



Avances en el estudio de la bacteria de la necrosis hepatopancreática (NHP)



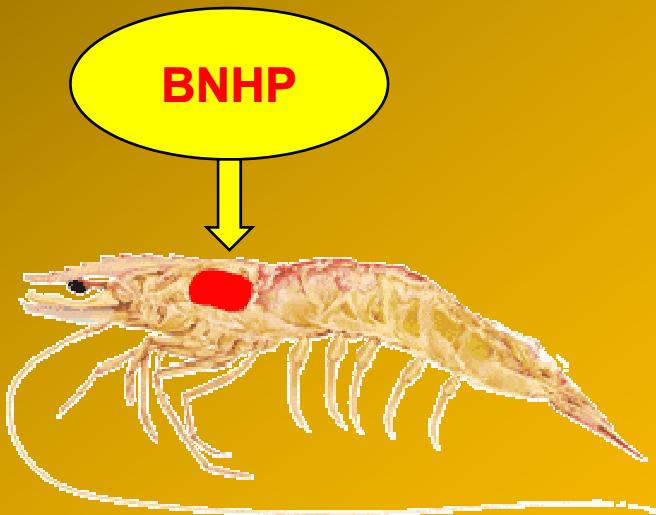
Dra. Teresa Gollas Galvan

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CIAD Hermosillo*

Necrosis Hepatopancreática (NHP)



Agente causal



Mortalidades 95 %

Litopenaeus vannamei
Litopenaeus setiferus
Litopenaeus stylirostris
Farfantopenaeus aztecus
Farfantopenaeus californiensis

Frelier, P. F., Sis, R. F., Bell, T. A., Lewis, D. H. 1992. Microscopic and ultrastructural studies of necrotizing hepatopancreatitis in pacific white shrimp (*Penaeus vannamei*) cultured in Texas. *Vet. Pathol.* 29, 269-277.

the Texas pond mortality syndrome. Though a definitive cause for the disease is unknown, recent studies suggest a bacterial etiology. Two intracellular Gram-negative bacteria have been consistently associated with this disease (Krol *et al.*, 1991; Frelier *et al.*, 1992).

BNHP

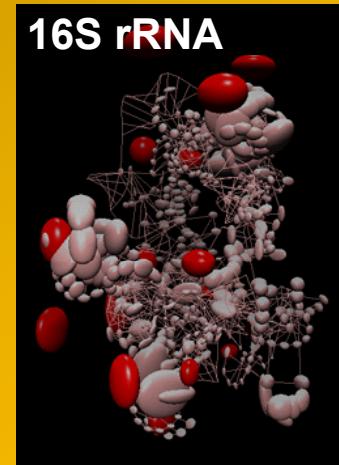
- Gram negativa
- Intracelular
- Pleomórfica



Krol y cols., 1991; Frelier y cols., 1992; Lightner y cols., 1992; Lightner, 1996



