pathway (Kucharski y Da Silva, 1991; Vinagre y Da Silva, 1992; Oliveira y Da Silva, 1997). This hypothesis explains the source of high protein hemolymph concentration in shrimps of both treatments at day 21 of starvation period.

In shrimp fed previously 5% DPL, glycogen reserves were depleted fast, evidencing that hemolymph reserves were not enough to compensate the starvation conditions, inducing the use of muscle reserves from the first starvation week. In this context, the constant titer of glucose and lactate during this last experimental part, suggests that the metabolic pathways are mainly regulated by protein catabolism in shrimp tissue via crustacean hyperglycemic hormone (CHH). (Santos y Nery, 1897) and Santos and Keller (1993) observed that CHH levels in the crab *Carcinus maenas* might be controlled by positive and negative feedback loops modulated by lactate and glucose respectively stimulating glycogenolysis (i.e., lactic acid formation from glucose) or inhibiting glycogen synthesis and finally controlling the glycogen metabolism (Lüschen et al., 1993).

Most studies on nitrogen metabolism during starvation of decapods indicate that total protein values generally show a significant reduction, confirming that shrimp, as other crustaceans, are well adapted to use protein as a source of energy (Claybrook, 1983). Results obtained in different starved shrimp species have suggested that the main mechanism used by shrimp to obtain energy is through the catabolism of the free amino acid pool (FAAP) (Cuzon et al., 1980; Dall and Smith, 1986,(Cuzon y Guillaume, 1987; Comoglio et al., 2004). In this pathway, amino acids, such as glutamic acid, glycine, and alanine, could feed into the glycolytic pathway or the TCA cycle, producing the energy needed during fasting periods such as has been observed in other crustacean species (Kucharski y Da Silva, 1991; Vinagre y Da Silva, 1992; Oliveira y Da Silva, 1997). If these mechanisms are working in *L. vannamei,* those findings could explain why the osmoregulatory capacity and osmotic pressure was maintained without significant changes during starvation: amino acids were used as a source of energy and were used also to maintain the osmotic pressure of shrimp.

Although in both experimental groups there was a reduction in oxyhemocyanin concentration (OxyHc), shrimp previously fed 40% DPL maintained OxyHc concentrations significantly higher than shrimp fed previously with the low protein

diet. These results indicate that dietary protein level modulated the protein reserves accumulated in hemocyanin and many other physiological functions in which participates. Hc has been identified as oxygen transporter, storage protein, osmolyte, and ecdysone transporter (Cuzon et al., 1980; Pascual et al., 2004c). Besides, Hc could play an important role in immune reactions like the precursor of anti-fungal peptides (Destoumieux et al., 2001) and the phenoloxidase-like enzyme (Adachi et al., 2003). Based on the aforementioned, several authors have proposed that Hc could be a useful field tool to measure crustacean health and nutritional condition or to identify when shrimp nutrition is deficient (Spicer y Baden, 2000; Rosas et al., 2004; Pascual et al., 2004b).

The shrimp immune system has a solid protein base; some of them are protein involved in recognizing foreign agents (Vargas-Albores y Yepiz-Plascencia, 2000). A clotting protein is involved in engulfing foreign invading organisms and prevents blood loss upon wounding (Hall et al., 1999; Montaño-Pérez et al., 1999). Defense reactions in shrimp are often accompanied by melanization; prophenoloxidase activating system are associated with oxidation of phenols to quinones leading to synthesis of melanin (Sritunyalucksana y Söderhall, 2000). Conversion of ProPO to PO occurs through a serine protease called prophenoloxidase-activating enzyme (ppA) regulated by another protein, -2 Macroglobulin, a trypsin inhibitor (Perazzolo y Barracco, 1997). The innate immune response of shrimp also relies upon the production, in hemocytes, of antimicrobial peptides called peneidins (Destoumieux et al., 2001). In consequence, it is not surprising that shrimp fed with an optimal protein diet presented significantly higher values of immune variables, before and after the food deprivation period.

Movement of reserves and immune variables of shrimp fed either diet before starvation suggest a strong association between catabolism of protein and immune depression. The metabolic effort to compensate a long period of food deprivation, at expense mainly of protein catabolism, compromised the immune condition of *Litopenaeus vannamei* juveniles previously fed with either diet. The

difference was that shrimp fed 40% DPL were able to maintain constant values of immune variables for one week of food deprivation. Results of respiratory burst (basal and activated), phenoloxidase activity and hemocytes count reveal that the shrimp maintained both indicators during 7 to 14 days of starvation. In contrast, immune variables of shrimp fed with a low dietary protein level decreased from the first starvation week, revealing that a weak nutritional condition is capable of limiting physiological compensation when catabolism of energetic reserves and food depletion are present. The results of this study agree with previous research, in which we found that sub-optimal dietary protein levels (5 and 15 g DPkg^{-1} BW^{-1} d^{-1}) induced not only a severe reduction in growth and assimilation efficiency but also in immune capacities via a reduction of OxyHc, hemocytes concentration, phenoloxidase activity and phagocytosis capacity of *L. vannamei* juveniles.

The present results also demonstrate that shrimp are well adapted to tolerate food deprivation for some time but that this tolerance is closely related to its previous nutritional condition. In the case of shrimp fed 40% DPL, wet weight, nutritional and immune condition were significantly affected after 14 days of starvation. In shrimp previously fed 5% DPL, tolerance to the starvation condition was limited to only a few days (7 days) as a result of low reserves of tissue and circulation protein.

All these results demonstrate that dietary protein levels can governor the immune condition of shrimp through the management reserves metabolism, indicating that a shrimp with a good nutritional condition can tolerate until 14 days without modify the evaluated immune responses. In this sense it can be conclude that protein metabolism have a central role for shrimp.

Acknowledgment

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Capítulo5

Discusión General

En la presente investigación se abordaron algunas adaptaciones fisiológicas de los juveniles de *Litopenaeus vannamei* relacionadas al proceso de domesticación, el tipo de cultivo (experimental, 90 l, y de cultivo, 20 t), y la condición nutricional. Los resultados señalan que los componentes de la hemolinfa reflejan los ajustes fisiológicos asociados al tipo de cultivo, el proceso de domesticación, y a las transformaciones de la energía derivada del alimento, lo cual repercute directamente en el crecimiento y la respuesta inmunitaria de los organismos. Tomando esto en consideración, se elaboró un modelo conceptual en el que se integran las rutas metabólicas y diversos mecanismos fisiológicos involucrados en el mantenimiento de la homeostasis de los juveniles de camarón.

1. Componentes de la hemolinfa y actividad metabólica

Los componentes de la hemolinfa han sido ampliamente utilizados para determinar el estado fisiológico de los crustáceos. En numerosas investigaciones la concentración de hemocianina, metabolitos plasmáticos y algunos elementos del sistema inmunitario han permitido identificar cambios metabólicos asociados con el proceso de muda (Dall y Smith, 1986; Chan et al., 1988), la presencia de contaminantes (Chen y Cheng, 1995), el efecto de la manipulación (Racotta y Palacios, 1998), hipoxia (Le Moullac et al., 1998b), exposición al aire (Santos y Keller, 1993a) cambios de salinidad y temperatura (Ferraris et al., 1994; Vargas-Albores et al., 1998; Pascual et al., 2003; López et al., 2003), y la condición nutricional de los juveniles de camarón (Angus, 1989; Lignot et al., 1999; Rosas et al., 2001a).

A pesar de lo mucho que se ha avanzado en la estandarización de técnicas sencillas y el uso de los componentes de la hemolinfa para determinar el estado fisiológico de los camarones, poco se conoce acerca de los patrones de variación a nivel poblacional, por lo cual, un objetivo de este trabajo fue elaborar una base de datos que pudiera servir de referencia para diagnosticar el estado de salud de los juveniles de *L. vannamei*. Se utilizó estadística descriptiva para

caracterizar los datos de las proteínas, glucosa, acilglicéridos, lactato y la oxihemocianina de juveniles cultivados en condiciones controladas (90 l) y a nivel piloto comercial (20 t). La población de datos se construyó con mínimo 60 camarones en intermuda para cada evaluación por condición de cultivo. Los resultados de la presente investigación indican que la evaluación de proteínas, glucosa, acilglicéridos y lactato, es de gran ayuda para conocer las rutas metabólicas involucradas en el aprovechamiento y el uso de la energía. Paralelamente, la concentración de OxiHc puede ser un indicativo de la reserva de proteínas en la hemolinfa, lo cual esta directamente relacionado con el catabolismo de proteínas y la ruta gluconeogénica de los camarones (Hagerman, 1983; Chen y Cheng, 1993b).

La concentración de los metabolitos y la OxiHc de *L. vannamei* fueron comparados con los resultados reportados en la literatura para juveniles de la misma especie, lo cual evidenció similitud en las técnicas y también en los valores (Charmantier et al., 1994; Racotta y Palacios, 1998; Rodríguez et al., 2000; Rosas et al., 2001a; 2001b). El análisis de la frecuencia de distribución de los componentes de la hemolinfa reveló que solo las proteínas de los camarones mantenidos en condiciones controladas presentaron una curva normal, mientras que el resto de los metabolitos y la concentración de oxihemocianina OxiHc tuvieron curvas de distribución sesgadas hacia alguno de los lados.

Estos resultados concuerda con otras dos investigaciones, en donde la frecuencia de distribución de la OxiHc y los metabolitos plasmáticos (proteínas, glucosa, lactato, colesterol y acilglicéridos) de poblaciones silvestres de crustáceos (*Litopenaeus setiferus, Nephrops norvergicus, Liocarcinus depurator* y *Hyas araneus*) presentaron distribuciones no normales (Spicer y Baden, 2000; Rosas et al., 2004). Los autores de estas investigaciones señalan que las formas de las curvas de distribución de los componentes de la hemolinfa podrían estar afectados por factores intrínsecos (tamaño, sexo, condición nutricional, estadio de muda, condición reproductiva, etc.), y también, por factores extrínsecos

(temperatura, salinidad, oxigeno disuelto, disponibilidad de alimento, etc.), lo cual indica su posible uso como indicadores del estado fisiológico, al evidenciar los ajustes metabólicos que resultan de la interacción dinámica entre los organismos y su ambiente. Debido a que los componentes de la hemolinfa responden a innumerables ajustes funcionales, la determinación adecuada del estado fisiológico de los organismos dependerá de la integración de varios indicadores, tal como se realiza en los análisis de clínica humana, en donde el diagnóstico requiere de un conjunto de datos para poder realizarse.

De acuerdo con Mágnum y Hochachka (1998), a través del estudio de variaciones interindividuales es posible identificar caracteres fisiológicos y bioquímicos que puedan correlacionarse al desempeño y supervivencia de los organismos. Las variaciones interindividuales representan por lo tanto, uno de los puntos de mayor interés para la fisiología y genética contemporánea, ya que también se pueden aplicar para genotipos específicos provenientes de programas de mejoramiento genético y/o ingeniería genética.

Bajo un enfoque comparativo, los estudios acerca de la variación fisiológica entre especies ofrecen una buena oportunidad para abordar el significado adaptativo de las características bioquímicas de los organismos. En la presente investigación se compararon los datos obtenidos de *L. vannamei* cultivados en condiciones controladas y a nivel piloto comercial, con los datos reportados para otros crustáceos, silvestres o mantenidos en cautiverio bajo condiciones similares. Los resultados señalan que los componentes de la hemolinfa varían con la actividad, consumo de oxígeno, amplitud metabólica y el grosor del caparazón (Castille y Lawrence, 1989; Sánchez et al., 1991; Rosas et al., 1992). Las especies con mayor actividad natatoria presentaron una mayor concentración en la hemolinfa de proteínas, glucosa, colesterol y OxiHc. En este sentido, los componentes de la hemolinfa parecen reflejan también las adaptaciones fisiológicas y morfológicas de los crustáceos, por ser el resultado de la regulación metabólica y la demanda de energía de los organismos.

Desde el punto de vista aplicativo la hemolinfa de los camarones es fácil de obtener y sus componentes brindan importante información biológica, por lo cual, podría utilizarse para monitorear la condición fisiológica de organismos silvestres y cultivados. No obstante, es necesario hacer numerosas evaluaciones bajo diferentes condiciones con la finalidad de entender como las características ambientales modulan la variación de cada parámetro sanguíneo. Los estudios hematológicos de un espectro más amplio de especies de crustáceos permitirían a su vez, esclarecer el significado adaptativo de las características bioquímicas de la hemolinfa en uno de los grupos más diversificado y exitoso del reino animal.

2. *Metabolismo de proteínas y homeostasis*

El alimento representa una de las mayores fuerzas selectivas, ya que los flujos de materia y energía que intercambian los organismos con el ambiente constituyen la causa y el efecto global de su metabolismo. En la presente investigación, la privación de alimento y las dietas experimentales constituyeron una forma útil para estudiar los ajustes fisiológicos involucrados con el aprovechamiento y uso de la energía. Los resultados de los diversos capítulos indican que la respuesta inmunitaria de los camarones está directamente relacionada con el estado nutricional, y que éste depende fuertemente del metabolismo de las proteínas (Pascual et al., 2004b). El estudio realizado con organismos en ayuno prolongado (21 días) permitió demostrar que las proteínas dietéticas (PD), aportadas previamente (5 y 40 %), están asociadas con el manejo de las reservas, determinando el estado nutricional e inmunológico de los camarones (Pascual et al. aceptado).

Debido a la importancia económica y ecológica de los camarones peneidos se ha logrado un gran avance en el conocimiento de su metabolismo. Con base en esta información y en los resultados que se desprenden de la presente investigación se elaboró un modelo conceptual acerca del aprovechamiento del

alimento en organismos silvestres y cultivados, y su relación con la condición nutricional y el sistema inmunitario de juveniles de *L. vannamei* (Fig. 1).

El ambiente y la variabilidad genética constituyen la base del modelo, ya que como ha sido mencionado, la interacción entre estos dos elementos afecta la capacidad digestiva, el flujo de energía, la síntesis de proteínas, la osmorregulación, la resistencia a enfermedades y el grado de control homeostático. Dentro de este marco están incluidos los principales órganos y sistemas de los juveniles de camarón: glándula digestiva, branquias, músculo, cutícula, sistema nervioso, tejido hematopoyético e interconectando a todos ellos, se encuentra la hemolinfa. Los intercambios de materia entre el camarón y el ambiente que incluye el esquema están relacionados con el agua, los iones, la entrada de patógenos, el alimento y los desechos nitrogenados.