Frelier y cols., 1993

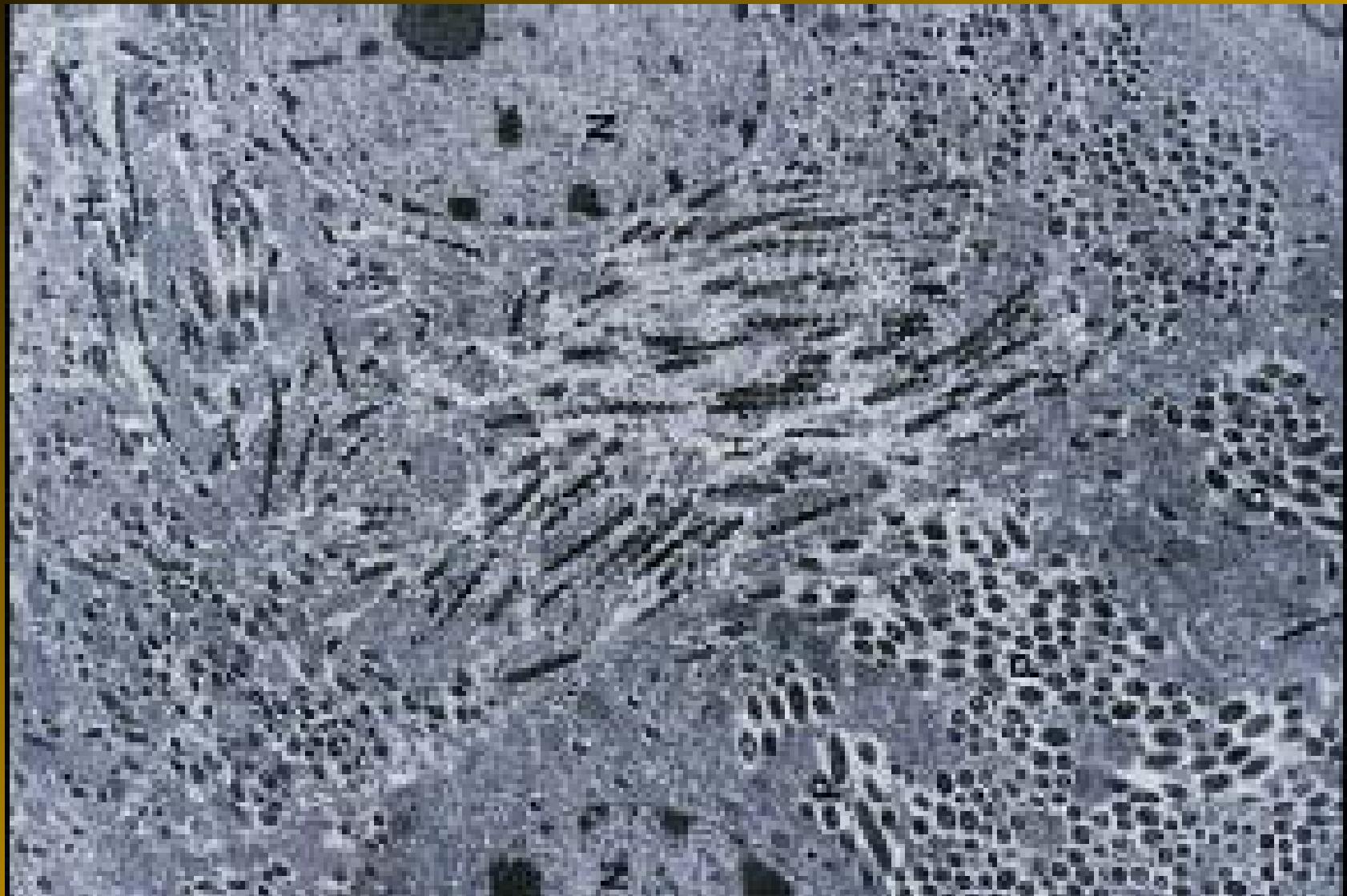


α -Proteobacterias
Loy y cols., 1996

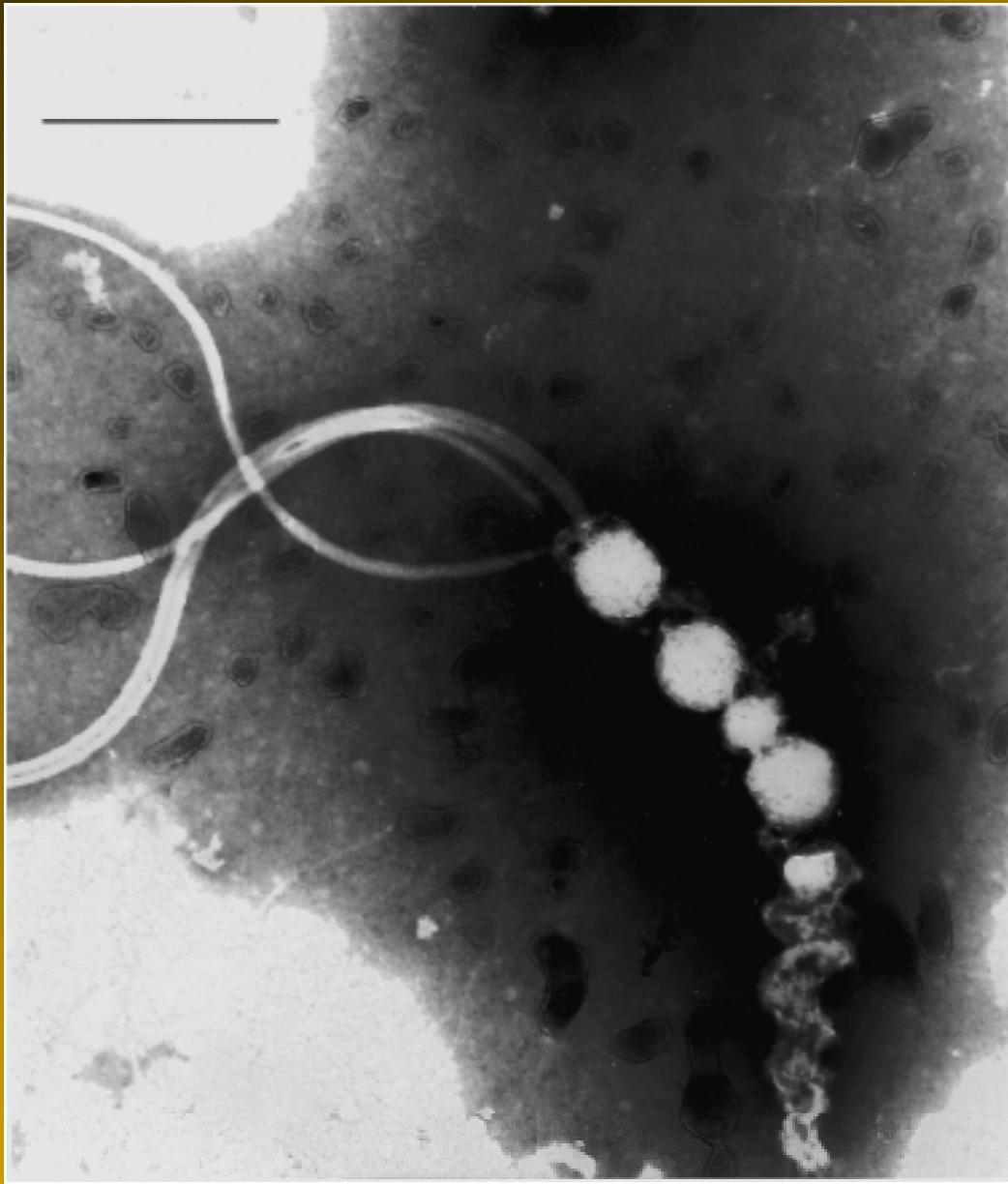
NOTE

Morphological evidence for a single bacterial etiology in Texas necrotizing hepatopancreatitis in *Penaeus vannamei* (Crustacea: Decapoda)D. V. Lightner¹, R. M. Redman¹, J. R. Bonami²

Discussion. Morphological characteristics presented here for the agent of TNHP support the theory that a single intracellular bacterium is present in shrimp hepatopancreatocytes with the disease. This is contrary to the conclusions reached in earlier descriptive papers by Krol et al. (1991) and Frelier et al. (1992), in which the morphological forms illustrated in the present paper were considered to represent at least 2 different species of intracellular bacteria. Our findings also support the contention of Frelier et al. (1992) that the helical form is not a spiroplasm belonging to the class Mollicutes, but rather that it is a true Gram-negative bacterium.

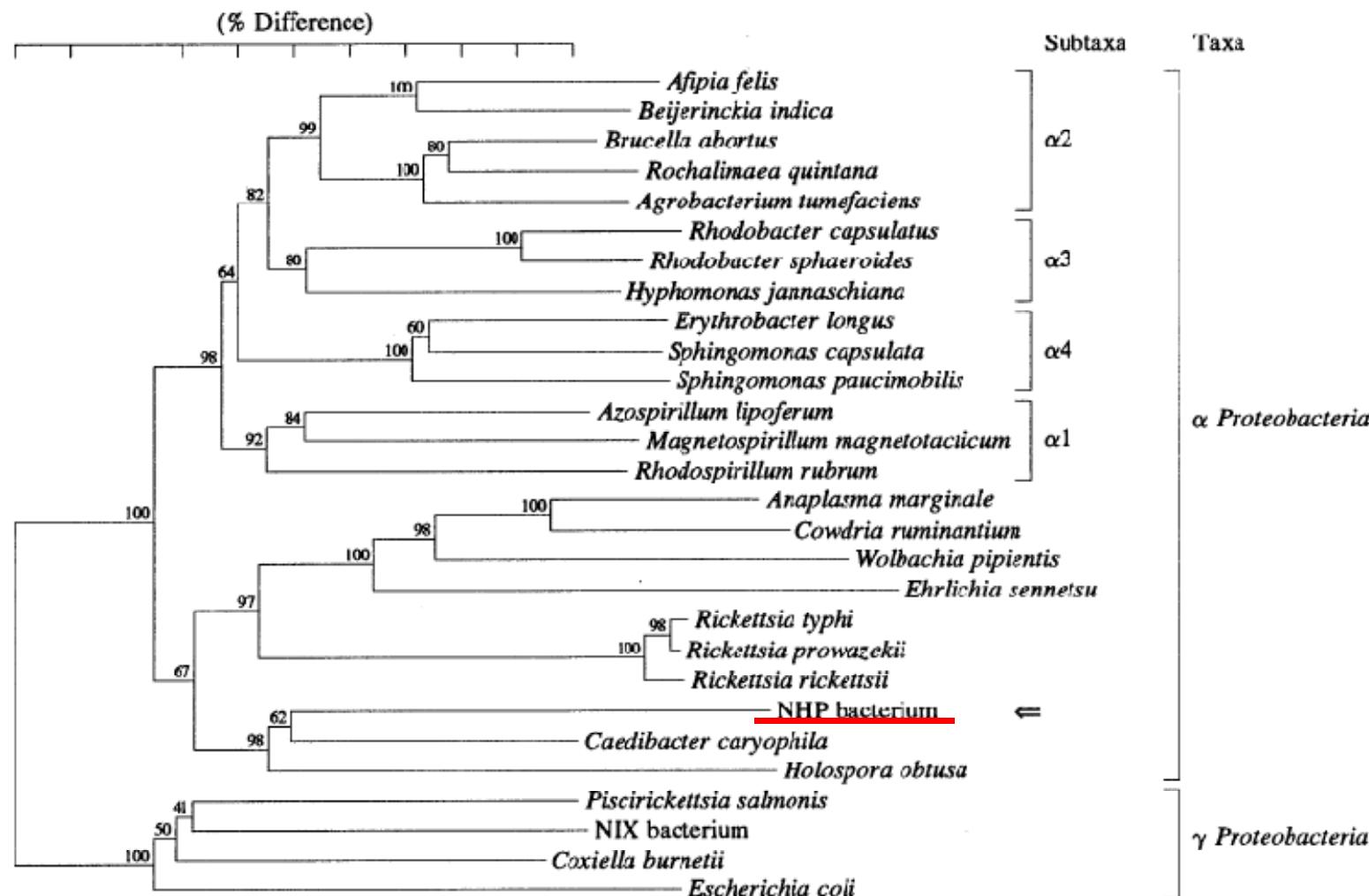




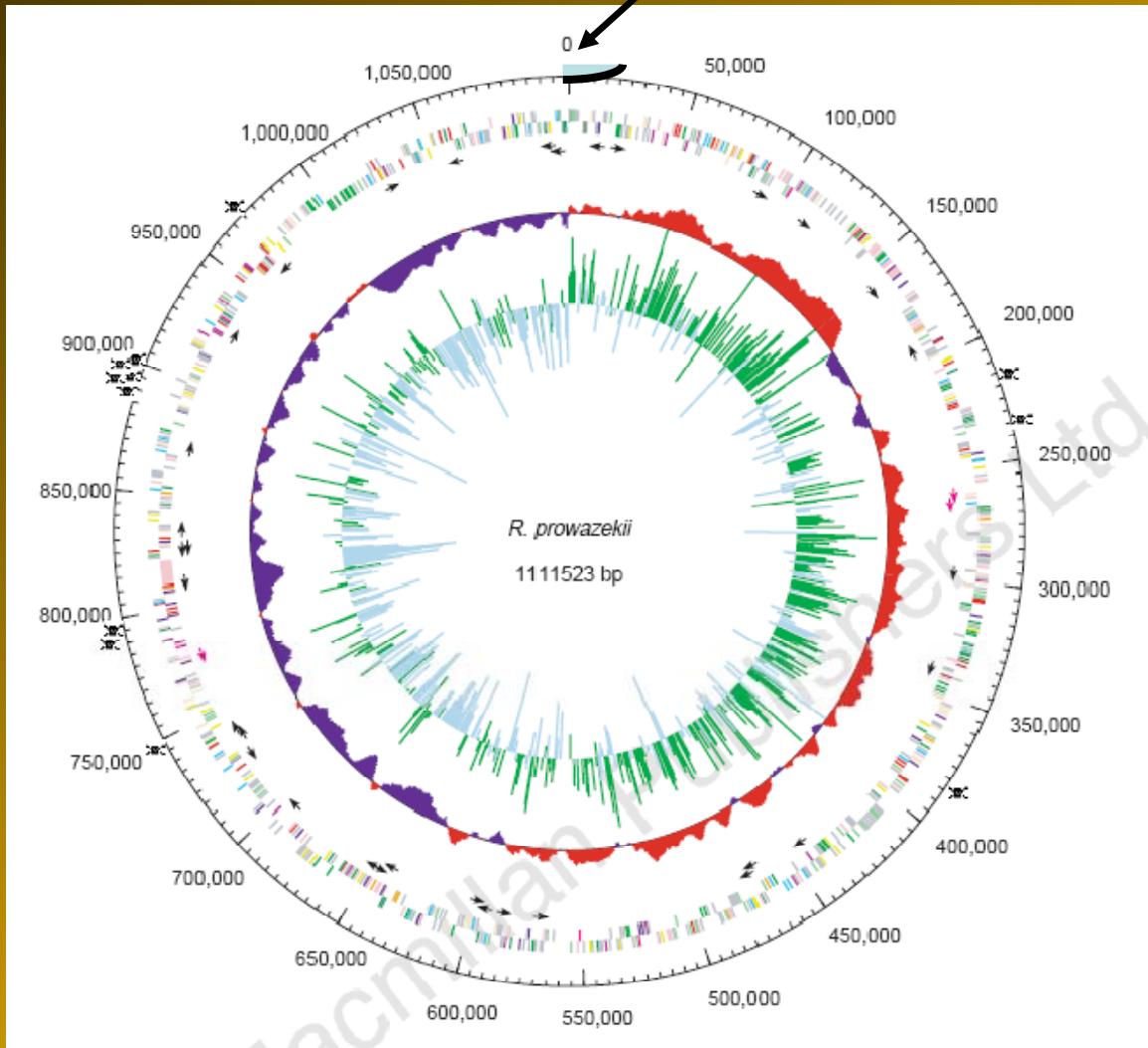


Molecular Phylogeny and In Situ Detection of the Etiologic Agent of Necrotizing Hepatopancreatitis in Shrimp

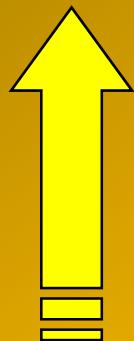
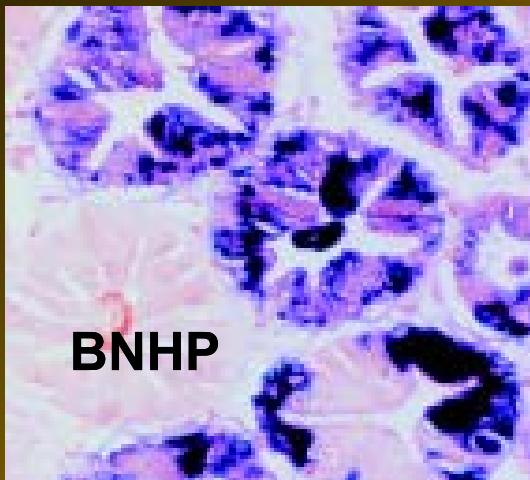
JAMES K. LOY,^{1*} FLOYD E. DEWHIRST,² WILLIAM WEBER,³ PAUL F. FRELIER,¹
THEODORE L. GARBAR,¹ SERBAN I. TASCA,¹ AND JOE W. TEMPLETON¹



Secuencia reportada para NHP



Signos clínicos



Temperaturas (29 a 35°C)
Salinidades 20 a 38 ppm
Hembras ablacionadas

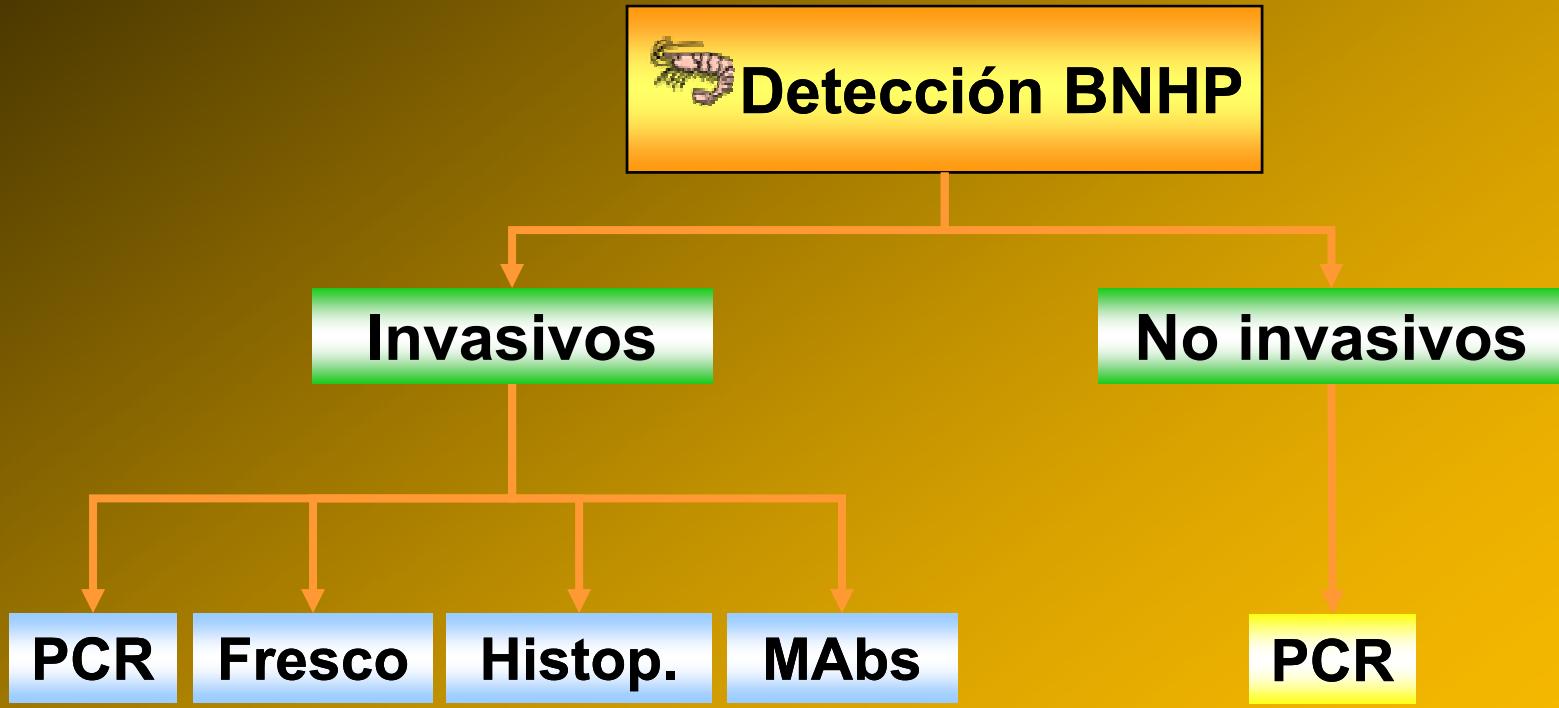
Effect of salinity on transmission of necrotizing hepatopancreatitis bacterium (NHPB) to Kona stock *Litopenaeus vannamei*.

Amanda G. Vincent, Jeffrey M. Lotz*

Department of Coastal Sciences, The University of Southern Mississippi, Gulf Coast Research Laboratory, PO Box 7000,
Ocean Springs, Mississippi 39566-7000, USA

The NHPB was transmissible regardless of salinity: nearly a quarter of susceptible shrimp exposed to NHPB at the lowest (10 per thousand) and highest (40 per thousand) salinity examined acquired NHPB. Transmission rates were highest at the intermediate salinities of 20 and 30 per thousand, suggesting that those salinities are optimal for NHPB transmission. The observed association between high salinity and NHP outbreak in a shrimp pond is not explained by these results because reduced transmission occurred at very low and very high salinities.

Métodos de diagnóstico para NHP



Briñez y cols., 2003

Diagnóstico

Presuntivo (Apariencia y análisis en fresco)

- Cutícula blanda, melanizada, pleópodos negros.
- HP atrofiado, blancuzco, melanizado, acuoso.
- Reservas bajas de lípidos.
- Atrofia de la mucosa epitelial del HP, túbulos melanizados.



Diagnóstico

Confirmatorio

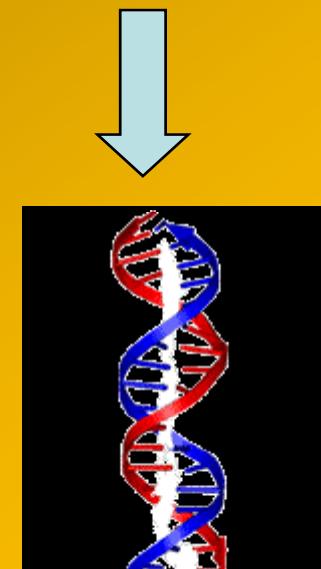
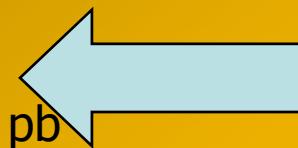
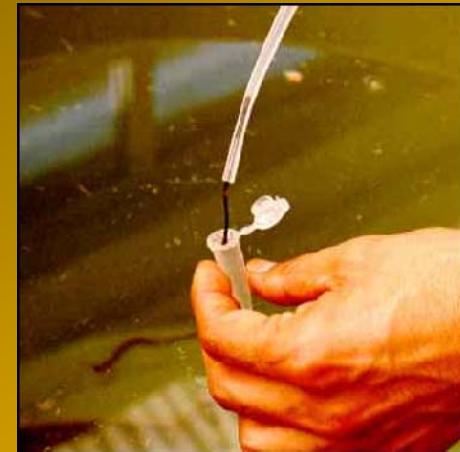
- Histología por Heosina & Ematosilina, Giemsa o tinción de Steiner & Steiner.
- Hibridación *in situ* con sondas de DNA específicas.
- Por medio de PCR.