En el caso de los camarones, la degradación del alimento se inicia con la absorción, cuando el quimo y las partículas finas son introducidos al lumen de la glándula digestiva (Lucas, 1993). El alimento es degradado en sus componentes primarios: aminoácidos, monosacáridos, ácidos grasos, vitaminas y minerales, los cuales son procesados, almacenados, y también pueden ser transportados a otros tejidos vía la hemolinfa (Al-Mohanna y Nott, 1987). El modelo incluye la esquematización de las principales vías metabólicas, sus interconexiones y productos (letras negras), lo cual representa el flujo de la energía química desde el alimento hacia los procesos fisiológicos, la respuesta inmunitaria, la incorporación de biomasa y las reservas.

Los resultados del presente estudio indican que las proteínas son el eje principal del metabolismo de los juveniles de *L. vannamei* domesticados (F_7 y F_9) ya que el crecimiento, las reservas y el estado inmunológico de los camarones estuvieron directamente asociados al nivel de las proteínas en la dieta. La concentración de lo hemocitos circulatorios y la actividad fagocítica de los juveniles de *L. vannamei* (F_9) varió de manera directa y significativa ($P < 0.05$) con el nivel de proteínas

dietéticas (5, 15 y 40 % PD) (Pascual et al., 2004b). Cuando los juveniles de *L. vannamei* fueron alimentados con 5 % de PD lograron mantenerse e incorporar una ligera biomasa (1.15 $\pm$ 0.06) durante los 50 días experimentales. No obstante, la energía asimilada fue insuficiente para mantener al sistema inmunitario (cantidad significativamente menor de hemocitos, actividad de fenoloxidasa y estallido respiratorio), y también, para generar reservas (bajos niveles de glucógeno en la GD y proteínas de reserva en la hemolinfa, OxiHc) (Pascual et al., 2004b).

El modelo señala la relación entre el aprovechamiento del alimento y el manejo de la energía. Las proteínas dietéticas primeramente son hidrolizadas en peptidos y finalmente en aminoácidos, los cuales pueden ser oxidados para obtener energía o utilizados para sintetizar glucógeno. Muchos aminoácidos son utilizados para la síntesis de proteínas, como enzimas digestivas y metabólicas, proteínas del sistema inmunitario y hemocianina, la cual parece tener un importante papel en la respuesta inmunitaria de los camarones. Algunos peptidos pueden afectar al tejido hematopoyético y alterar la tasa de proliferación de hemocitos (TPH), o ser utilizados para sintetizar neuropeptidos, y lipoproteínas. Los aminoácidos también pueden ser transportados a la hemolinfa, al pool de aminoácidos libres (AAL), y de estos, al músculo.

De acuerdo con los trabajos realizados por Vinagre y Da Silva (1992) y Rosas et al. (2001a) los camarones están adaptados para sintetizar CHO a partir de proteínas. Los resultados señalan que esta adaptación se ha favorecido en poblaciones seleccionadas por tamaño y sujetas a un programa nutricional basado en las proteínas (Arena et al., 2003; Pascual et al., 2004a). Actualmente los alimentos formulados utilizados durante el cultivo contienen un constante y elevado nivel de proteínas (25 a 45 %), por lo cual, un programa de selección por tamaño, implica la selección de organismos que metabolizan preferentemente a las proteínas.

El resultado después de varias generaciones es una reducción en la capacidad para aprovechar mayores niveles de CHO en la dieta y camarones metabolitamente dependientes a las proteínas dietéticas (Arena, 2004; Pascual et al., 2004a). El estudio genético de los camarones realizado por Arena et al (2003) señala que los organismos silvestres presentan una mayor frecuencia alélica para el gen de la alfa amilasa, y también una mayor actividad enzimática. En los camarones cultivados la frecuencia alélica y la actividad de la amilasa disminuye directamente con el número de ciclos cerrados (F_7 y F_{25}) ($P < 0.05$), revelando que la reducción de la variabilidad genética afecta la capacidad digestiva de los camarones y con ello, el aprovechamiento de los alimentos, lo cual compromete la condición inmunológica de los organismos.

La degradación de los almidones dieteticos conduce a la absorción de glucosa, la cual puede ser trasportada directamente a la hemolinfa o metabolizada por medio de la glucólisis (GL), subsecuentemente el piruvato a través de la Acetil-Coenzima-A (A-CoA) puede ingresar a la cadena respiratoria (CR) para la obtención de energía (ATP). Bajo condiciones de estrés metabólico, es decir, súbita actividad y deficiencia de oxígeno metabólico, la glucosa es metabolizada por medio de la fermentación, y el lactato generado puede ser transportado a la hemolinfa.

La capacidad digestiva de los camarones silvestres revela la importancia de aprovechar los CHO dietéticos, lo cual puede estar asociado a los diversos puntos de regulación metabólica relacionada con la ruta glucolítica. Nueve de los diez aminoácidos no esenciales se pueden sintetizar a partir de la glucosa, donde el glutamato proporciona el grupo amino. En dirección opuesta, el catabolismo de los aminoácidos glucogénicos representa la base de la ruta gluconeogénica para obtener CHO metabólicos, los cuales pueden ser utilizados como sustrato energético a través de la glucólisis. El metabolismo de los lípidos esta conectado a la ruta glucolítica a través de Acetil-CoA; alta concentración de ATP o NADH favorece la síntesis de ácidos grasos desde la A-CoA, y la ß-oxidación de los

lípidos del alimento o de las reservas representa la dirección inversa para generar energía metabólica.

El modelo contempla la participación de la hormona hiperglicemiante de los crustáceos (HHC), ya que genera la liberación de glucosa desde el glucógeno almacenado, al activar a la glucógeno fosforilasa en la GD y en el músculo (Lüschen et al., 1993). La acción de este neuropeptido incluye un efecto secretagoge que controla la liberación de la alfa-amilasa en la GD, la regulación de la síntesis de ecdisteroides (Santos y Keller, 1993b), y modulación durante el proceso de muda. La liberación de la CHH desde el órgano X puede ser inhibida por alta concentración de glucosa en la hemolinfa, promoviendo la glucogénesis. En dirección contraria, alta concentración de lactato puede promover la liberación de la HHG y favorecer la hidrólisis del glucógeno (Santos y Keller, 1993a). Esta regulación señala claramente que las rutas metabólicas obedecen a la demanda energética de los tejidos.

El estudio de los juveniles de *L. vannamei* sometidos a un periodo de inanición prolongado (21 d) señala una disminución importante en los indicadores fisiológicos e inmunológicos de los camarones; en los camarones alimentados previamente con 5 % de PD el abatimiento de las reservas de energía y la respuesta inmunitaria fue acelerado (7 d), en comparación con los camarones alimentados con 40 % PD, los cuales lograron compensar el ayuno por mas tiempo (14 d) (Pascual et al. aceptado).

El rápido agotamiento de los lípidos en la GD y en la hemolinfa, señala su uso como sustrato energético, lo cual indica que independientemente a la condición nutricional, los camarones utilizan de manera inicial los lípidos y después de una semana de inanición pueden prácticamente terminar con las reservas lipídicas de la GD. Este patrón de comportamiento pudiera estar relacionado con la capacidad adaptativa que presentan los camarones para tolerar periodos cortos

de inanición asociada a las fluctuaciones en la disponibilidad de alimento natural y al proceso de muda.

Los componentes de la hemolinfa y las reservas de glucógeno en la glándula digestiva de los juveniles mantenidos en ayuno prolongado revelaron el uso paralelo de otros sustratos energéticos. Las fluctuaciones en la concentración de glucosa, lactato, proteínas y la OxiHc, así como el glucógeno señalan el papel central del catabolismo de las proteínas y la ruta gluconeogénica. Esto significa que los camarones alimentados previamente con 40 % de PD tienen una mayor oportunidad de compensar la privación de alimento, a través de la hidrólisis de las proteínas de la hemolinfa y del músculo, lo cual se ve reflejado en el mantenimiento de la respuesta inmunitaria durante 15 días de inanición.

La glándula digestiva (GD) y el músculo constituyen los principales centros metabólicos en los camarones, por lo cual el modelo contempla rutas metabólicas y manejo de sustratos similares, pero con algunas diferencias. En la GD se acumulas las reservas de lípidos y probablemente se ensamblen las lipoproteínas, LP (apoliproteinas, fosfolipidos, acilglicéridos y en menor cantidad colesterol). En el músculo la mayor parte de los lípidos son componentes del tejido muscular, y en casos extremos de demanda de energía, algunos de ellos (fosfatidilcolina y ésteres de esterol) pueden ser utilizados como sustrato energético (Perez-Velazquez et al., 2002). En cuanto al metabolismo de proteínas, el trabajo biosintético del músculo esta relacionado principalmente con la incorporación de biomasa, mientras que en la GD es el sitio de síntesis de proteínas por excelencia.

La hemolinfa está directamente relacionada con la regulación metabólica, ya que constituye el vínculo entre los principales órganos y sistemas. El proceso de osmorregulación esta esquematizado en el modelo como una zona donde interaccionan el músculo, la hemolinfa y las branquias. La enzima relacionada con la regulación del volumen celular es la glutamato deshidrogenasa (GDH), la

cual controla la incorporación o remoción del amonio desde el "pool" de aminoácidos libres al glutamato e ingresar al ciclo de Krebs (CK) a través de la alfa-cetoglutarato. El ciclo del glutamato (CG) esta representado por un círculo acoplado al CK. El grupo amino puede ser transferido a la glutamina, la cual es menos toxica que el NH_4, por lo cual está involucrada en la excreción nitrogenada a través de las branquias. El grupo amino también puede ser acoplado a un carbohidrato para la formación de glucosamina, que es una molécula relacionada con la formación de quitina, componente principal de la cutícula de los crustáceos.

El tejido hematopoyético y los hemocitos están representados en el modelo bajo una profunda interacción con la GD y el sistema nervioso, puesto que la condición nutricional y algunos neurotrasmisores pueden afectar la tasa de proliferación de los hemocitos y con ello, el estado de la respuesta inmunitaria.

La concentración de hemocitos en la epidermis durante algunas etapas de muda pudiera estar relacionada con la liberación de glucógeno para la síntesis de quitina, lo cual concuerda con la capacidad de los hemocitos de sintetizar glucógeno y almacenar enormes cantidades, proporcionalmente mayor a la de la GD (Loret, 1993). Por otro lado, la gran cantidad de aminoácidos en el interior de los hemocitos pudiera señalar una posible participación en la liberación de aminoácidos a la hemolinfa asociada a la regulación osmótica del medio interno y/o a la demanda de energía (ClaybrooK, 1983). Puesto que las funciones metabólicas de los hemocitos no han sido demostradas se señalan en el modelo con una flecha de línea discontinua.

Los resultados de la presente investigación concuerdan con otras investigaciones que señalan al metabolismo de proteínas como la clave para entender el manejo y destino de la energía en los camarones (Deshimaru, 1978; Rosas et al., 2002). El alto requerimiento de proteínas dietéticas, y la limitada capacidad de almacenar reservas de lípidos y carbohidratos (Dall y Smith, 1986), concuerda con la

habilidad de los camarones para usar a las proteínas como principal sustrato energético (Rosas et al., 1998; Kureshy y Davis, 2002). No obstante, altos niveles de proteínas en la dieta suelen afectar la calidad de agua en los sistemas de cultivo. Las proteínas que son utilizadas como sustrato energético, contribuyen con la liberación de los productos nitrogenados a los estanques, lo cual puede generar una eutrificación eventual. Importantes descargas de materia orgánica a los afluentes naturales están asociadas con el crecimiento de las áreas de engorda y el manejo de altas densidades durante el cultivo. Esto ha generado un deterioro importante de las condiciones ambientales dentro y fuera de las granjas, favoreciendo la incidencia de enfermedades y afectando negativamente a la producción y al medio natural.

Por todo esto, existe un marcado interés por desarrollar alimentos para la acuacultura que sean amigables con el medio, es decir, dietas formuladas con la cantidad mínima de proteínas y que permitan un óptimo crecimiento. El inconveniente es el alto requerimiento de proteínas y su papel central en el metabolismo de los camarones. No obstante, un mayor conocimiento sobre la bioquímica fisiológica y sobre el sistema inmunitario podría ser de gran ayuda para determinar cuales insumos alternativos como; diferentes fuentes de proteínas de origen vegetal, aditivos, inmunomoduladores, inmunoestimulantes, antioxidantes, hidolozados, entre otros, podrían satisfacer las necesidades metabólicas, sin comprometer a la respuesta inmunitaria. Bajo esta perspectiva, los programas de domesticación con criterios de selección que incluyan variabilidad genética, capacidad digestiva y condición fisiológica inmunológica, podrían representar las bases para el desarrollo de una camaronicultura sustentable y amigable con el ambiente.

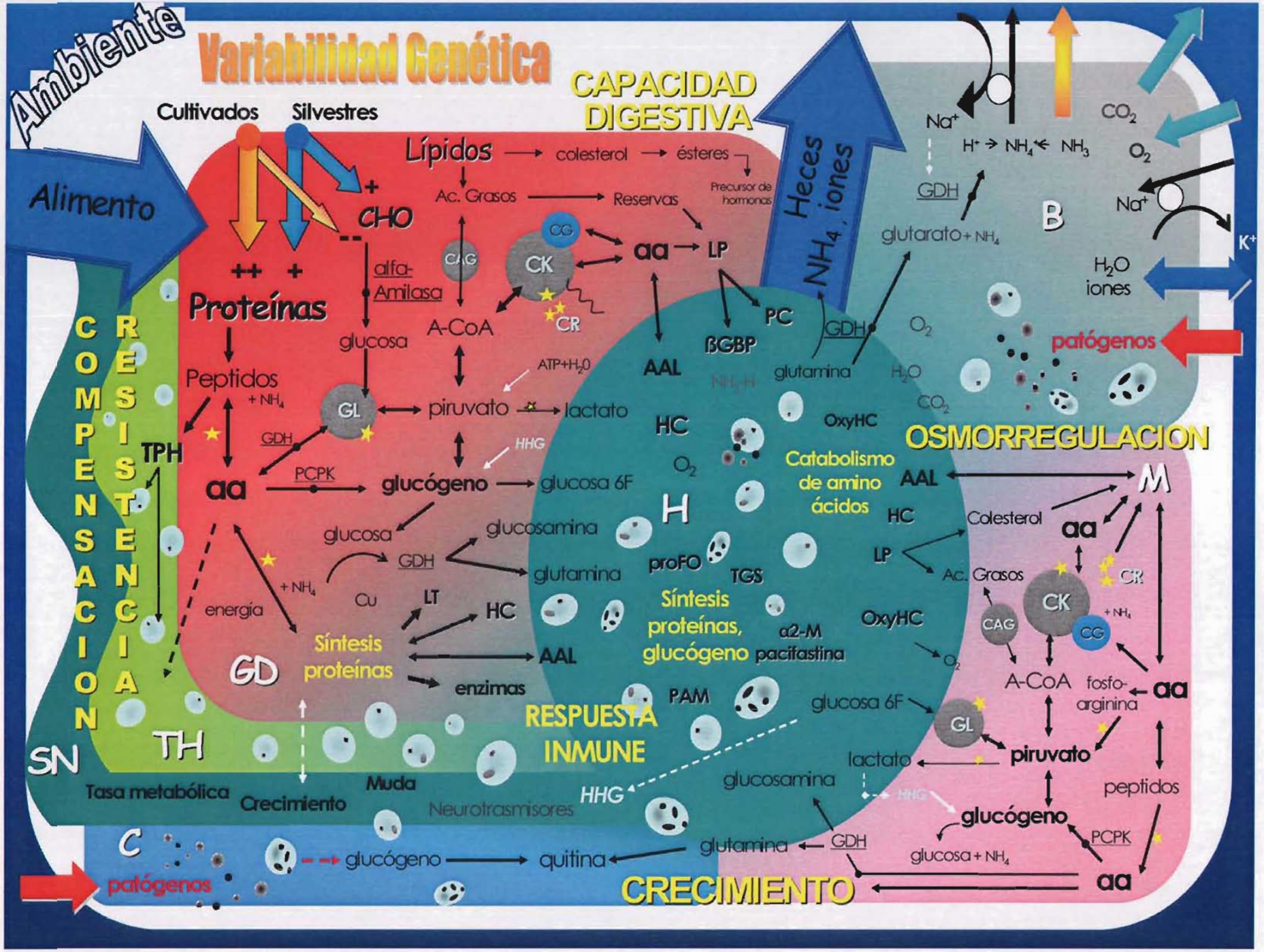
Ambiente
Variabilidad Genética
CAPACIDAD DIGESTIVA
Cultivados
Silvestres
Alimento
Heces
NH4, iones
Lípidos
colesterol
ésteres
Precursor de hormonas
Ac. Grasos
Reservas
CHO
alfa-Amilasa
CAG
CK
CG
CR
aa
LP
Proteínas
A-CoA
glucosa
PC
ßGBP
AAL
GDH
glutamina
Peptidos
+ NH4
GL
piruvato
lactato
ATP+H2O
HHG
HC
OxyHC
TPH
PCPK
glucógeno
glucosa 6F
Catabolismo de amino ácidos
glucosamina
proFO
TGS
Síntesis proteínas, glucógeno
α2-M
pacifastina
energía
Cu
LT
Síntesis proteínas
enzimas
PAM
RESPUESTA INMUNE
GD
TH
SN
Tasa metabólica
Crecimiento
Muda
Neurotrasmisores
HHG
C
patógenos
glucógeno
quitina
CRECIMIENTO
Na+
H+ → NH4 ← NH3
CO2
O2
GDH
glutarato + NH4
B
Na+
K+
H2O iones
patógenos
OSMORREGULACION
M
AAL
HC
Colesterol
aa
LP
Ac. Grasos
CK
CR
CAG
CG
+ NH4
A-CoA
fosfo-arginina
aa
GL
piruvato
lactato
peptidos
glucógeno
PCPK
glucosa + NH4
aa
COMPENSACION
RESISTENCIA

Figura 1. Modelo conceptual: el metabolismo de proteínas y homeostasis.

El esquema incluye los principales órganos y sistemas de los juveniles de camarón: glándula digestiva, **GD**; branquias, **B**; músculo, **M**; cutícula, **C**; sistema nervioso, **SN**; tejido hematopoyético, **TH**; y la hemolinfa, **H**. La variabilidad genética representa la base del modelo ya que afecta al flujo de la energía química desde el alimento hacia los procesos fisiológicos. Los juveniles de *Litopenaeus vannamei* cultivadas en ciclo cerrado y seleccionados por tamaño (F_7, F_9) presentan una menor capacidad de aprovechar los carbohidratos (**CHO**), y un mayor uso de las proteínas. La degradación de las proteínas genera peptidos y aminoácidos (**aa**) que son oxidados o también pueden ser utilizados para sintetizar glucógeno. Los **aa** también pueden ser utilizados para la síntesis de **enzimas digestivas y metabólicas, proteínas del sistema inmunitario** (como las del sistema profenoloxidasa, **proFO**; lectinas, **LT**; lipoproteínas de reconocimiento, **BGBP**; proteína de coagulación, **PC**; péptidos antimicrobianos, **PAM**; de regulación de la respuesta inmunitaria, **2-macroglobulina** y **pacifastina)**, y hemocianina, **Hc**, la cual parece tener una participación en la respuesta inmunitaria de los camarones, además de funcionar como una proteína de reserva en la hemolinfa. Los aminoácidos también pueden ser transportados a la hemolinfa para conformar el pool de aminoácidos libres (**AAL**), que pueden ser utilizados en el músculo para la generación de tejido.

La degradación de los almidones conduce a la absorción de glucosa, esta es trasportada directamente a la hemolinfa o metabolizada por medio de la glucólisis (**GL**), subsecuentemente el piruvato, a través de la Acetil-Coenzima-A (**A-CoA**), puede ingresar al ciclo de Krebs (**CK**) y proseguir con la cadena respiratoria (**CR**) para la obtención de energía (**ATP**). Bajo condiciones de deficiencia de oxígeno metabólico, se lleva a cabo la fermentación, y el lactato generado puede ser transportado a la hemolinfa. La capacidad digestiva de los camarones silvestres revela la importancia de aprovechar los CHO dietéticos, lo cual puede estar asociado a los diversos puntos de regulación metabólica relacionada con la ruta glucolítica. Nueve de los diez aminoácidos no esenciales se pueden sintetizar a partir de la glucosa, donde el glutamato proporciona el grupo amino. En dirección opuesta, por ejemplo en condiciones de ayuno, el catabolismo de estos aminoácidos representa la base de la ruta gluconeogénica para obtener CHO que pueden ser utilizados como sustrato energético. Los lípidos (ciclo de los ácidos grados: **CAG**) están conectados a la ruta glucolítica a través de A-CoA; alta concentración de **ATP o NADH** favorece la síntesis de ácidos grasos desde la A-CoA. La **ß-oxidación** de los lípidos del alimento o de las reservas representa la dirección inversa para generar energía metabólica. El modelo incluye el efecto de regulación de glucosa y lactato sobre la hormona hiperglicemiente de crustáceos (**HHC**), y a su vez, el efecto que tiene esta hormona sobre la segregación de la -amilasa y el glucógeno.

La regulación de la presión osmótica está asociada principalmente a la bomba **Na-K atpasa**, intercambio de iones, al catabolismo de los aa y la actividad de la glutamato deshidrogenasa (**GDH**), la cual controla la incorporación o remoción del amonio desde AAL al glutamato. El ciclo del glutamato (**CG**) esta representado por un círculo acoplado al CK. El grupo amino puede ser transferido a la glutamina, la cual es menos toxica que el NH_4, por lo cual está involucrada en la excreción nitrogenada a través de las branquias. El grupo amino también puede ser acoplado a un carbohidrato para la formación de glucosamina para la formación de quitina. Por lo cual, el crecimiento, las reservas y el estado inmunitario de los juveniles de *L. vannamei* están vinculadas al metabolismo de las proteínas.

Conclusiones

- La línea base de los metabolitos de la hemolinfa de *Litopenaeus vannamei* presentaron una concordancia con los valores reportados en la literatura para esta misma especie, lo cual señala su utilidad como un indicador de referencia del estado fisiológico de los camarones.

- La concentración de oxihemocianina, proteínas y glucosa de los camarones cultivados en condición piloto comercial (20 t) fue mayor a la los camarones cultivados en condiciones controladas (90 l), lo cual refleja una condición nutricional distinta asociada al alimento vivo en los estanques de 20 t.

- El estudio comparativo de los metabolitos en la hemolinfa de *L. vannamei* y lo reportado para otros crustáceos señaló que la concentración de proteínas, oxihemocianina, glucosa, acilglicéridos y lactato presenta una relación con la actividad, consumo de oxígeno, amplitud metabólica y el grosor del caparazón. En este sentido, los componentes de la hemolinfa parecen reflejan las adaptaciones fisiológicas por ser el resultado de la demanda de energía de los organismos.

- El estudio de los componentes de la hemolinfa de los camarones silvestres y cultivados (F_7) provenientes de un programa de selección por tamaño señaló una reducción en la capacidad para aprovechar mayores niveles de carbohidratos dietéticos (44 % CHO) y un metabolismo sesgado al manejo de las proteínas, lo cual afecta la condición nutricional y compromete el estado inmunológico de los camarones.

- El estudio del balance energético, el estado nutricional y la respuesta inmunitaria en juveniles de *L. vannamei* (F_9) alimentados con 5, 15 y 40 % de proteínas dietéticas reveló el carácter central del metabolismo de proteínas en los camarones cultivados y seleccionados por tamaño, ya que el crecimiento, las reservas y el estado inmunológico de los juveniles de *L. vannamei* estuvieron asociados al nivel de las proteínas en la dieta.

- Un ayuno prolongado de 21 días permitió demostrar que las proteínas dietéticas, aportadas previamente (5 y 40 %), están asociadas con el manejo de las reservas, determinando el estado nutricional e inmunológico de los juveniles de *L. vannamei* cultivados (F_9).

- Desde el punto de vista aplicativo la hemolinfa de los camarones es fácil de obtener y sus componentes brindan importante información biológica, por lo cual, podría utilizarse para monitorear la condición fisiológica e inmunológica de organismos silvestres y cultivados.

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Anexos

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PHYSIOLOGICAL AND GENETIC VARIATIONS IN DOMESTICATED AND WILD POPULATIONS OF *LITOPENAEUS VANNAMEI* FED WITH DIFFERENT CARBOHYDRATE LEVELS

LETICIA ARENA,[1] GERARD CUZON,[2] CRISTINA PASCUAL,[1] GABRIELA GAXIOLA,[1] CLAUD SOYEZ,[2] ALAIN VAN WORMHOUDT,[3] AND CARLOS ROSAS[1]*

[1]*Laboratorio de Biología Marina Experimental, Apdo. Post 69, Cd. del Carmen, Camp., México;* [2]*Centre Océanologique du Pacifique, BP 7004, Taravao, Tahiti, French Polynesia; and* [3]*Station du Biologie Marine du Museum National d'Histoire Naturelle et du Collége de France, BP 225, 29900, Concarneau, France*

ABSTRACT The relationship between polymorphism of α-amylase and physiologic and biochemical behavior of *L. vannamei* was used to determine whether artificial selection based on body weight and body size affect the adaptation ability of shrimp to use dietary carbohydrates as a source of energy. Shrimp fitness was addressed by measurement of energy balance using growth (P), oxygen consumption (R), and ammonia excretion (U) of juveniles from wild, 7th, and 25th generations of cultured shrimp. Hemolymph glucose, digestive gland glycogen, amylase activity, and amylase polymorphism was also evaluated in the three shrimp populations. Heterozygosity, amylase activity, and starch metabolism were affected by artificial selection of *L. vannamei*. Shrimp from a 25th-cultured generation had less heterozygosity and physiologic alteration than did wild shrimp. Shrimp from a 7th-generation cultured shrimp population showed an intermediate state of genetic and physiologic alteration. Although a statistical comparisons cannot be made between the three studied populations, it is evident that there is a reduction in amylase activity related to shrimp domestication, with high values in wild shrimp (between 24 to 39 IU mg^{-1} protein), intermediate in 7th-generation cultured shrimp (between 16 to 25 IU mg^{-1} protein), and low in 25th-generation cultured shrimp (between 3.6 to 15.8 IU mg^{-1} protein). A reduction in the frequency of alleles of amylase genes possibly related to domestication of shrimp was also demonstrated. It appears that the reduction of allele frequency of amylase genes affected the adaptative ability of shrimp to use dietary carbohydrates as a source of energy and molecules and caused farmed populations to be protein dependent. Results of energy balance studies indicate that there are differences in production efficiency (P/AS) between populations: a reduction in P/AS as a function of generations of farmed shrimp suggests that efficiency with which shrimp transform energy into biomass is reduced with artificial selection.

KEY WORDS: *Litopenaeus vannamei*, physiology, genetics, populations, domestication, bioenergetics, blood parameters

INTRODUCTION

The Pacific white shrimp *L. vannamei* (Boone) is the most important shrimp species cultivated in the Americas and the second in word production (Benzie 2000). More than 90% of the shrimp cultivated in 1998 on the American continent were *L. vannamei* (132 000 t; Rosenberry 1998). For that reason, shrimp farmers are establishing selective breeding programs for *L. vannamei* throughout the natural range of the species, as well as the US Atlantic coast and Brazil (Sunden & Davis 1991, Paiva-Rocha 2001, García-Culleja 2000). These programs are motivated in part by the serious disease problems caused by uncontrolled farmed population movements (Wyban et al. 1993, Bedier et al. 1998) and are focused to obtain better profitability through the selection of body weight or body size for optimal harvest. Although a better growth rate has been observed in breeding programs with *L. vannamei*, the impact of reported reduction of genetic diversity (Sunden & Davis 1991) on the general physiology of shrimp is not known (Benzie 1998). In a recent study Xu et al. (2001) showed a reduction in genetic diversity in cultured *P. monodon* compared with wild populations. That genetic differentiation pattern among populations was related to the prevalence of IHHNV viral disease in the same populations, indicating that the change in genetic diversity of shrimp could change the disease susceptibility of cultured or wild shrimp, affecting their fitness.

Assimilation (As) is the key characteristic of living organisms because it is a direct index of the energy allocated to body weight or gametes or to maintain homeostasis. According to Lucas (1993), As = P + R, where P is the energy allocated to production of biomass or gametes and R is the metabolizable energy. Although the fitness of a population has reproductive consequences, in a practical sense many researchers have been using the energy balance on juvenile forms to determine how the environmental fluctuations or types of food affect the energy allocation in Crustacea trying to predict the environmental or nutritional consequences in energy partitioning (Mayzaud & Conover 1988, Stickle et al. 1989, Du-Preez et al. 1992, Koshio et al. 1992, Hopkins et al. 1993, Rosas et al. 1993, Rosas et al. 1995, Guerin & Stickle 1997, Rosas et al. 1998, Rosas et al. 2001).

The energy derived from food depends on mechanisms of transformation of dietary components that, in turn, depends on the ability of organisms to hydrolyze, absorb, and assimilate those dietary nutrients (Ceccaldi, 1998). In a series of recent articles, we have demonstrated that energy allocation derived from dietary carbohydrates (CHO) has been found to be a limiting factor in *L. stylirostris*, *L. vannamei*, and *L. setiferus* (Rosas et al. 2000a, Rosas et al. 2000b, Rosas et al. 2001). In these works, we reported that glucose uptake in metabolism was limited because of saturation of α-amylase when shrimp are fed with diets above 33% CHO. At the same time, the digestive gland was saturated with glycogen in shrimp fed with diets >33% CHO, affecting nutrient absorption and in consequence growth rate and biomass production. Shrimp fed without dietary CHO can produce their own CHO using the gluconeogenic pathway, demonstrating that shrimp protein metabolism is well adapted to produce its own metabolic energy despite energy lost through ammonia excretion.