NOTE

Fecal samples as DNA source for the diagnosis of Necrotizing Hepatopancreatitis (NHP) in *Penaeus vannamei* broodstock

Boris Bríñez, Fernando Aranguren, Marcela Salazar*

Análisis no invasivo por medio de PCR



Presencia de NHP y WSSV en granjas de Sonora

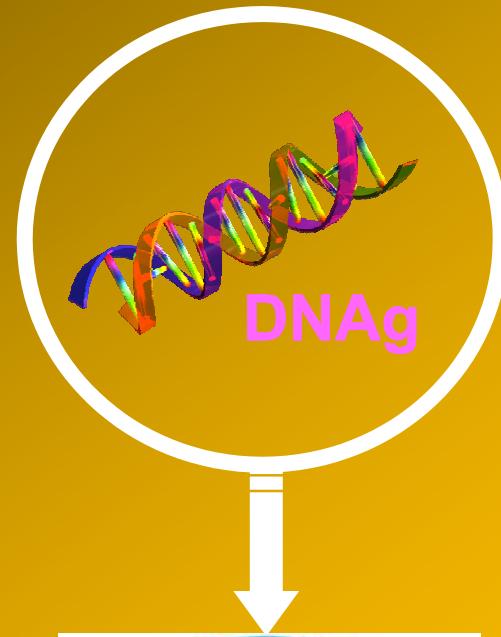


2005
128 granjas

73 NHP (Necrosis
Hepatopancreática)



Estandarización del Método de Detección



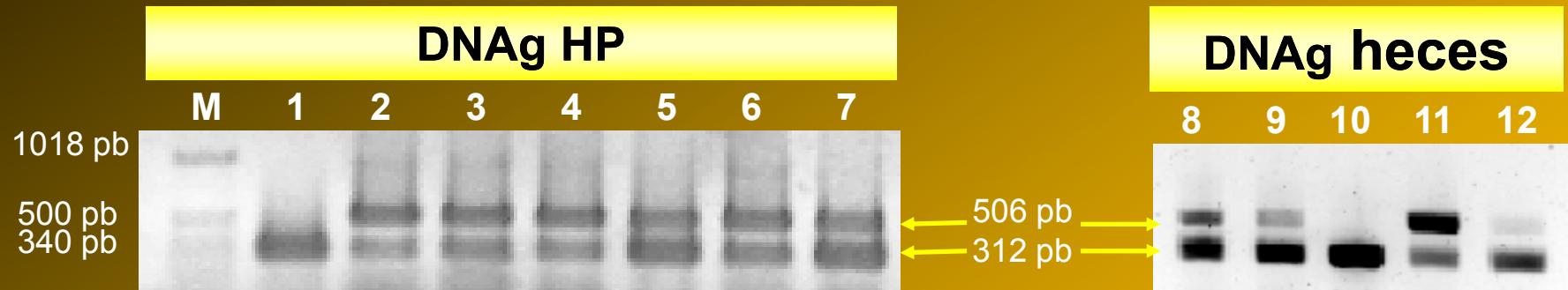
Sistema simple iniciadores NHP, DiagXotics® Inc.

Iniciadores diseñados (Loy y cols., 1996)

PCR

Iniciadores DiagXotics® Inc.

Temperatura alineación = 60°C



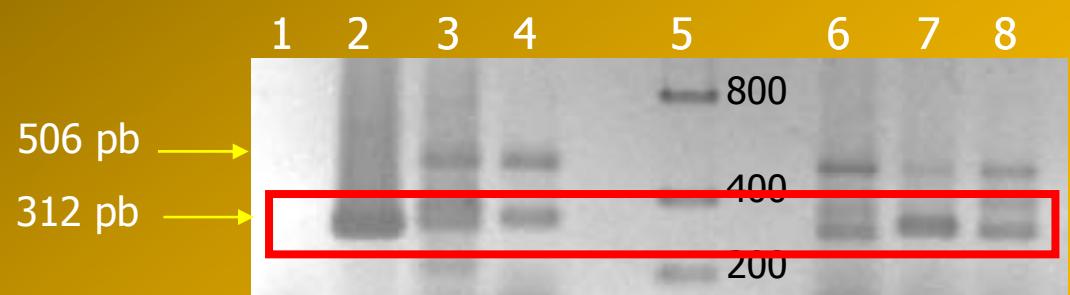
Detección de la BNHP por PCR. M) Marcador de PM. 1) Control positivo BNHP (DiagXotics®), 2 - 4) DNAg de HP sanos, 5 - 7) DNAg de HP con NHP, 8 y 9) DNAg heces de camarones sanos, 10) control positivo BNHP, 11 y 12) DNAg de heces de camarones con NHP.

Reacciones Inespecíficas PCR invasivo y no invasivo

Reacciones inespecíficas

Sistema simple iniciadores NHP,
DiagXotics® Inc.

- 1) Control (-) NHP
- 2) Control (+) NHP
- 3) *Vibrio spp*
- 4) *M. lysodeikticus*,
- 5) Marcador de PM
- 6) Zooplacton
- 7) Probiótico
- 8) *E. coli*.



Corte de bandas para clonación



Sistema de Purificación GFX™ DNA bandas gel,
Amersham Biosciences

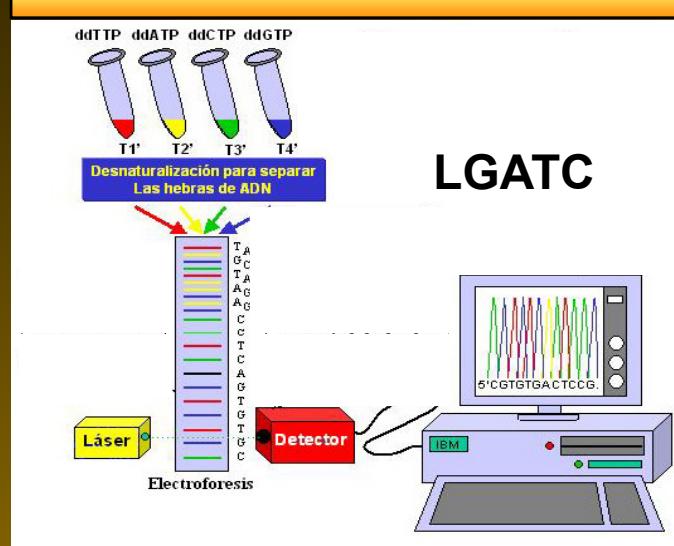


Sistema de Purificación GFX™ DNA bandas gel,
Amersham Biosciences



Secuenciación

Secuenciación automática

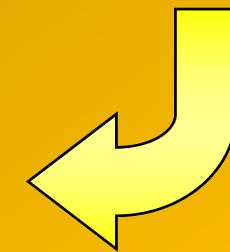
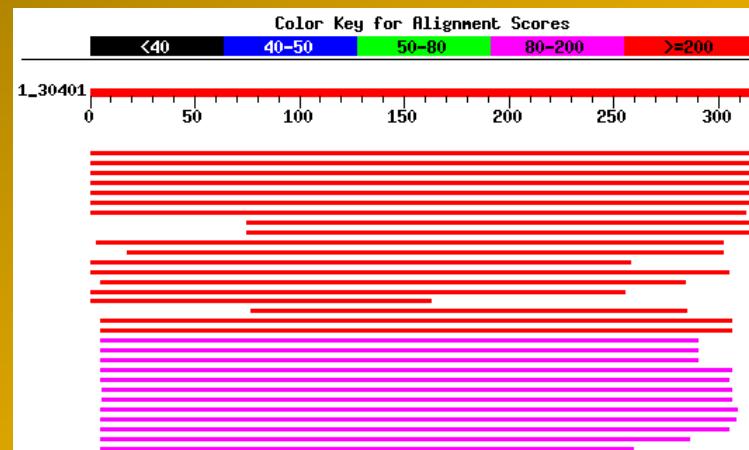


LGATC



- Blast Seq2
- Blast N
- Genomic Blast

Sanger y cols., 1997



24

Altschul y cols., 1990

Reacciones inespecíficas

**Secuencia parcial, gene RNAr 16S α -Proteobacteria
no identificada (bases 1 - 1415)**

GenBank U65509

3' GAATGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGCAATAGGTTGCCATTGAGTGGCAGACGGG
TGAGTAATACACGGGAATCTACCTCTAGGTAAGGAATAGCTACGGGAAACTGTAGGTAATACCTTATAATATGTGC
AAACATCAAAGATTATCGCCTAGAGAGGGAGCCGTGCCAGATTAGGTCGTTGGTGGGTAACAGCCTACCAAGC
CTATGATCTGTAGTTGGTCTGAGAGGGATGATCAGCCACACTGGGACTGAGAACGGCCCAGACTCCTACGGGGGGC
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TTCTTAAGGCGATGAGCTAACAGCAATGGAGGCAGTCGACCACAGAAAAGGTGATGACTGGGGTT 5'