Shrimp α-amylase is one of the best-studied polymorphic digestive enzymes in shrimp. Two allelic forms were measured in *Asellus aquaticus*, four isoforms in *Palaemonetes varians*, seven

*Corresponding author. E-mail: crv@hp.felenciat.unam.mx

isoforms in *P. elegans* three isoforms in *P. serratus* and *L. vannamei*, and three in *Farfantepenaeus notialis*, in *L. schmitti*, and in *L. setiferus* (Lomholt & Christensen 1970, Christensen & Lomholt 1972, Van Wormhoudt 1983, Van Wormhoudt & Favrel 1988, Diaz et al. 1995, Le Moullac et al. 1996, Ball et al. 1998, Arena 1999, García-Machado et al. 2001). This enzyme can be induced or repressed by dietary CHO, protein levels, or by circadian, annual, or moult cycles (van Wormhoudt 1974, van Wormhoudt 1977). Van Wormhoudt et al. (1980) reported a reduction in amylase activity in *Palaemon serratus* as a function of the increase in dietary glucides. Rosas et al. (2000a) showed an increase in α-amylase of *L. stylirostris* as a function of an increase in dietary CHO levels. Lovett and Felder (1990) stated that a significant increase in amylase activity of *L. setiferus* postlarvae might be a response to low levels of CHO in the postlarval diet. Le Moullac et al. (1996) reported a reduction of amylase activity in *L. vannamei* when the amount of this protein increased in diets, showing that α-amylase gene expression could be repressed by casein, reflecting the control that diet has on activity of amylase isoforms. In the present research, a relation between polymorphism of α-amylase and physiologic and biochemical behavior of *L. vannamei* was used to study whether artificial selection based on body weight and body size affected the ability of shrimp to use dietary CHO as a source of energy. Shrimp fitness was addressed through measurement of energy balance using growth, oxygen consumption, and ammonia excretion of juveniles from wild, 7th, and 25th generations of cultured shrimps. Hemolymph glucose, digestive gland glycogen, amylase activity, and amylase polymorphism was also evaluated in the three shrimp populations.

MATERIAL AND METHODS

The study was divided between two experiments. The first was conducted in Mexico where comparisons were made wild and 7th-generation specimens of *L. vannamei*. The second experiment was conducted at the French Marine Research Institute (IFREMER) Tahiti facilities with 25th-generation specimens of *L. vannamei*. Both experiments were conducted under the same basic conditions and with the same experimental diets.

Experimental Conditions

For experiment 1, live wild *L. vannamei* (n = 200; 0.8 ± 0.1 g wet weight) were collected from Hurizache and Caimanero Lagoon on the Pacific Coast of México. Shrimp were transported by plane in plastic bags with cool sea water (30‰ salinity; 20°C) to the Experimental Marine Biology Laboratory of National Autonomous University of Mexico in Cd. del Carmen, Campeche, México. Shrimp were acclimated to laboratory conditions for 2 wk before any experimental procedure was initiated. During this period shrimp were maintained in a circular external pond (20 m^2) with aerated (O_2 > 5.0 mg/L) natural seawater (32‰; 29 ± 2°C). During acclimation, shrimp were fed twice each day on a commercial shrimp diet containing 45% protein (Api Aba camarón ultra, Malta Clayton SA®). At the same time, a sample of 7th-generation cultured shrimp (n = 200; 0.03 ± 0.03 g live weight) from a farm located in Sisal, Yucatán, was transported to the laboratory in cool sea water (35‰ salinity, 24°C) and acclimated under identical conditions to those described above.

After 2 wk of acclimation, a sample of each population was removed and distributed in 90-l plastic tanks. For experiment one, shrimp were reared for 55 to 58 days in a flow-through sea water system (32‰ salinity) at a density of 10 shrimp per tank. For experiment 2, we used 1600 postlarvae (0.009 ± 0.001 wet weight) of 25th-generation *L. vannamei* obtained in the IFREMER hatchery facilities. In IFREMER shrimp were reared in 800-L tanks for 36 days in a flow-through sea water system (36‰ salinity) at a density of 100 shrimp per tank.

In both experiments shrimp were fed three times a day (0800, 1400, and 2000 h), uneaten food particles were removed twice a day (0730 and 1700 h) and water quality variables were maintained as temperature 28 ± 1°C, dissolved oxygen >5.0 mg/L, and pH >8.2 ± 0.3. In both locations the photoperiod was set at 12h/12h. Samples of digestive gland for biochemical and genetic analysis from experiment 1 were stored at –80°C and then freeze-dried until analysis. Digestive glands from 25th-generation cultivated shrimp were freeze-dried at the IFREMER facilities in Tahiti before analysis.

Diets

L. vannamei juveniles were fed practical diets, formulated with two levels of carbohydrate (CHO): 3% and 44%. Experimental diets were prepared by thoroughly mixing dry ingredients with oil and then adding water until a stiff dough resulted. The dough was passed through a mincer with a 2-mm die, and the resulting spaghetti-like strings were air dried at 60°C. After drying, the strings were broken up and sieved to a convenient pellet size and stored at –4°C.

Growth and Survival

The growth rate was evaluated as the difference between wet weight at the beginning and end of the experiment and calculated as daily growth coefficient (DGC,%; Cho 1992):

$$DGC = 100 \times \{[\text{final weight (g)}]^{1/3} - [\text{initial weight (g)}^{1/3}]\}/\text{time (days)}$$

The DGC measure was chosen to make comparisons in growth tests because initial weights were different between treatments (Bureau et al. 2000, Cho 1992). The survival rate was calculated as the difference between the number of live animals at the beginning and end of the experiment.

Amylase Activity

At the end of growth trials, digestive glands from fasting (12 h) shrimp (40 per treatment) were dissected immediately, quickly frozen in liquid nitrogen, and then kept at –80°C for subsequent analysis. Frozen samples were homogenized in 500 μL of ice-cold, deionized water. Homogenates were centrifuged at 16000 g for 6 min at 8°C. Part of the supernatant was diluted in 10 volumes of ice-cold deionized water. Homogenates (crude or diluted) were immediately used for enzyme analysis (Brito et al. 2001). The soluble-protein content was measured in diluted homogenates by the Bradford (1976) method using the Sigma Micro Protein Determination Kit (Procedure No. 610). Samples were read in a Bio-Rad model 550 microplate reader at 495 nm. Duplicate assays for each sample were made. Amylase activity was assayed in diluted homogenates according to the method Bernfeld (1955) with 1% oyster glycogen (Sigma G8751) as substrate in 10 mM phosphate buffer, pH 7. One unit of amylase activity was defined as 1 mg of maltose liberated in 1 min at 30°C. Each sample was assayed in duplicate. Activity was expressed in units of μM substrate cleaved

per minute, based on an extinction coefficient $\varepsilon_{410} = 18000$ L mol^{-1} cm^{-1}. Each sample was assayed in duplicate.

Hemolymph Glucose

Blood glucose measurements were made in the same shrimp sampled for amylase activity. Before sampling, shrimp were placed in chilled (18°C) and aerated seawater for 5 min to reduce the effect of manipulation before the hemolymph extraction (Rosas et al. 2000a). Only shrimp in intermolt stage (C stage) were used. Hemolymph (approximately 200–300 µL per shrimp) was individually sampled through a chilled syringe needle inserted at the base of the fifth pereiopod after the shrimp had been dried with a paper towel. The individual weight (±0.05 g) was measured. Molting stages were identified by uropod examination (Drach & Tchernigovtzeff 1967). Commercial kits were used for glucose (GH; GOD-PAD, Merck-740393) determinations and were read with a microplate using 20 µL of plasma (obtained after 8000 *g* centrifugation) and 200 µL of enzyme chromogen reagent. Absorbance was recorded in a microplate reader (Bio-Rad model 550) and concentrations were calculated from a standard solution of substrate.

Glycogen Concentration in Digestive Gland (DGG)

Glycogen was extracted in the presence of sulfuric acid and phenol (Dubois et al. 1965). The digestive gland was first homogenized in trichloroacetic acid (5%) for 2 min at 6,000 rpm. After centrifugation (7000 *g*), the supernatant was quantified. This procedure was done twice. One milliliter of trichloroacetic acid was pipetted into a tube and mixed with 5 volumes of 95% ethanol. The tubes were placed in a oven at 37–40°C for 3 h. After precipitation, the tubes were centrifuged at 7000 *g* for 15 min. The glycogen (pellet) was dissolved by addition of 0.5 mL of boiling water and then 5 mL of concentrated sulfuric acid and phenol (5%) were added and mixed. The content of the tubes was transferred to a cuvette and read at 490 nm in a spectrophotometer.

Amylase Allozyme Analysis

Digestive glands from each population were homogenized in 500 µL of TRIS-phosphoric acid buffer (0.06 Mol /L, pH7) and centrifuged at 12000 rpm (4°C, 20 min). We used conventional 10% vertical polyacrylamide gel electrophoresis with TRIS-glycine as the running buffer (Davis 1964). Polyacrylamide gels were run at 250 V for 4 h. Band staining was done using an agar gel (1%) with 1% amylose, 1 M Ca, and 1 M Mg, phosphate buffer (pH 7), and 250 mM NaCl. The acrylamide–agar gel matrix was incubated at 37°C for 20 min and then stained with lugol solution (1:5) to obtain the bands. Alleles were coded by letters according their relative migration on gels (García-Machado et al. 2001). A locus was considered polymorphic when the frequency of the most common allele in the population did not exceed 95% and rare when the frequency was <0.005%. Genetic variability deviations of Hardy-Weinberg (H_e) expectations were determined using Wright's F statistics (Wright 1965).

Energy Balance

Energy balance was estimated using the equation of Lucas (1993):

$$Ab = R + U + P$$

where *Ab* is the absorbed energy (joules day^{-1} gww^{-1}), *R* is routine respiration, *U* is the energy lost through ammonia excretion, and *P* is the energy invested in production of biomass. Assimilated energy (*AS*) was estimated using the equation (Rosas et al., 1998):

$$AS = P + R$$

Production (*P*) was obtained from the growth rate of the shrimp. The mean value of 4900 ± 147 J gdw^{-1} was used to transform the growth data into production units (P: J g^{-1}dw d^{-1}). This value was obtained from analyzing the energy content of the muscle of 25 shrimp by means of a calorimeter (Parr), previously calibrated with benzoic acid.

Respiration (Rrout) or basal metabolism (Hem) was obtained through oxygen consumption measurements in nine fasting (12 h) shrimp on each dietary regimen. Oxygen consumption was measured on individual shrimp in a continuous flow respirometer (Rosas et al. 1998). Oxygen consumption was calculated as follows:

$$VO_2 = O_{2e} - O_{2ex} \times Fr$$

where VO_2 is oxygen consumption (mg O_2 h^{-1} animal^{-1}), O_{2e} indicates oxygen concentration at the entrance to the metabolic chamber (mg L^{-1}), O_{2ex} is oxygen concentration at the exit (mg L^{-1}), and Fr is the flow rate (L h^{-1}). Oxygen concentration was measured using a digital oximeter (YSI 50B digital, Dayton, OH) with a polarographic sensor (±0.01 mg L^{-1}), previously calibrated with oxygen-saturated seawater at 28°C. The shrimp were then fed food pellet fragments of 0.06 ± 0.002 g each in the respirometric chambers. The same amount of food was placed in a control chamber without an organism to estimate the oxygen lost by food decomposition. Oxygen consumption of fed shrimp was measured every hour for 4–6 h between 0800 and 1300–1500 h. Once the experiment was concluded, the shrimp were weighed. Specific routine oxygen consumption rate (mg g^{-1} h^{-1}) was estimated from the VO_2 of the unfed shrimp. The specific rate of the apparent heat increase (AHI), mg g^{-1} h^{-1}, was estimated from the difference between VO_2 of the unfed shrimp and the maximum value attained after feeding. A 14.3 J mg^{-1} conversion factor of oxygen consumption was used to transform the unfed and fed VO_2 to J g^{-1} dry weight (dw; Lucas 1993).

Along with the oxygen consumption measurements, water samples for ammonia excretion were obtained. Ammonia excretion was determined as the difference between the ammonia concentration at the entrance and the exit of each respirometric chamber and multiplied by the rate of water flow. The concentration of ammonia (total ammonia: $NH_4^+ + NH_3$) was measured using a flow injection-gas diffusion system (Hunter and Uglow, 1993). This technique consists of a carrier stream of NaOH (0.01 M) separated from an indicator solution (bromothymol blue 0.5 g L^{-1}) by a gas permeable membrane (PTFE). All ammonia in the sample is converted to gaseous NH_3, which diffuses across the membrane and reacts with the indicator to produce a pH-dependent color change that is detected by a photometer. A calibration curve was made using different concentrations of $(NH_4)_2SO_4$. The ammonia excretion of unfed and fed shrimp (postprandial nitrogen excretion; PPNE) was converted to energy units using the value of 20.5 J per mg N-NH_3 excreted (Lucas 1993) and defined as U_{prot} for the energy lost before feeding and U_{ppne} the energy lost after feeding. Total ammonia excretion was defined as U_{Total}.

R_{AHI} and U_{ppne} (J g^{-1} ww day^{-1}) were estimated considering the time needed for peak oxygen consumption after feeding and the number of rations fed to the shrimp per day ($Rt = 3$),

TABLE 1.

Daily growth coefficient of *L. vannamei* juveniles from wild and cultivated populations: experiment 1: wild vs. 7th-cultured generation comparisons.

	Wild		7th Generation	
	HCHO	LCHO	HCHO	LCHO
Initial weight, g	1.31 ± 0.02	1.39 ± 0.02	0.06 ± 0.05	0.07 ± 0.01
Final weight, g	8.42 ± 0.19	8.54 ± 0.38	4.10 ± 0.34	6.0 ± 0.22
Survival, %	78 ± 6[a]	60 ± 6[a]	68 ± 10[a]	68 ± 6[a]
Time, days	55	57	55	58
DGC, %	1.71 ± 0.4[a]	1.63 ± 0.4[a]	2.20 ± 0.2[b]	2.42 ± 0.2[c]
N	6	6	6	6

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

$$R_{AH1} = [(VO_2 \text{ af} - VO_2 \text{ bf} \times 14.3 \text{ J mg}^{-1}) \times (T \times Rt)]$$
$$U_{prot} = [(N - NH_3 \text{ af} - N - NH_3 \text{ bf} \times 20.5 \text{ J mg}^{-1}) \times (T \times Rt)]$$

where VO_2 or $N\text{-}NH_3$ are the oxygen consumption after (af) and before (bf) feeding. 14.3 J mg^{-1} and 20.5 J mg^{-1} are the constant to convert VO_2 or $N\text{-}NH_3$ in energy units. T is time (h) to reach the peak after feeding.