Reacciones inespecíficas

Organismo	% Identidad	Región secuencia U65509	Región secuencia problema	Error
<i>E. coli</i>	89%	264-372	1-110	2e -27
<i>M. lysodeikticus</i>	87%	190-372	141-324	3e -51
<i>Vibrio spp.</i>	80%	191-372	185-1	1e -28
Zooplacton	92%	190-372	184-1	7e -67
Control positivo BNHP	99%	7-372	19-312	0

Comparaciones de secuencias nucleotídicas de microorganismos contra la secuencia U65509, reportada de BNHP

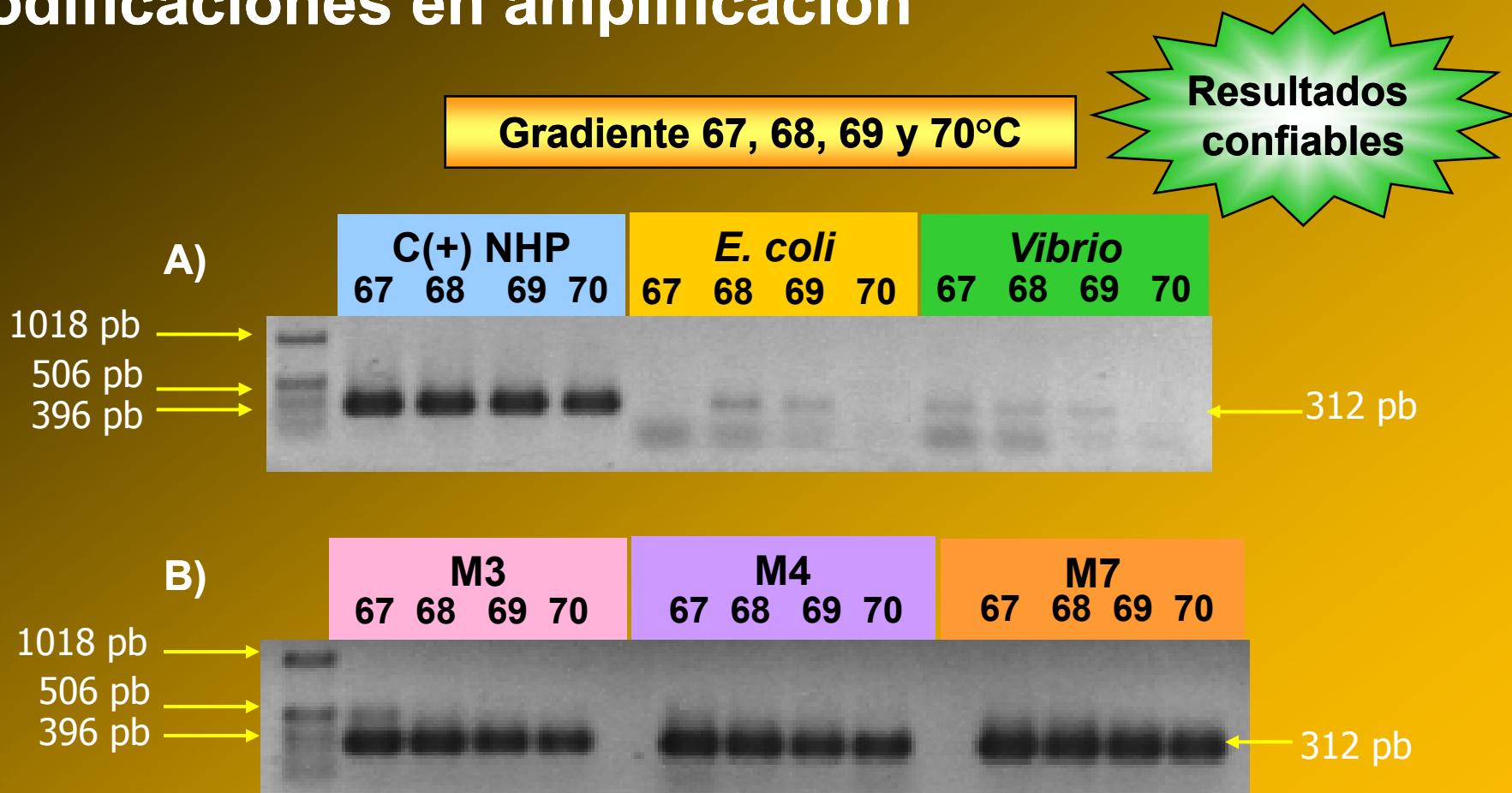
Genes ribosomales ↑↑ Conservados

Mathews y cols., 2003; Sambrook y Russell, 2001

Vibrios
Flora normal estanques e intestinal
Leaño y cols., 1998



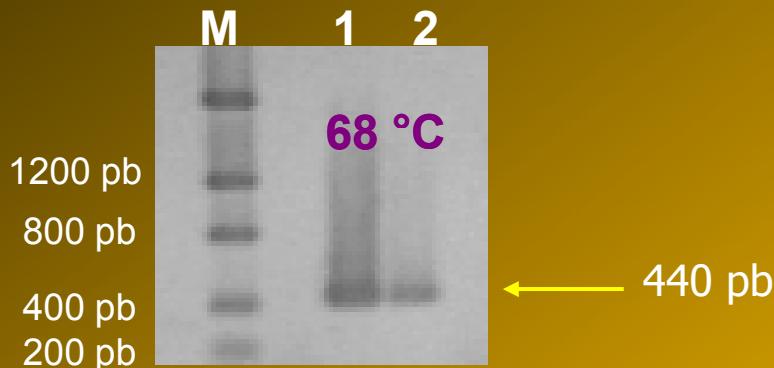
Modificaciones en amplificación



Gradientes de temperatura de alineación en PCR con iniciadores DiagXotics®. A) DNAg de *E. coli* y *Vibrio spp.* B) DNAg M3, M4 y M7 de camarones infectados con NHP.

Modificaciones en amplificación

Iniciadores diseñados pf-1 y pr-1



Detección de la BNHP con iniciadores pf-1 y pr-1. 1)Marcador PM, 1 y 2) Muestras positivas NHP

Temperatura alineación 58 °C a 68 °C

Loy y cols., 1996; Briñez y cols., 2003; Vincent y cols., 2004

Estandarización de métodos de diagnóstico



Prevenir diagnósticos falsos positivos

Evitar \$\$ innecesarios en OTC



Evaluación del estado fisiológico del camarón Infectado con NHP



Estudios sobre modelos de infección NHP



BNHP gradiente @

Frelier y cols., 1993



Extracción HP

Proliferación
Agudo
Crónico

Vincent y cols., 2004

Experimental infection of Pacific white shrimp *Litopenaeus vannamei* with Necrotizing Hepato-pancreatitis (NHP) bacterium by per os exposure

Amanda G. Vincent, Verlee M. Breland, Jeffrey M. Lotz*

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Ocean Springs, Mississippi 39566-7000, USA

Time course of necrotizing hepatopancreatitis (NHP) in experimentally infected *Litopenaeus vannamei* and quantification of NHP-bacterium using real-time PCR

Amanda G. Vincent, Jeffrey M. Lotz*

Department of Coastal Sciences, The University of Southern Mississippi, Gulf Coast Research Laboratory, PO Box 7000,
Ocean Springs, Mississippi 39566-7000, USA

Clasificación del desarrollo de la infección por NHPB

Grado I Fase inicial

No se observa atrofia del HP, pero los organismos presentan deformación tubular y estrangulamiento de los túbulos

Grado II Fase aguda

Atrofia del HP, mayor desprendimiento celular, células con nucleos hipertrofiados, coloración pálida y necrosis.