R_{raat} and U_{raat} (J g^{-1} ww day^{-1}) were estimated as:

$$R_{raat} = [(VO_2 \text{ bf} \times 14.3 \text{ J mg}^{-1}) \times (T_{wf} \times Rt)]$$
$$U_{raat} = [(N - NH_3 \text{ bf} \times 20.5 \text{ J mg}^{-1}) \times (T_{wf} \times Rt)]$$

where T_{wf} is the difference between time of one day (24 h) and $(T \times Rt)$.

Statistical Analysis

Statistical analyses were used separately in each experiment. Analysis of growth rates was performed independently for each population to emphasize dietary influence. Student *t*-tests were used on final average weight gains. The effect of dietary carbohydrate was analyzed for physiologic and genetic data using 2-way analysis of variance in Experiment 1, and one-way analysis of variance in Experiment 2. Arc sine transformation was used prior to analysis of survival data expressed in percentages. Homogeneity of variances of all distributions was verified with Cochran's test. Means obtained during the treatment were compared by using Duncan's multiple range test (Zar 1974).

RESULTS

Growth and Survival

Experiment 1

The daily growth coefficient (DGC%) was affected by dietary CHO and was higher in shrimp from the 7th generation than in wild shrimp (Table 1; $P < 0.05$). The DGC of 7th-generation shrimp was higher in shrimp fed with low dietary CHO than that in shrimp fed with high dietary CHO ($P < 0.05$). No differences were observed between wild shrimp fed with high or low dietary CHO levels (Table 1; $P > 0.05$). Survival was not affected by dietary CHO in either of the shrimp populations. A mean value of 69% survival was obtained in all treatments (Table 1).

Experiment 2

L. vannamei juveniles from Tahiti population (25th generation) were not affected by dietary CHO (Table 2; $P > 0.05$).

Amylase Activity

Experiment 1

The amylase activity was affected by dietary CHO and was higher in wild shrimp than in 7th-generation shrimp (Fig. 1A; $P < 0.05$). A higher amylase activity was observed in wild shrimp fed with high dietary CHO (35.0.1 IU/mg protein) than in wild shrimp fed with low dietary CHO (26.8 IU/mg protein). A significantly lower mean value of amylase activity was obtained in shrimp from the 7th-generation population (21 IU/mg protein) than in wild shrimp (Fig. 1A; $P < 0.05$).

Experiment 2

Dietary CHO levels significantly affected the amylase activity with high values in shrimp fed with low dietary CHO (13.5 IU/mg protein) and low values in shrimp fed with high dietary CHO (4.1 IU/mg protein) ($P < 0.05$; Fig. 1B).

Hemolymph Glucose

Experiment 1

A lower glucose hemolymph level was measured in wild shrimp fed with low dietary CHO (0.13 mg/mL) compared with that measured in wild and 7th-generation cultured shrimp (mean value of 0.28 mg/mL; Fig. 2A, $P < 0.05$).

TABLE 2.

Daily growth coefficient for juveniles of *L. vannamei* from 25th-cultured generation: Experiment 2.

	HCHO	LCHO
Initial weight, g	0.009 ± 0.001[a]	0.009 ± 0.001[a]
Final weight, g	0.72 ± 0.04	1.02 ± 0.05
Survival, %	83 ± 5	88 ± 6
Time, days	36	36
DGC, %	1.91 ± 0.7[a]	2.21 ± 0.78[a]
N	8	8

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

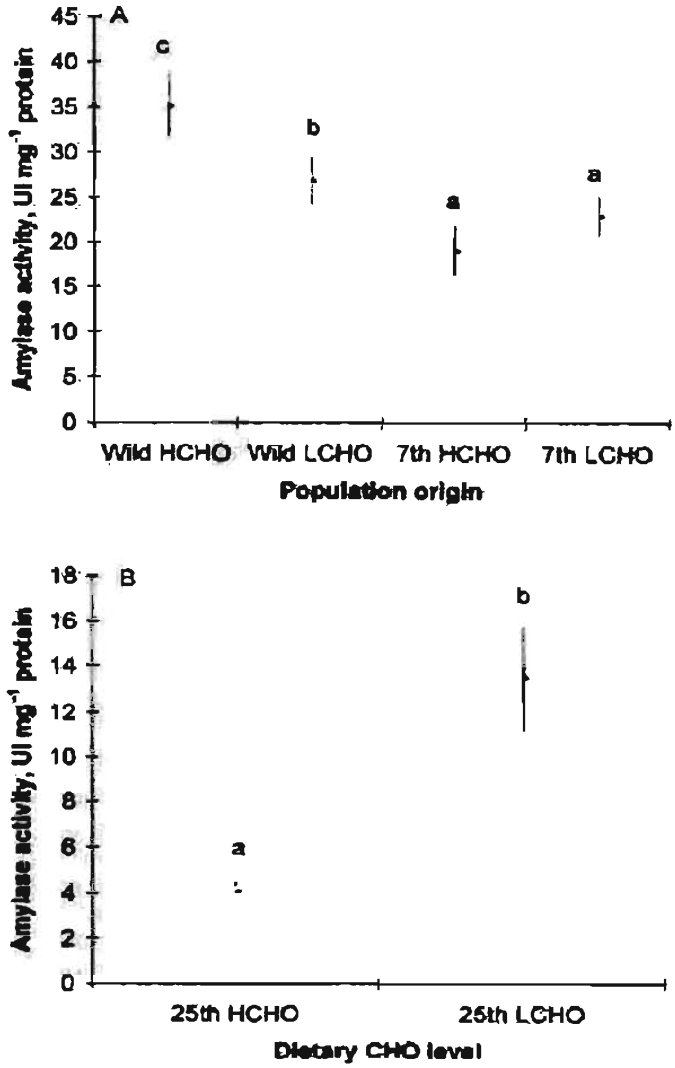

Figure 1. Amylase activity by wild and 7th-generation cultured *L. vannamei* (A) and 25th-generation cultured *L. vannamei* (B). Mean ± SE. Different letter means statistical differences at $P < 0.05$ level.

Experiment 2

A significantly high glucose hemolymph level was measured in 25th-generation shrimp fed with high CHO (1 mg/mL) that was 2.6 times the value in shrimp fed with low CHO (0.39 mg/mL) (Fig. 2B; $P < 0.05$).

Digestive Gland Glycogen

Experiment 1

Digestive gland glycogen concentration was affected by dietary CHO and the origin of shrimp (Fig. 3A). In wild and 7th-generation cultured shrimp, a high glycogen concentration was measured in shrimp fed with low dietary CHO ($P < 0.05$).

Experiment 2

In 25th-generation cultured shrimp, the high dietary glycogen level was measured in shrimp fed with high dietary CHO (2.0 mg/g) that was 30% higher than that in shrimp fed with low dietary CHO (1.4 mg/g; $P < 0.05$; Fig. 3B).

Pattern of Allozyme Variation

An eight-band pattern was observed in the electrophoretic analysis of amylase. These patterns were classified into two systems; system 1 with alleles a, b, and c and system two with five alleles: a, b, c, d, and e (Fig. 4). Both systems were polymorphic (Table 3). System 1 was more conservative than system 2. In such a system, alleles a, b, and d were rare with an allelic frequency <0.05. A reduction in H in system 2 was observed into domesticated populations, with high values in wild shrimp (H = 0.29) and low values in 25th-generation cultured shrimp populations (H = 0.08), reflecting a high percentage of homozygosity. Amylase loci from wild and 7th-generation cultured shrimp were in equilibrium. Locus from the 25th-generation of cultured shrimp showed significant deviation from Hardy–Weinberg proportions (heterozygosis deficit; $P < 0.05$) (Table 4).

Energy Balance

Experiment 1

Oxygen consumption of 12-h fasting shrimp was affected by dietary CHO in both wild and cultured populations (Table 5). The

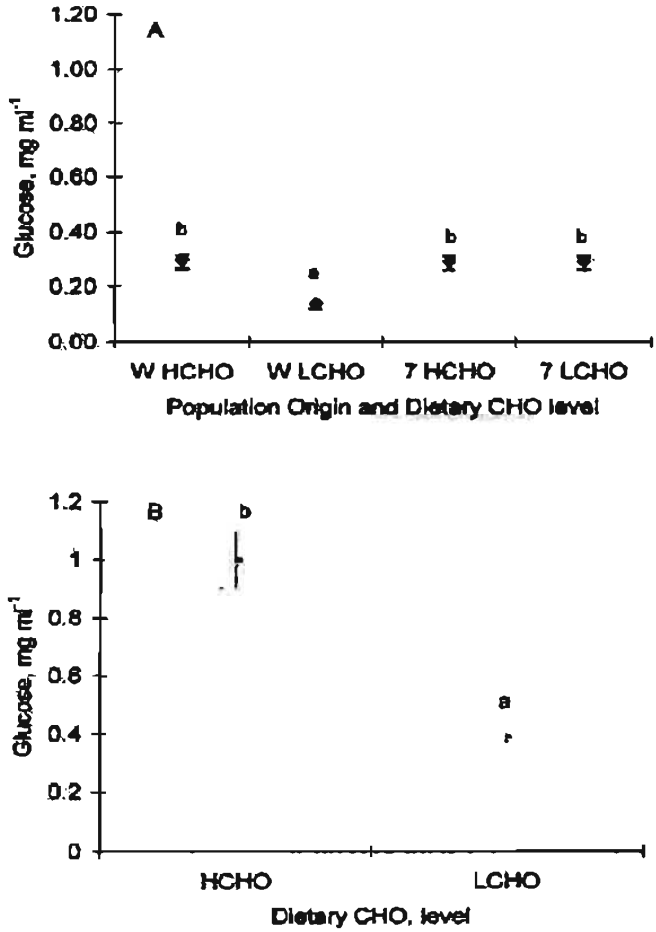

Figure 2. Glucose hemolymph level of wild and 7th-generation cultured *L. vannamei* (A) and 25th-generation cultured shrimp (B). Mean ± SE. Different letter means statistical differences at $P < 0.05$ level.

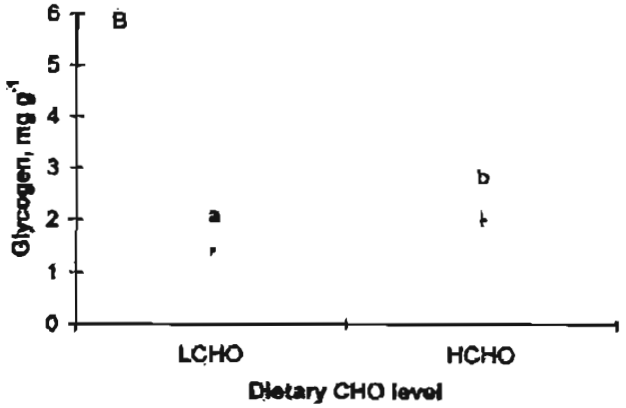

Figure 3. Digestive gland glycogen of wild and 7th-generation cultured *L. vannamei* (A) and 25th-generation cultured shrimp (B). Mean ± SE. Different letter means statistical differences at $P < 0.05$ level.

highest oxygen consumption was measured in 7th-generation cultured shrimp (0.65 mg O_2/h/g wet weight) fed with high dietary CHO ($P < 0.05$). The lowest oxygen consumption value was in wild shrimp fed with low dietary CHO (0.19 mg O_2/h/g wet weigh; $P < 0.05$). The oxygen consumption rate increased after feeding in each treatment (Table 5). Oxygen consumption of shrimp during feeding followed either of two patterns; one for wild shrimp fed with low dietary CHO and the other for the remaining shrimp groups. During feeding, oxygen consumption of wild shrimp fed with low dietary CHO was significantly lower than in wild shrimp fed high dietary CHO shrimp and 7th-generation shrimp fed with high or low dietary CHO. In each, oxygen consumption increased rapidly after feeding and decreased afterwards until reaching levels similar to those at the start of experiment. The time required to achieve oxygen consumption peak was higher in 7th-generation shrimp fed with high dietary CHO (2 h) than in all remaining shrimp groups (0.5 to 1 h).

Ammonia Excretion

Ammonia excretion in fasting wild shrimp (mean value of 0.06 mg N-NH_3/h/g wet weight) was significantly lower than in 7th-generation shrimp (mean value of 0.15 mg N-NH_3/h/g wet

TABLE 3.

Genetic diversity (H_e) in wild and cultured populations of *L. vannamei*.

Population	System 1 He	System 2 He
Wild	0.66	0.29
7th generation	0.51	0.27
25th generation	0.51	0.08

weight). Ammonia excretion increased after feeding, reaching a maximum value between 0.5 to 3 h after feeding depending on shrimp group (Table 6). The highest postprandial ammonia excretion value was recorded in 7th-generation shrimp fed with low dietary CHO and the lowest in wild shrimp fed with high dietary

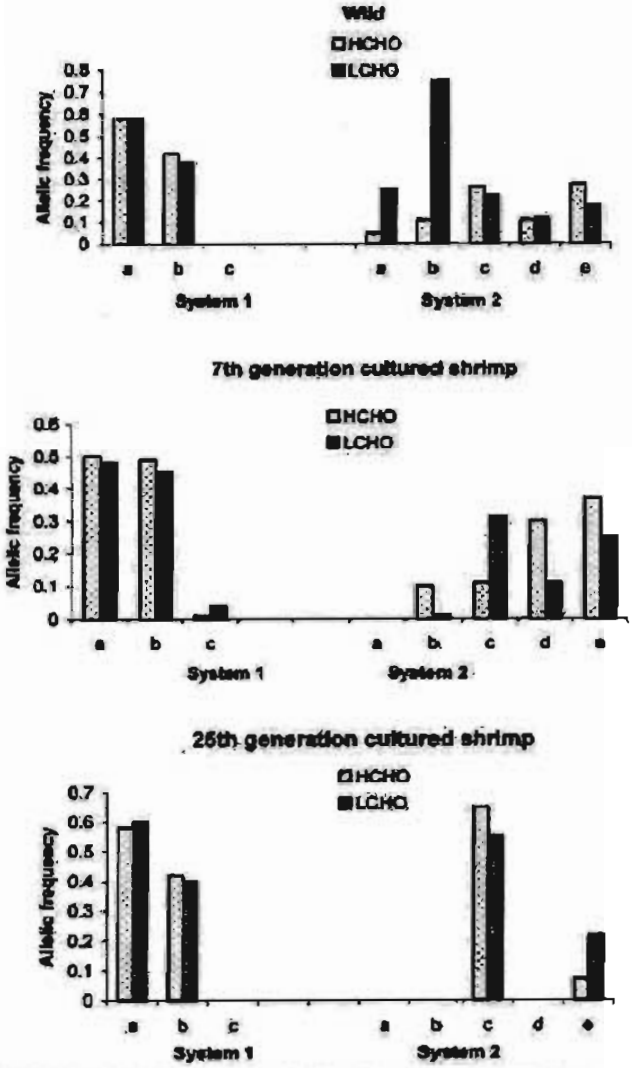

Figure 4. Allele frequencies of wild, 7th-, and 25th-generation cultured *L. vannamei* fed with high dietary CHO (HCHO) and low dietary CHO (LCHO).