Grado III Fase crónica

Se observa una mayor cantidad de nódulos hemocíticos melanizados

Modelo de Infección



Alimentación forzada
Homogenizados de HP + BNHP



15 camarones

40 μ L inóculo

Control
Tejido infectado
Asegura reproducción

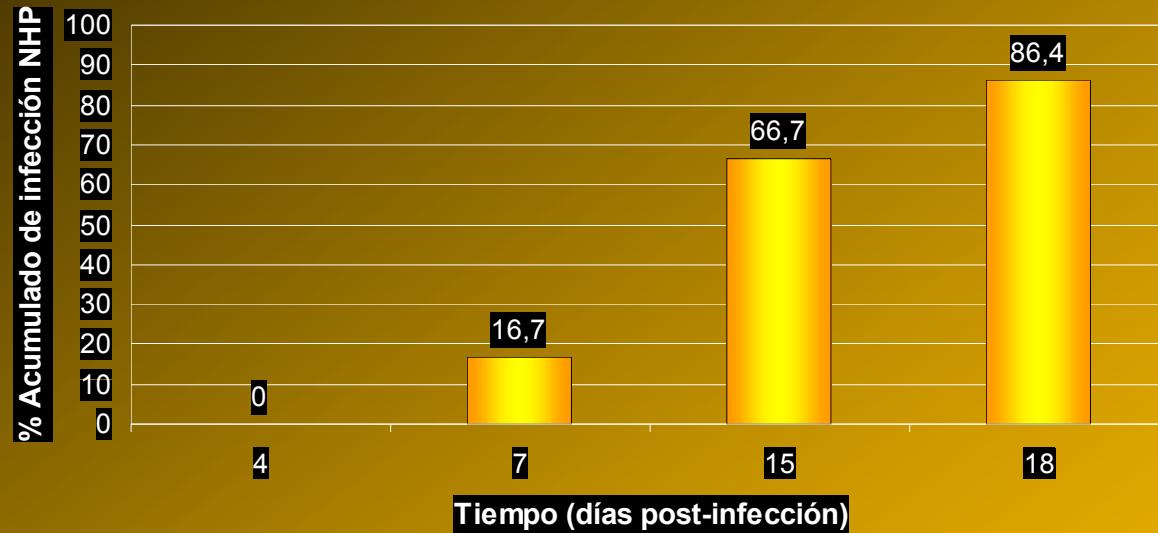
(Avila Villa 2006)

Infección experimental de organismos

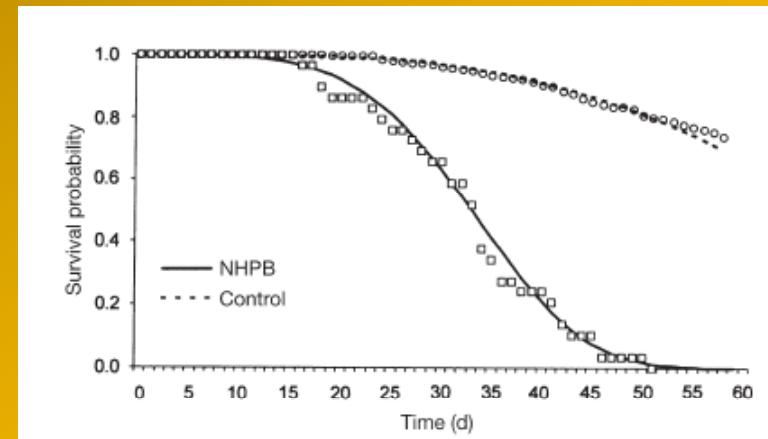


Control: Realimentación con 40 µL homogeneizado de HP sano
Infectado: Reinfección con 40 µL inóculo

Infección experimental de organismos



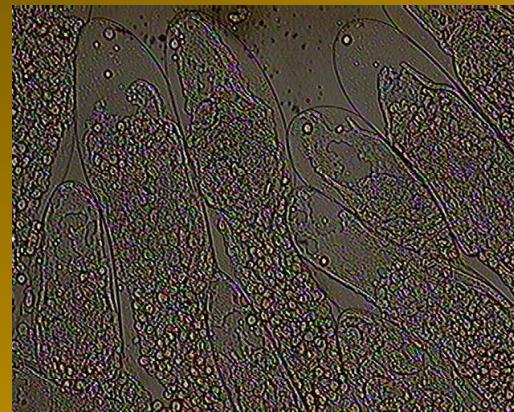
No mencionan detección
temprana de BNHP



Grado de infección en los túbulos del hepatopáncreas por análisis en fresco



Necrosis



Disminución de vacuolas
lipídicas



Estrangulamiento de
túbulos

Grado II (Fase aguda)



Día 19

NOTE

Preservation of necrotizing hepatopancreatitis bacterium (NHPB) by freezing tissue collected from experimentally infected *Litopenaeus vannamei*

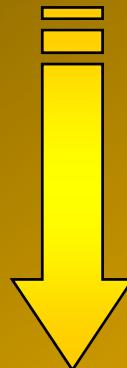
Brenda G. Crabtree¹, Matthew M. Erdman¹, D. L. Harris^{1,2,*}, Isabel Turney Harris¹

¹Department of Animal Science and ²Veterinary Diagnostic and Production Animal Medicine, Iowa State University,
11 Kildee Hall, Ames, Iowa 50011, USA

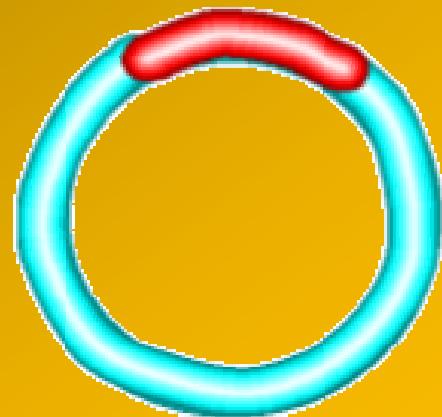
ABSTRACT: Necrotizing hepatopancreatitis (NHP), a severe disease of penaeid shrimp, is caused by bacteria (NHPB) that have previously been demonstrated to reside in tubular epithelial hepatopancreatic (HP) cells of infected shrimp. There has yet to be a successful *in vitro* culture method to grow the intracellular organism; therefore, it must be propagated *in vivo* via transmission from NHPB-infected shrimp to healthy individuals. In our studies, NHPB propagation tanks containing infected shrimp were used to maintain a constant supply of organisms for experiments. In order to develop a method for storing infectious NHPB material for future challenge studies, we collected HP tissue containing NHPB by flash freezing whole, fresh HPs at -80°C for up to 80 d and used it to successfully infect specific pathogen-free *Litopenaeus vannamei* per os in controlled experiments. HP tissue samples were collected from dead shrimp, and PCR was performed to confirm the presence of NHPB. Our results demonstrate that the infectivity of NHPB in tissue is not altered after being frozen at -80°C when compared to NHPB in fresh tissue. Thus, the continual propagation of NHPB *in vivo* is not required to assure a source of the infectious agent.

KEY WORDS: Aquaculture · NHPB · *Litopenaeus vannamei* · Frozen tissue · Rickettsiae

Estabilidad de la BNHP en inóculos



Temperaturas

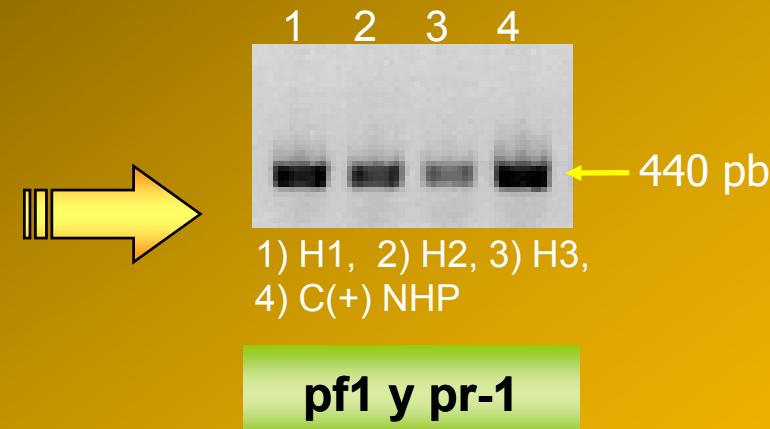
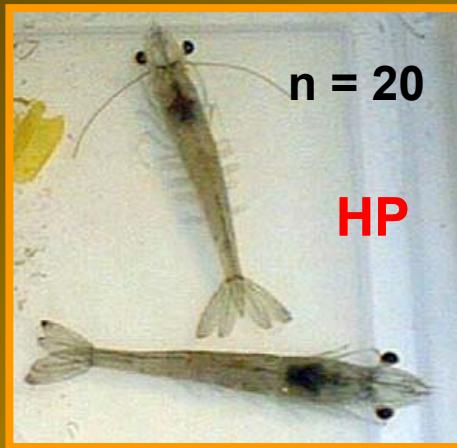
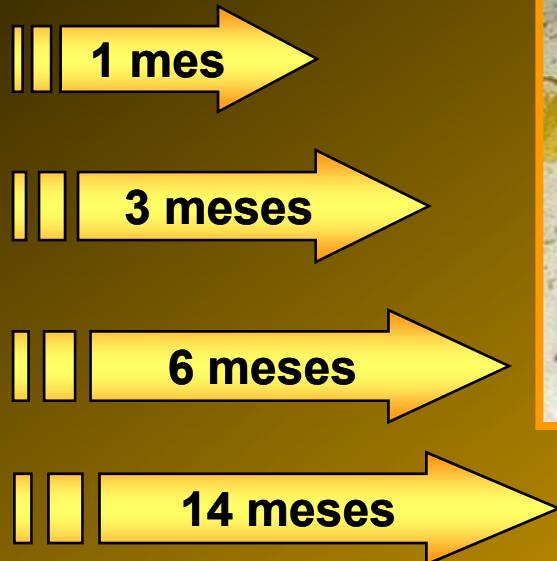