TABLE 4.

Allelic frequencies comparison among different populations of *L. vannamei* from wild (México), 7th-generation cultivated shrimp and 25th-generation cultivated shrimp.

	Wild		7th Generation		25th Generation	
Population	S1	S2	S1	S2	S1	S2
Wild	—		NS	*	*	*
7th generation	—		—		NS	*
25th generation	—		—		—	

NS, without significant statistical difference. *Statistical differences at $P < 0.05$ level.

CHO. Intermediate values were recorded in the remaining shrimp groups ($P < 0.05$).

The respiratory energy (R_{Total}) varied between populations and was affected by dietary CHO (Table 7). Of the R_{Total}, 17% was wasted in R_{AHI} in wild shrimp fed with low dietary CHO in comparison with the 3.4–4% waste as R_{AHI} in the remaining shrimp groups (Table 7). R_{rout} was observed between 83–97% of R_{Total} with the lowest value in wild shrimp fed with low dietary CHO. There were statistical differences between U_{Total} between populations and between treatments in 7th-generation cultured shrimp (Table 7; $P < 0.05$).

The percentage of U_{Total} that was U_{rout} varied between shrimp populations with the lowest value in wild shrimp fed with low dietary CHO (37%) and the highest (82%) in 7th-generation shrimp fed with low dietary CHO. The energy wasted after feeding (U_{pp}) was higher in wild shrimp fed with low dietary CHO (63% of U_{Total}) than that in 7th-generation cultured shrimp fed with the same diet (18% of U_{Total}). Absorbed energy ($Ab = P + R + U$) showed differences between shrimp groups and was affected by dietary CHO with high values in wild shrimp fed with high dietary CHO (824 J^{-1} g^{-1} ww day^{-1}) and low values in 7th-generation cultured shrimp fed with same diet (598 J^{-1} g^{-1} ww day^{-1}; Table 7). U_{Total} varied between 5–11% of Ab with low values in wild shrimp fed with high dietary CHO and high values in 7th-generation cultured shrimp fed with high and low dietary CHO (11% and 10%). Between 89 and 95% of Ab was assimilated. The energy assimilated (*AS*) was the result of adding *R* to *P*. The *As* value was affected differently in each shrimp population. In wild shrimp the highest value was observed in shrimp fed with high dietary CHO whereas in 7th-generation cultured shrimp the highest value was observed in shrimp fed with low dietary CHO (Table 7). Respiratory efficiency (*RIAS*) was lower in wild than in 7th-generation cultured shrimp and was affected by dietary CHO in each shrimp group (Table 6). Inversely, growth efficiency (*PIAS*) was higher in wild than in 7th-generation shrimp and highest in shrimp fed with low dietary CHO in both shrimp groups.

Experiment 2

Oxygen Consumption

No difference was measured in 12-h fasting oxygen consumption values between treatments (mean value of 0.23 mg O_2/h/g wet weight; Table 8; $P > 0.05$). A similar maximum oxygen consumption value was observed in both dietary shrimp groups (0.32 mg O_2/h/g wet weight). The time to reach the peak was different between treatments with 1 h for shrimp fed with high dietary CHO and 2 h for shrimp fed with low dietary CHO (Table 8).

Ammonia Excretion

In 25th-generation shrimp, 12-h fasting shrimp had similar values of ammonia excretion between treatments (mean value of 0.022 mg N-NH_3/h/g wet weight; $P > 0.05$; Table 9). After feeding, the ammonia excretion increased. The time to reach the peak was similar in both treatments with high values in shrimp fed with high dietary CHO (0.040 mg N-NH_3/h/g wet weight) and low values in shrimp fed with low dietary CHO (0.035 mg N-NH_3/h/g wet weight; $P < 0.05$).

Dietary CHO affected R_{Total} (Table 10). Shrimp fed with high dietary CHO had the higher proportion of energy from R_{Total} that was channeled to R_{rout} (96%) and at the same time the lower proportion of R_{Total} that was used in R_{AHI} (4%). In contrast the higher proportion of energy of U_{Total} that was lost as U_{rout} was in

TABLE 5.

Oxygen consumption (mg O_2/h/gww) of *L. vannamei* after 12-h fasting (time = 0) and at time increments after feeding: Experiment 1.

Time After Feeding, h	Wild		7th Generation	
	HCHO	LCHO	HCHO	LCHO
0	0.49 ± 0.09^a	0.19 ± 0.05^a	0.65 ± 0.06^b	0.54 ± 0.05^a
0.5	0.59 ± 0.11^b	0.44 ± 0.04^c	0.61 ± 0.09^b	0.57 ± 0.08^a
1	0.61 ± 0.11^b	0.46 ± 0.07^c	0.65 ± 0.09^b	0.70 ± 0.09^b
2	0.58 ± 0.07^b	0.28 ± 0.05^b	0.72 ± 0.04^b	0.59 ± 0.11^a
3	0.52 ± 0.08^a	0.39 ± 0.04^c	0.48 ± 0.05^a	0.59 ± 0.06^a
4	0.46 ± 0.06^a	0.22 ± 0.03^a	0.35 ± 0.04^a	0.61 ± 0.14^a

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

TABLE 6.

Ammonia excretion (mg N-NH_3/h/gww) of *L. vannamei* after 12-h fasting (time = 0) and at time increments after feeding; Experiment 1.

Time After Feeding, h	Wild		7th Generation	
	HCHO	LCHO	HCHO	LCHO
0	0.067 ± 0.002[a]	0.05 ± 0.008[a]	0.15 ± 0.02[b]	0.14 ± 0.02[a]
0.5	0.18 ± 0.004[b]	0.15 ± 0.02[a]	0.15 ± 0.05[b]	0.34 ± 0.03[c]
1	0.12 ± 0.003[b]	0.19 ± 0.03[b,c]	0.16 ± 0.007[b]	0.35 ± 0.04[c]
2	0.19 ± 0.003[b]	0.21 ± 0.03[c]	0.16 ± 0.03[b]	0.24 ± 0.03[b]
3	0.08 ± 0.003[a]	0.22 ± 0.06[c]	0.25 ± 0.03[c]	0.33 ± 0.05[c]
4	0.11 ± 0.003[a]	0.07 ± 0.009[a]	0.06 ± 0.01[a]	0.14 ± 0.04[a]

Different letter means statistical differences. $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

shrimp fed with low dietary CHO (87%) in comparison to 75% lost in U_{rest} in shrimp fed with high dietary CHO. Inversely the proportion of U_{Total} that was lost as U_{pp} was higher in shrimp fed with high dietary CHO (25%) than in shrimp fed with low dietary CHO (13%; Table 10). In both treatments 95% of energy absorbed (*Ab*) was assimilated (*AS*). Dietary CHO affected *AS* and growth and respiratory efficiencies. Shrimp fed with low dietary CHO showed the higher *AS* and growth efficiency (72%) compared with shrimp fed with high dietary CHO (61%; Table 10).

DISCUSSION

In *L. vannamei* shrimp from the 25th-cultured generation exhibited less heterozygosity than did wild shrimp. From results obtained, the 7th-generation cultured shrimp showed an intermediate genetic and physiologic alteration. Although results demonstrate significant genetic differentiation among cultured and wild populations when based upon only an amylase allozyme marker, we acknowledge the necessity to confirm such differences at the mtDNA level through sequence variation of the amylase gene as recommended by Xu et al. (2001) and García-Machado et al. (2001). A more detailed study involving molecular biology and genetic alterations by domestication of *L. vannamei* is in process. As a consequence of selection in cultured populations, carbohydrate metabolism routes (hydrolysis, absorption, and synthesis) in shrimp fed with different dietary CHO was affected. A different enzyme activity-dietary CHO relation was observed depending on population characteristics; wild shrimp amylase activity was induced by high dietary CHO whereas low dietary CHO induced a high amylase activity in cultured shrimp. If reduction of heterozygosis means a reduction in amylase genes, then amylase activity induction was a compensatory response to obtain the highest possible glucose from the diet, increasing enzyme synthesis when shrimp are fed with low dietary CHO. On the contrary, in wild shrimp an excess of dietary CHO induced amylase activity because those shrimp have all the isoforms of the amylase enzyme to respond directly to the dietary starch. If amylase production in domesticated shrimp is efficient enough to process dietary CHO, it can be analyzed in a general context. Although a statistical comparison cannot be done among the three studied populations, it is evident there is a reduction in amylase activity as a function of domestication, with high values in wild shrimp (between 24 to 39 IU mg^{-1} protein), intermediate in 7th-generation cultured shrimp (between 16 to 25 IU mg^{-1} protein), and low in 25th-generation cultured shrimp (between 3.6 to 15.8 IU mg^{-1} protein; Fig. 1). Such reduction indicates that the reduction of allel frequency of amylase genes affected the adaptative ability of shrimp to use

TABLE 7.

Energy balance in juveniles of *L. vannamei*; Experiment 1.

	Wild		7th Generation	
	HCHO	LCHO	HCHO	LCHO
R_{rut}, J/day/gww	147.1 ± 1.9	57.1 ± 10.7	167.3 ± 18.8	162.1 ± 26.7
R_{AHE}, J/day/gww	5.2 ± 0.8	11.6 ± 1.1	6.0 ± 0.8	6.8 ± 0.8
R_{Total}, J/day/gww	152.3	68.7	173.3	168.9
U_{rest}, J/day/gww	24.7 ± 0.73	18.5 ± 2.70	46.1 ± 6.1	60.3 ± 8.6
U_{PPNB}, J/day/gww	13.8 ± 1.9	31.4 ± 4.7	18.5 ± 2.4	12.9 ± 1.6
U_{Total}, J/day/gww	38.5	49.9	64.6	73.2
P, J/day/gww	633.4 ± 70	614.6 ± 73	359.9 ± 46	500.9 ± 65
Absorption (*Ab*) J/day/gww	842.2	732.6	597.8	743.0
Assimilation (As) J/day/gww	785.7	683.3	533.3	669.8
Ef assimilation, *As/Ab*	95.3	93.3	89.2	90.1
Respiratory efficiency, % *R/As*	19.4	10.1	32.5	25.2
Production efficiency, % *P/As*	80.6	89.9	67.5	74.8

Mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

TABLE 8.

Oxygen consumption (mg O_2/h/gww) of *L. vannamei* (25th generation) 12 h fasting (time = 0) and at time increments after feeding: Experiment 2.

Time After Feeding, h	HCHO	LCHO
0	0.24 ± 0.02[a]	0.21 ± 0.01[a]
1	0.31 ± 0.02[b]	0.31 ± 0.02[d]
2	0.29 ± 0.02[b]	0.33 ± 0.02[d]
3	0.28 ± 0.02[b]	0.27 ± 0.02[c]
4	0.26 ± 0.02[ab]	0.27 ± 0.02[c]
5	0.25 ± 0.02[a]	0.28 ± 0.02[c]
6	0.27 ± 0.02[b]	0.25 ± 0.02[b]

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

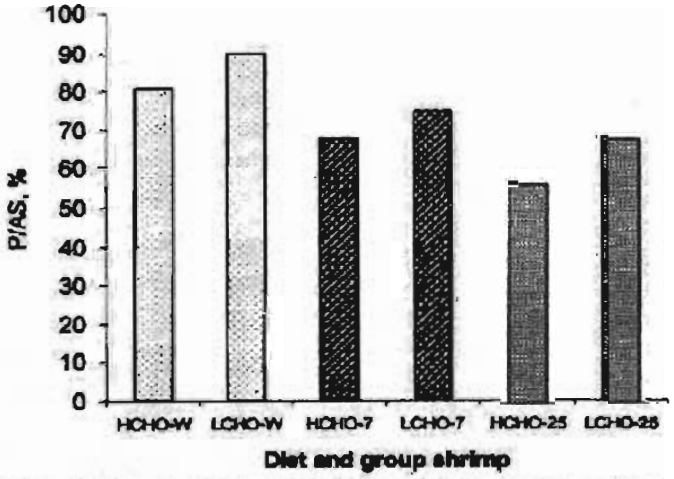

Figure 5. Growth efficiency (P/AS,%) of wild (W) 7th (7)-, and 25th (25)-generation cultured *L. vannamei* fed with different carbohydrates levels. HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

dietary CHO as a source of energy and molecules, which could cause farmed populations to be protein dependent.

Juveniles of *Litopenaeus vannamei* can synthesize their own glucose from protein through a gluconeogenic pathway (Rosas et al. 2001). Shrimp fed with low dietary CHO had digestive gland glycogen levels that were higher than when fed with high dietary CHO because the enzymatic system is induced to synthesize CHO from protein (Cuzon et al. 2001). In the present study, an increase in digestive gland glycogen was measured in wild and 7th-generation shrimp fed with low dietary CHO indicating that an induction mechanism is working. In contrast, in the 25th-generation farmed shrimp, that mechanism appears to be working in the opposite direction, producing more digestive gland glycogen in shrimp fed with high dietary CHO than in shrimp fed with low dietary CHO. If Amylase genes are repressed after 25th generations of selection then a high probability exists that other genes could be repressed also, producing changes and reducing the gluconeogenic route in shrimp.

This indicates that artificial selection of shrimp favored more than size and harvest weight, as it also favored protein metabolism by acting on shrimp digestive capacity. The use of high levels of animal protein in shrimp feeds in all phases of shrimp culture, from larvae to broodstock (including *Artemia*, krill, *Cyclops*, high quality fish meal, and squid) is responsible for activation and repression of genes. For amylase, Le Moullac et al. (1996) reported a reduction of enzyme activity in *L. vannamei* after an increase in dietary protein, which was related to a regulating role of amino acids on amylase expression. They observed a disappearance of one amylase mRNA associated with a high protein level suggesting that a regulation of amino acids would take place at the transcriptional level. Because, in selected shrimp, protein metabolism was favored and growth rate depended on dietary protein (Andrews et al. 1972), one can explain why 7th and 25th-generation farmed shrimp possess a higher growth rate than wild shrimp (Tables 1 and 2).

There are several costs that are necessary to take into account with the breeding programs that only take into account the size of shrimp at harvest, which is also related to growth efficiency. From results on energy balance, there are differences in production ef-

TABLE 9.

Ammonia excretion (mg N-NH_3/h/gww) of *L. vannamei* (25th generation) 12-h fasting (time = 0) and at time increments after feeding: Experiment 2.

Time After Feeding, h	HCHO	LCHO
0	0.020 ± 0.002[a]	0.024 ± 0.0004[a]
1	0.030 ± 0.002[b]	0.028 ± 0.001[b]
2	0.040 ± 0.002[c]	0.035 ± 0.001[c]
3	0.030 ± 0.001[b]	0.029 ± 0.001[b]
4	0.037 ± 0.002[bc]	0.026 ± 0.001[b]
5	0.029 ± 0.001[b]	0.030 ± 0.0009[b]
6	0.03 ± 0.001[b]	0.027 ± 0.0008[b]

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

TABLE 10.

Energy balance of *L. vannamei* (25th generation): Experiment 2.

	Dietary CHO	
	HCHO	LCHO
R_{rest}, J/day/gww	72.07 ± 6.0[a]	54.1 ± 2.6[b]
R_{ASD}, J/day/gww	3.0 ± 0.85[a]	10.3 ± 1.7[b]
R_{Total}, J/day/gww	75.1	64.4
U_{net}, J/day/gww	7.4 ± 0.7[a]	8.9 ± 0.2[b]
U_{FENG}, J/day/gww	2.5 ± 0.3[a]	1.4 ± 0.1[b]
U_{Total}, J/day/gww	9.84	10.2
P, J/day/gww	96.78 ± 14[a]	137.6 ± 20.6[b]
Absorption (*Ab*), J/day/gww	181.7	212.2
Assimilation (*As*), J/day/gww	171.8	202.0
Ef assimilation *As*/*Ab*	94.6	95.2
Respiratory efficiency, % *R*/*As*	43.7	31.9
Production efficiency, % *P*/*As*	56.3	68.1

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

ficiency between populations (Fig. 5); a reduction of the P/AS ratio depending on the generations of farmed selected shrimp indicate that efficiency with which shrimp transform energy into biomass is reduced with artificial selection. That situation has several implications on coastal ecology. When selected shrimp are lost by pond break caused by floods or hurricanes they could be liberated to surroundings environment. If those shrimp are from a breeding program based on size only, they shrimp could growth faster and consume more protein than wild shrimp, wasting energy due to its reduced assimilation efficiency and wasting other nutrients offered by the natural environment in the form of CHO and in consequence changing the relation between nutrients and consumers. In this same sense a reduction in *P/AS* ratio could have implications on the shrimp industry if is considered that a reduction in production efficiency could means the use of foods with more and more fish meal to satisfy the protein requirement of shrimp provoking that the shrimp industry to compete with other industry that use fish meal to produce meat for human consumption.

On the other hand, selection shrimp programs could have relevance for the health of farmed shrimp. Recently, Xu et al. (2001) showed that there is a relation between genetic diversity and IHHNV sensitivity of *P. monodon* from Philippines. Although such relation is not understanding at all it could means that at the same time that shrimp are selected for size some other genes related with virus tolerance could be selected as well, provoking a segregation of the genes involved in virus resistance. If such concepts are applied to *L. vannamei* from breeding programs we could help to develop an industry based on rapid growth, low efficiency and vulnerable shrimp. It will necessary change looking for an shrimp based in the conception of breeding program that try to select shrimp that have wider adaptative ability to respond demands including all that are related to feed composition, productivity, and sustainability (Fenucci et al. 1982, Bureau et al. 2000), and biosecurity (Xu et al. 2001).

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Respuesta fisiológica de los juveniles de *Litopenaeus vannamei*, silvestres y cultivados, alimentados con bajos y altos carbohidratos (3 y 44 %)

Cristina Pascual, Carlos Rosas, Gabriela Gaxiola y Leticia Arena

Laboratorio de Ecología y Biología Marina Experimental, Facultad de Ciencias, UNAM

Introducción

Investigaciones recientes indican que el proceso de domesticación de las poblaciones cultivadas de *L.vannamei* conduce a la perdida de alelos del gen de la alfa amilasa (Arena, *et al.*,en impresión). Esto podría significar un cambio en la capacidad de los camarones para degradar a los carbohidratos dietéticos (CBH), reduciendo así, el espectro de posibilidades para el aprovechamiento de los alimentos formulados.

En el presente estudio se utilizaron a los componentes de la hemolinfa para identificar cambios metabólicos entre las poblaciones de *L. vannamei*, silvestres y cultivados (F7), alimentados con bajos y altos CBH (44 y 3 %). Se incluyeron además, evaluaciones imunológicas (la actividad de la profenoloxidasa evaluada en el degranulado de hemocitos, el conteo y la caracterizacion de los hemocitos), con la intención de lograr una visión del estado de salud de los organismos expuestos a un proceso de domesticación y la relación con el estado nutricional.

Material y Métodos

Los camarones cultivados en ciclo cerrado (F7) fueron obtenidos de Industrias Pecis S.C., y los organismos silvestres de la Laguna de Huizache y Caimanero, México. Por 60 días, los camarones fueron alimentados con peletizado de alto y bajo nivel de CBH (44 y 3 %) (Tabla 1). Cada tratamiento contó con 6 réplicas, donde se mantuvieron 10 juveniles en estanques de 0.28 m^2, con sistema de agua de mar de flujo constante. Para comparar los patrones de crecimiento se utilizó el Coeficiente de Crecimiento Diario (%DGC). Al término del experimento se utilizaron 40 organismos por tratamiento para determinar la concentración en la hemolinfa de glucosa, proteínas, colesterol, y la concentración de glucógeno en la glándula digestiva (GGD). Se realizó el conteo y caracterización de los hemocitos, la actividad de profenoloxidasa fué evaluada en el degranulado de hemocitos y se determinó el estadio de muda. Los datos de los organismos en intermuda se utilizaron para identificar el efecto del origen de las poblaciones y del nivel de CBH dietéticos, a través, de un análisis de varianza de dos vías.

Tabla 1. Composición de los alimento experimentales.

Ingredientes (%)	*Altos CBH*	*Bajos CBH*
Caseína	19	55
Calamar	20	25
Aceite de harina de bacalao	8	8
Lecitina de soya	2	2
Almidón de trigo	44	3
Premezcla de vitaminas	2	2
Rodimix (C) *	2	2
Minerales	1	1
Colesterol	0.2	0.2
Celulosa	1.8	1.8
Composición (%)		
Proteínas	30	66.5
Lípidos	10	10
CBH	44	3
Energía Digerible *	17 Kj/g	19 Kj/g

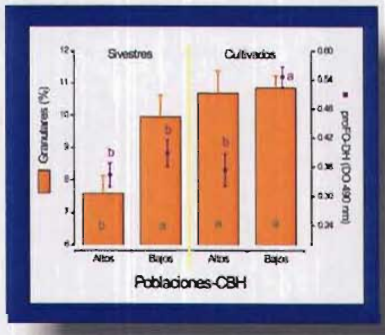

Figura 4. Hemocitos granulares y actividad de profenoloxidasa de los juveniles de *L. vannamei*, silvestres y cultivados, alimentados con bajos y altos CBH (3 y 44 %). Promedio ± ES. Letras distintas indican diferencia significativa (P<0.5) entre los tratamientos.

Abstract

The hemolymph components were used to identify metabolic changes between cultivated (F7) and wild juveniles of *Litopenaeus vannamei* shrimp, fed with low and high carbohydrates (CBH) (3 y 44 %). The daily growth coefficent was highest in cultivated shrimp fed with low CBH. The results obtained demonstrated that wild shrimp were more able to use dietary CBH than cultivated shrimp. The total hemocites count and the protein level in hemolyph show a similar behavior. Both indicators has been used like health state indices and were highest in wild shrimps fed high CBH. That results reflects some effects of the domestication process on physiological state of *L. vannamei*. Furthermore, the components of hemolymph show the diversity of the metabolic response in the wild shrimps.

Resultados y Discusión

Figura 1. Coeficiente de Crecimiento Diario de los juveniles de *L. vannamei*, silvestres y cultivados, alimentados con bajos y altos CBH (3 y 44 %). Promedio ± ES. Letras distintas indican diferencia significativa (P<0.5) entre los tratamientos.

El patrón de crecimiento fue significativamente mayor en los camarones cultivados que en los silvestres. El nivel de carbohidratos (CBH) de la dieta afectó el crecimiento de los juveniles cultivados, siendo significativamente mayor el DGC % de los camarones alimentados con la dieta de bajos CBH. El crecimiento de los juveniles silvestres no fue afectada por el nivel de CBH en el alimento (Fig. 1). El mayor número de alelos del gen de la alfa-amilasa reportado en las poblaciones silvestres de *L. vannamei* puede ser relacionado con un manejo adecuado de CBH dietéticos en un rango de 3-44 %. Mientras que la disminución de los alelos del gen de la alfa-amilasa de las poblaciones cultivadas parece representar una desventaja en el aprovechamiento de alimento con alto nivel de CBH (44%).

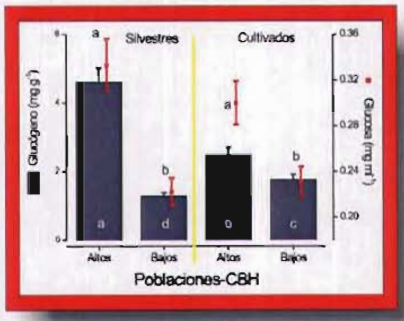

Figura 2. Concentración de glucógeno en la glándula digestiva y nivel de glucosa en la hemolinfa de los juveniles de *L. vannamei*, silvestres y cultivados, alimentados con bajos y altos CBH (3 y 44 %). Promedio ± ES. Letras distintas indican diferencia significativa (P<0.5) entre los tratamientos.

En ambas poblaciones el nivel de glucosa en la hemolinfa y la concentración de glucógeno en la glándula digestiva estuvo en concordancia con el nivel de CBH del alimento (Fig. 2). Sin embargo, en los camarones silvestres la concentración de GGD fue significativamente mayor cando fueron alimentados con altos CBH. Considerando que los organismos silvetres cuentan con un mayor número de isoformas de la enzima alfa-amilasa, los presentes resultados concuerda con la relación observada entre la actividad de la alfa-amilasa y la curva de saturación de GGD de distintas especies de camarón (Rosas, *et al.*, 2002)

El nivel de colesterol en la hemolinfa fue significativamente mayor en los organismos silvestres e independiente al nivel de CBH en la dieta (Fig. 3). Ya que los camarones no sintetizan colesterol y puesto que las dos dietas experimentales contenían el mismo nivel de colesterol, las diferencias observadas entre las poblaciones de camarones indica diferencias en el metabolismo de los lípidos.

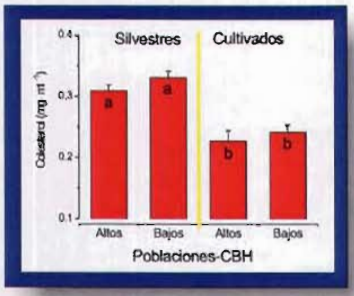

Figura 3. Concentración de colesterol en la hemolinfa de los juveniles de *L. vannamei*, silvestres y cultivados, alimentados con bajos y altos CBH (3 y 44 %). Promedio ± ES. Letras distintas indican diferencia significativa (P<0.5) entre los tratamientos.

La actividad de profenoloxidasa y la cantidad de hemocitos granulares fue mayor en los camarones cultivados (Fig. 4). El conteo total de hemocitos mostró un comportamiento similar al nivel de proteínas en la hemolinfa (Fig. 5), ambos parámetros son considerados indicadores del estado de salud y en el presente estudio se obtuvieron los mayores valores en los camarones silvestres alimentados con altos CBH.

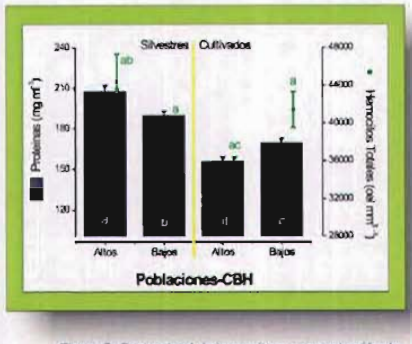

Figura 5. Conteo total de hemocitos y concentración de proteína en la hemolinfa de juveniles de *L. vannamei*, silvestres y cultivados, alimentados con bajos y altos CBH (3 y 44 %). Promedio ± ES. Letras distintas indican diferencia significativa (P<0.5) entre los tratamientos.

Conclusiones

Los procesos de selección normalmente aplicados en las granjas camaronicolas se basan principalmente en el crecimiento. Los resultados obtenidos con los camarones cultivados señalan un metabolismo en base a las proteínas, mientras que los componentes de la hemolinfa de los organismos silvestres indican un mejor aprovechamiento de otros nutrientes como CBH y lípidos. Los resultados reflejan que el proceso de domesticación de *L vanamei* a provocado cambios a nivel fisiológico y señalan la diversidad de la respuesta metabólica de los organismos silvestres.

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Indicadores de la condición fisiológica del camarón blanco *Litopenaeus vannamei*

Pascual Jiménez, Cristina; Sánchez Arteaga, Ariadna; Rosas Vázquez, Carlos y Gaxiola Cortés, Gabriela. Unidad Multidisciplinaria de Investigación y Docencia, Fac. Ciencias, UNAM, en Sisal, Yucatán, México.

Introducción

El cultivo de camarón representa una buena alternativa ante la disminución de la pesca en altamar, y ha ido adquiriendo mayor participación en el suministro del producto, ante un mercado mundial en crecimiento.

El camarón blanco del Pacífico, *Litopenaeus vannamei*, es la segunda especie más importante que se produce a nivel mundial y la más extensamente utilizada para su cultivo en América. No obstante para mantener e incrementar su producción, es necesario controlar la presencia de enfermedades.

El establecimiento de un cuadro infeccioso es altamente dependiente de la condición fisiológica de los animales. En este sentido, un mayor conocimiento de la bioquímica sanguínea de los camarones permitiría identificar las mediciones que pudieran proporcionar mayor información a cerca del estado de salud y la susceptibilidad de los organismos ante las condiciones de cultivo, alguna enfermedad o su estado nutricional. Por lo cual, el objetivo del presente estudio fue determinar si los componentes de la sangre (hemolinfa) reflejan la condición fisiológica de los juveniles de camarón asociados al estado nutricional (5, 15 y 40 % de proteínas dietéticas) y tipo de cultivo (condiciones controladas, 90 L y escala piloto comercial, 20 t). Al igual que en clínica humana, se evaluaron los metabolitos plasmáticos (proteínas, lactato y glucosa), la concentración del pigmento respiratorio (hemocianina) y el conteo y caracterización de células circulatorias (hemocitos).