HP + Sol
Crioprotectante

- 80°C, 2 años

Sambrook y Russell, 2001

Viabilidad de la BNHP en inóculos



HP congelación – 80°C
2.5 meses
Crabtree y cols., 2006

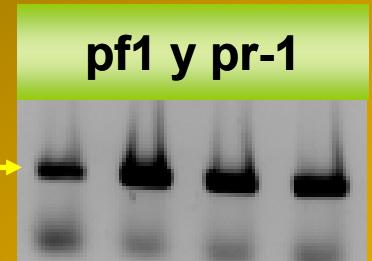
Inóculo viable
14 meses
A -20°C

Ventajas de almacenar BNHP

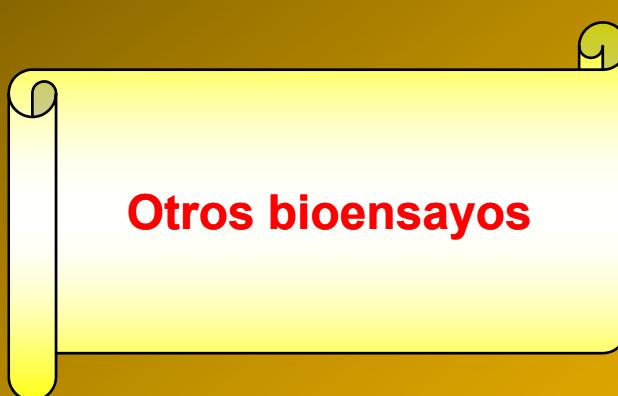
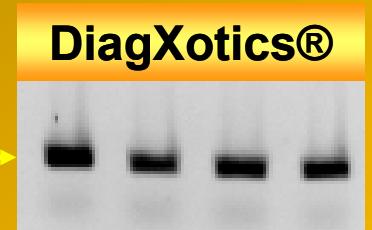


||| Reinfección

440 pb



312 pb



Evaluación del estado fisiológico del camarón



Parámetros bioquímicos

Respuesta inmune

Modificación de la expresión



Available online at www.sciencedirect.com



Aquaculture xx (2006) xxx–xxx

Aquaculture

www.elsevier.com/locate/aqua-online

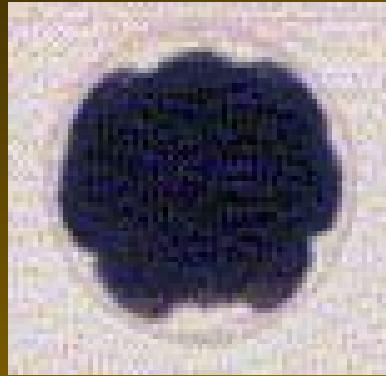
Necrotizing hepatopancreatitis (NHP) infected *Penaeus vannamei* female broodstock: Effect on reproductive parameters, nauplii and larvae quality

Luis Fernando Aranguren, Boris Briñez, Lácides Aragón, Camilo Platz, Xenia Caraballo,
Andres Suarez, Marcela Salazar *

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Received 11 May 2005; received in revised form 6 March 2006; accepted 29 March 2006

Parámetros bioquímicos



L. vannamei hembras de crianza

N = 55

NHP (-) = 23

NHP (+) grado1 = 20

NHP (+) grado2 = 12



Acilglicéridos: control vs NHP grado 2 (n = 12) DE

Colesterol y Glucosa NE

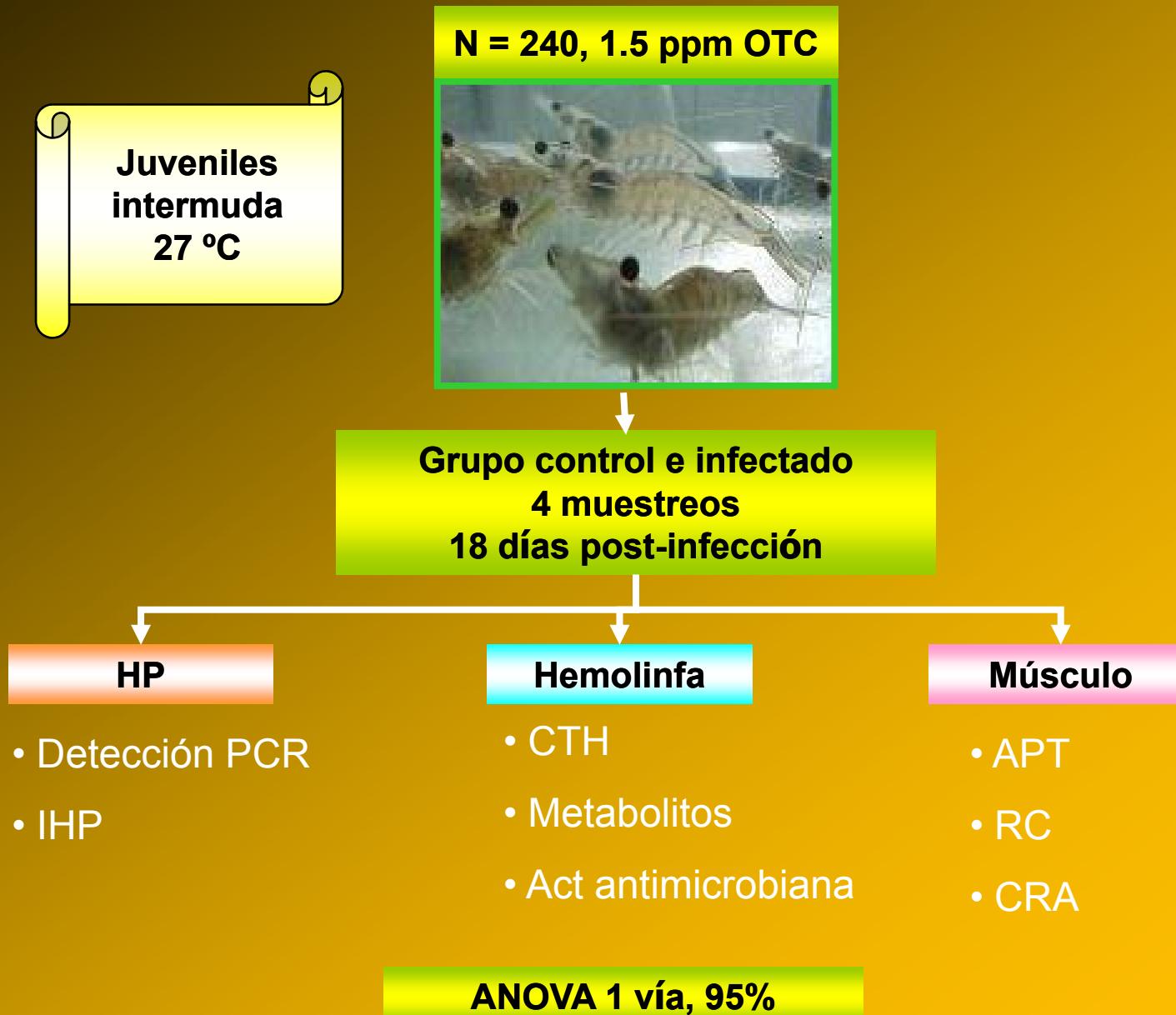
Aranguren y cols., 2006

386 Table 3. Differences in biochemical variables of nauplii II (means \pm SE) from females with mild,
387 moderate and no NHP disease.