Diseño experimental y métodos

Estado Nutricional

Tabla 1. Alimento experimental con tres niveles de proteínas (bajo, medio y óptimo).

Dietas experimentales	5 %	15%	40 %
Harina calAzochoveta	5.4	16.8	45
Harina de Calamar	1.25	3.75	10
Harina de Soya	2.25	6.75	18
Lecitina de Soya	2	2	2
A. de Hígado Bacalao	4.3	3.2	2.2
Vitaminas	1.7	1.7	1.7
Minerales	0.8	0.8	0.8
Colesterol	0.2	0.2	0.2
Ravimix-C	0.0266	0.0266	0.0266
Almidón de Trigo Nativo	60.91	49.54	19.28
Relleno	19.96	14.23	0
Alginato	1	1	1

El experimento tuvo una duración de 50 días, con un diseño completamente aleatorizado, con 6, 8 y 10 réplicas para el tratamiento de **40, 15 y 5 % de proteínas** respectivamente (Tabla 1). En cada una de ellas se mantuvieron 10 juveniles por tanque (90 litros), el sistema de agua de mar fue de flujo constante: temperatura de 28 ± 1°C, OD mayor a 5 mg/l, pH 7.9 a 8.2 y salinidad de 35 UPS.

Al termino de los experimentos se obtuvo la hemolinfa con un jeringa de 1 ml. Para el conteo de hemocitos la hemolinfa se mezcla con solución Alsever (10 % formol). La caracterización fue morfológica. La oxihemocianina se midió con el espectrofotómetro considerando su coeficiente de extinción. Los metabolitos se evaluaron en plasma, por lo cual, se mezcla la hemolinfa con una solución anticoagulante (1:3) y se centrifugó (800 g/3min).

Se utilizaron kits comerciales: proteínas por método Bradford (Biorad cat. 500-0006); glucosa (Bayer Sero Psk Plus cat. B014509-01) y lactato (Sigma-cat. 735). El estadio de muda fue determinado por el desarrollo de las setas y se utilizaron para el análisis solamente a camarones en intermuda.

Tipo de cultivo

Bajo **condiciones de laboratorio** (90 l) los camarones fueron alimentaron por 40 días con alimento formulado, otorgado a una tasa del 13 % del peso húmedo por día (Tabla 2). Los parámetros fisicoquímicos fueron muy constantes: temperatura de 28 ± 1 °C, OD mayor a 5 mg/l, pH > 8.0 y a una salinidad entre 32 a 36 UPS.

Tabla 2. Alimento utilizado para condiciones controladas.

Ingredientes	g/kg
Pasta de Soja	480
Levadura	340
Aceite de sardina	20
Aceite de soja	73
Celulosa	34
Premix (vitaminas)	20
Lecitina de Soja	1
Aceite de Bacalao	2
Na_2HPO_4	6
CaH_2PO_4	2
$CaCO_3$	1
$MgCl_2$	3
Composición g/kg	
Carbohidratos	21.7
Proteínas	45
Lípidos	10
Humedad	12.1

En **condiciones cercanas a cultivo comercial** (20 t) los camarones fueron alimentados por ±10 días con alimento comercial (35 % de proteína, Api camarón ultra de Malta Clayton®) suministrado a una tasa del 5 % del peso húmedo por día. Los parámetros fisicoquímicos fueron muy similares en ambos dispositivos, excepto por la temperatura que osciló entre 27 a 29 °C.

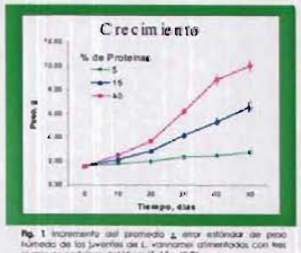


Fig. 1 Incremento del promedio ± error estándar de peso húmedo de los juveniles de *L. vannamei* alimentados con tres niveles de proteínas dietéticas (5, 15 y 40 %).

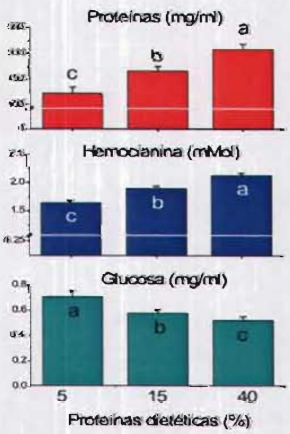


Fig. 3 Componentes de la hemolinfa (promedio ± E.S.) de juveniles de *L. vannamei* alimentados con tres niveles de proteínas dietéticas (bajo, medio y óptimo, respectivamente). Diferentes letras denotan diferencia significativa ($P < 0.05$).

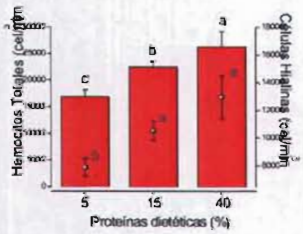


Fig. 2 Conteo total de hemocitos y células hialinas (promedio ± E.S.) de juveniles de *L. vannamei* alimentados con tres niveles de proteínas dietéticas (bajo, medio y óptimo, respectivamente). Diferentes letras denotan diferencia significativa ($P < 0.05$).

Resultados y Discusión

Tabla 3. Estadística descriptiva y prueba de normalidad de los componentes de la hemolinfa en juveniles mantenidos en estanques piloto comerciales (A) y condiciones controladas (B). Diferencias estadísticas a $P < 0.05$

	N	Promedio	Desviación Estándar	Mediana	Rango cuartil	Prueba de normalidad KS_d
Hemocianina [Hc], mmol/l						
A	70	1.93	0.33	1.98	0.48	0.02
B	83	1.52	0.39	1.59	0.55	0.001
A+B						
Proteínas, mg/ml						
A	60	217.5	47.08	224.2	64.19	0.01
B	211	102.8	17.4	98.2	19.2	0.2
A+B						
Glucosa, mg/ml						
A	213	0.42	0.16	0.38	0.21	0.0001
B	167	0.31	0.15	0.28	0.17	0.001
A+B						
Lactato, mg/ml						
A	205	0.14	0.13	0.09	0.10	0.0001
B	319	0.15	0.15	0.10	0.10	0.0001
A+B	532	0.15	0.14	0.11	0.11	0.0001

En el presente estudio el crecimiento, la condición nutricional y la respuesta inmune de los camarones presentaron el mismo patrón de resultados. El peso, los hemocitos, la hemocianina y las proteínas fueron significativamente más altos en juveniles alimentados con 40 % de proteínas dietéticos. En los camarones cultivados a escala piloto comercial los niveles de hemocianina, proteínas y glucosa fueron significativamente mayores que en condiciones controladas, lo cual se atribuyó a la productividad natural de los estanques. Estos resultados concuerdan con investigaciones anteriores donde se señala al metabolismo de proteínas como la clave para entender el manejo y destino de la energía en los camarones.

La hemolinfa constituye la vía de transferencia de energía, de intercambio de información para la regulación metabólica, el transporte de oxígeno y también, es la sede principal del sistema inmunológico de los camarones, por lo cual, los estudios sobre la dinámica se sus componentes pueden ser de gran importancia para entender las adaptaciones fisiológicas al proceso de domesticación y el tipo de cultivo. Las evaluaciones utilizadas en el presente estudio son sencillas y relativamente de bajo costo, y además, la obtención de la hemolinfa puede realizarse sin sacrificar al camarón (camarones con mas de 2 g), cumpliendo así, muchas de las características de la evaluaciones utilizadas para diagnóstico clínico.

Conclusiones

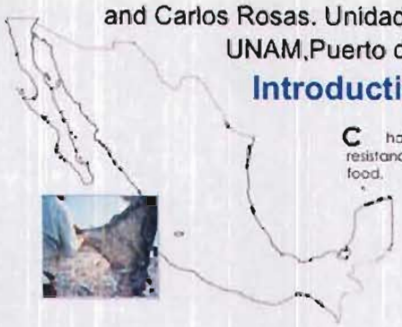
Los componentes de la hemolinfa reflejan el estado nutricional e inmunológico de los juveniles de *Litopenaeus vannamei*, por lo cual, pueden ser utilizados como indicadores del estado de salud en los programas de monitoreo y control de enfermedades en las granjas camaronícolas.

Energetic metabolism and immune response of *Litopenaeus vannamei* juveniles to dietary protein: feeding and starving conditions.

VII Simposio Internacional De Nutrición Acuícola

Cristina Pascual, Ariadna Sánchez, Jaime Suárez, Gabriel Taboada, Gerard Cuzon, Gabriela Gaxiola

and Carlos Rosas. Unidad Multidisciplinaria de Investigación y Docencia, Fac. Ciencias, UNAM,Puerto de abrigo, Sisal, Yuc. México. crispas6@yahoo.com



Introduction

Challenges of shrimp industry are directly related with resistance disease, good growth and low cost of artificial food.

Protein metabolism (requirement, use and turnover) is fundamental to the growth and immune condition of shrimp.

Therefore, the objective of the present study was to investigate the effect of dietary protein (5, 15 and 40%) on energetic metabolism, reserves management and immune response of *Litopenaeus vannamei* juveniles in feeding and starving conditions.

Methods

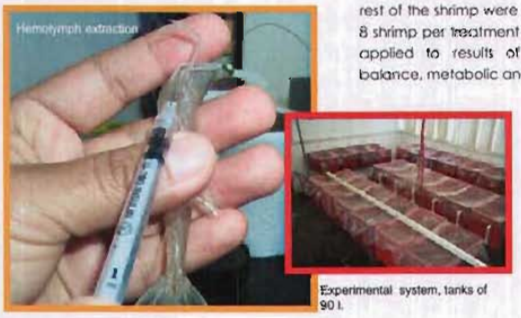
A group of 150 shrimp (1.59 + 0.07 g wet weight) were used in the first experiment. Shrimp were reared for 50 days in 90-l tanks (10 shrimp/tank) and fed different protein levels: 5, 15 and 40 % (Table 1). The effects of protein level on energetic balance were recorded by ingestion rate (I), respiratory rate (R) and biomass production of organism during its growing process (P). Energy lost from feces (H) and urine products (U) was calculated as (H+U)=I-R-P and assimilated energy (As) as R+P (1).

At second experiment 180 shrimp were reared for 21 days (60 shrimp in 200-l tank), and fed with two protein levels (5 and 40 %). After that time, blood metabolites (protein, glucose, cholesterol), oxy-hemocyanin (OxyHc), glycogen and total lipids of digestive gland (DG), and immune response (prophenoloxidase activity, respiratory burst, hemocytes count), were measured in 15 shrimp per treatment. Only shrimp in intermolt stage (C stage) were used.

These evaluations were considered as initial fasting values. The rest of the shrimp were not fed by other 21 days. During this time, 8 shrimp per treatment were sampled every 7 days. ANOVA was applied to results of growth rate, survival, and energetic balance, metabolic and immunological parameters.

Table 1. Composition of experimental diet (g kg^{-1})
Total energy of the three diet: 16 kJ g^{-1}

Experimental Diet	5 %	15 %	40 %
Anchovy fish meal	56	168	450
Squid meal	12.5	37.5	100
Soy bean meal	22.5	67.5	180
Soy bean lecithin	20	20	20
Cod liver oil	43	32	22
Vitamins	17	17	17
Minerals	8	8	8
Cholesterol	2	2	2
Rovimix-C	0.286	0.286	0.286
Native wheat flour	609.1	495.4	192.8
Filler	199.6	142.3	0
Alginate Na	10	10	10



Experimental system, tanks of 90 l.

Fig.1 Weight of Digestive gland and body of *L. vannamei* maintained in starving (21 days) and feeding previously with 5 and 40 % protein diet for three weeks.

Results and Discussion

Table 2. Energy balance of *L. vannamei* juveniles fed different dietary protein levels.

Energy Balance			
Proteins diet (%)	**5**	**15**	**40**
Ingestion rate, (I) J day^{-1} g^{-1} dw	3298 ^{a}	1954 ^{b}	1427 ^{c}
Respiration R_{ASS} J day^{-1} g^{-1} dw	6.86 ^{a}	49.7 ^{c}	60.1 ^{b}
Respiration R_{EXT} J day^{-1} g^{-1} dw	438.4 ^{a}	249.6 ^{b}	171.6 ^{c}
$R = R_{EXT} + R_{ASS}$	445.3 ^{a}	299.4 ^{b}	231.6 ^{c}
Production (P) J day^{-1} g^{-1} dw	155 ^{c}	664 ^{b}	1115 ^{a}
Assimilation (P+R)	600	964	1347
Assimilation efficiencies, As/I %	18 ^{b}	49 ^{b}	94 ^{a}
Feces energy content J g^{-1} dw	6243 ^{a}	6999 ^{b}	11809 ^{a}
R/As %	74	31	17
P/As %	25	68	82

Means with superscript letters in same row are statistical different.

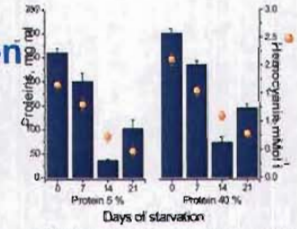


Fig.2 Blood protein and hemocyanin of *L. vannamei* maintained in starving for 21 days.

Growth of *L. vannamei* was high in shrimp fed 40% dietetic protein in comparison to shrimp fed 5 and 15%. An inverse relation between wastes (H+U) and dietary protein level was observed indicating that shrimp lost 81% of ingested energy when fed 5% protein, and only 5.6% when fed 40% (Table 2).

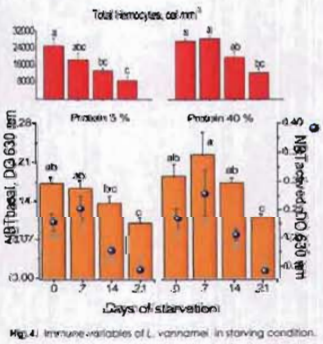


Fig.4 Immune variables of *L. vannamei* in starving condition.

The two weeks of fasting decreases significantly the hemolymph components (proteins and OxiHc) (Fig. 2), and reserves (glycogen and total lipids in DG) (Fig. 3). Shrimp fed with optimal protein diet (40%) presented significantly higher values of body and digestive gland weight (Fig. 1) and immune variables (hemocytes count and respiratory burst), before and after starving period (Fig. 4).

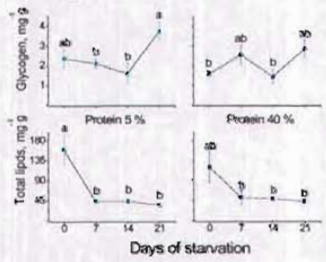


Fig. 3. Reserves of juveniles in starving condition (21 days).

Conclusions

Compensatory mechanism used by *L. vannamei* to respond sub-optimal dietary protein level (5 and 15 %) induced not only a severe reduction in growth rate and assimilation efficiency but also in immune capacities.

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