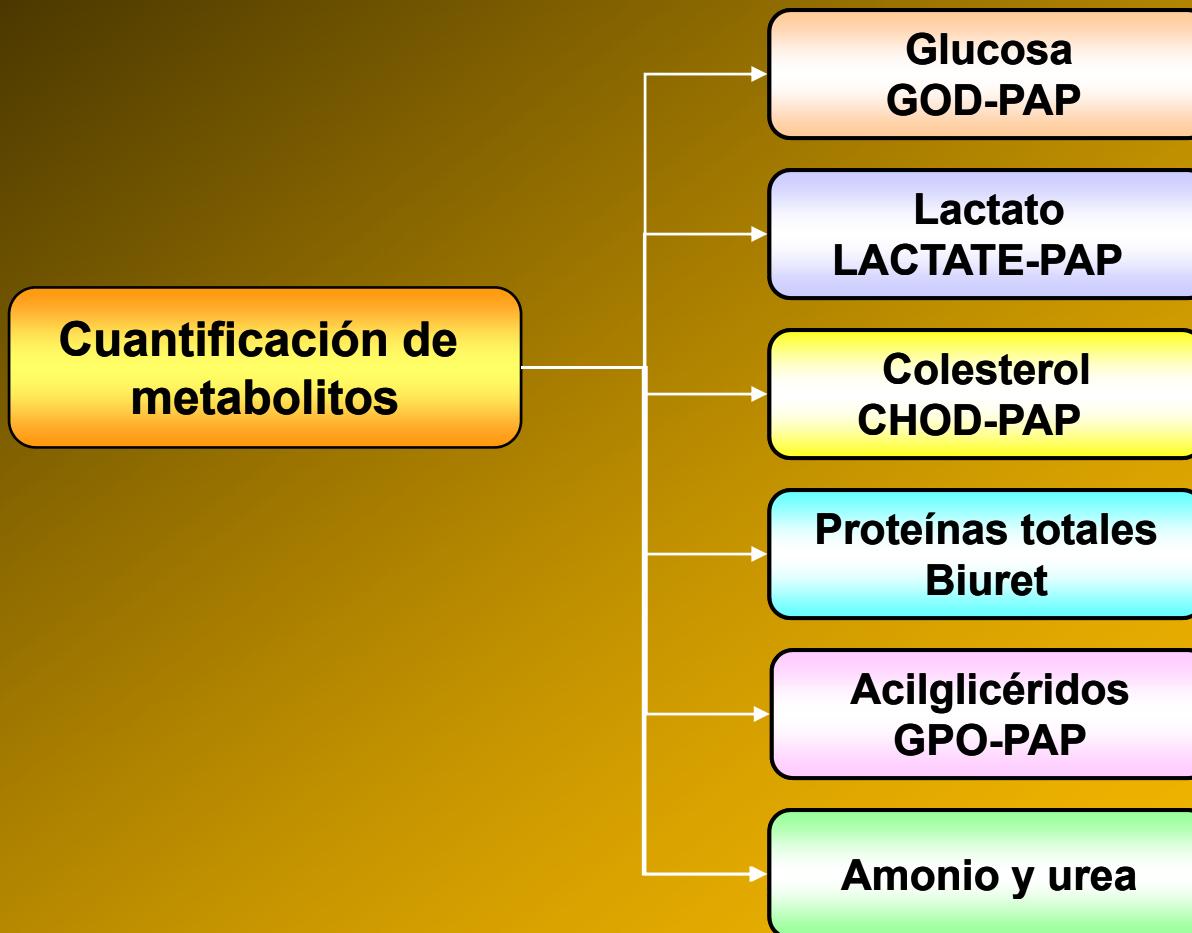
Biochemical parameters	NHP negative	Mild NHP	NHP Moderate
	n= 11	n= 7	n=6
Cholesterol (mg/g)	2,32 \pm 2,45 ^a	1,49 \pm 0,80 ^a	1,93 \pm 0,82 ^a
Glucose (mg/g)	3,35 \pm 2,18 ^a	1,77 \pm 0,53 ^a	3,22 \pm 0,6 ^a
Triglycerides (mg/g)	16,79 \pm 7,10 ^a	12,42 \pm 4,76 ^a	6,73 \pm 3,29 ^b
Triglyceride/cholesterol	8,39 \pm 4,80 ^a	5,26 \pm 2,83 ^a	5,39 \pm 2,46 ^a
Glucose/cholesterol	1,82 \pm 1,87 ^a	1,51 \pm 0,88 ^a	1,79 \pm 0,60 ^a

388 Values with a different superscript in the same row are significantly different (p<0.05)

Infección experimental de organismos



Metabolitos y Parámetros Fisiológicos



Variables fisiológicas en hemolinfa de camarón

Metabolito	Control				Infectados			
	4 d	7 d	15 d	18 d	4 d	7 d	15 d	18 d
Prot. tot.	87±15.2	79±12.8	89±17.8	89±21.9	86±19.2	79±12.3	96±15.6	93±22.5
Glucosa	0.28±0.1	0.21±0.05	0.45±0.2	0.24±0.1	0.23±0.09	0.31±0.1	0.35±0.2	0.21±0.07
Lactato	0.05±0.036	0.13±0.088	0.09±0.068	0.09±0.048	0.08±0.039	0.10±0.05	0.12±0.091	0.15±0.055

Medias ± D.E ($n_{control} = 16$, $n_{infectado} = 30$). Unidades mg/mL. No existen diferencias significativas ($p \geq 0.05$).

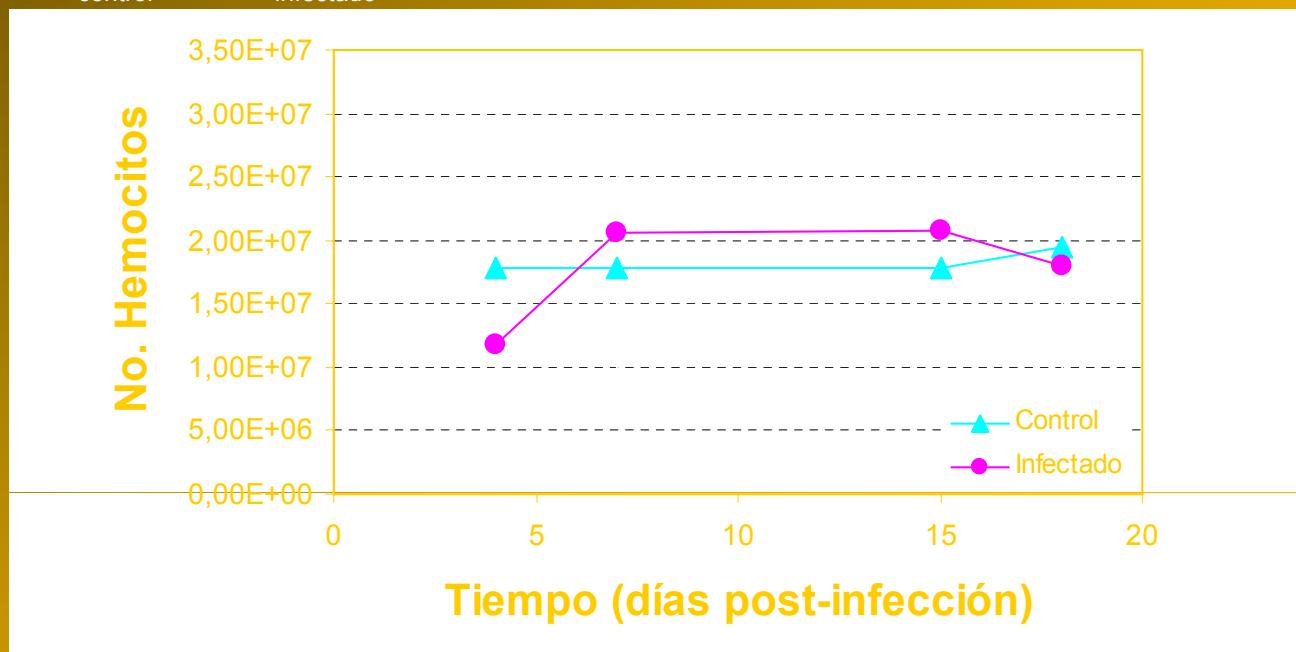
Metabolito	Control				Infectados			
	4 d	7 d	15 d	18 d	4 d	7 d	15 d	18 d
Acilglic.	0.038±0.01	0.032±0.003	0.032±0.003	0.035±0.006	0.034±0.005	0.032±0.003	0.031±0.003	0.032±0.002
Colesterol	0.14±0.04	0.27±0.12	0.23±0.08	0.23±0.09	0.16±0.05	0.26±0.07	0.25±0.09	0.24±0.08

Medias ± D.E ($n_{control} = 16$, $n_{infectado} = 30$). Unidades mg/mL. No existen diferencias significativas ($p \geq 0.05$).

Variables fisiológicas en hemolinfa de camarón

Metabolito	Control				Infectados			
	4 d	7 d	15 d	18 d	4 d	7 d	15 d	18 d
Amonio	45.8±8.3	39.1± 3.8	51.1±8.7	50.6±7.0	42±3.4	35.1±3.2	56.4±2.5	44.1±2.9
Urea	0.21±0.06	0.16±0.04	0.12±0.03	0.21±0.06	0.17±0.07	0.15±0.04	0.13±0.08	0.14±0.05

Medias ± D.E ($n_{control} = 16$, $n_{infectado} = 30$). Unidades mg/mL. No existen diferencias significativas ($p \geq 0.05$).



Variables relacionadas con el sistema inmune



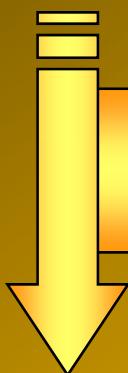
CTH

TSV y WSSV

(Song y cols., 2003; Yoganandhan y cols., 2003)

***V. alginolyticus* asociado a amonio y nitritos**

(Liu y Chen, 2004; Tseng y Chen, 2004; Cheng y cols., 2006)



Incrementos temperatura (Pascual y cols., 2003)

Efecto de aclimatación (Sánchez y cols., 2001)

Muda, sexo, desarrollo, estado reproductivo, nutrición y condiciones ambientales

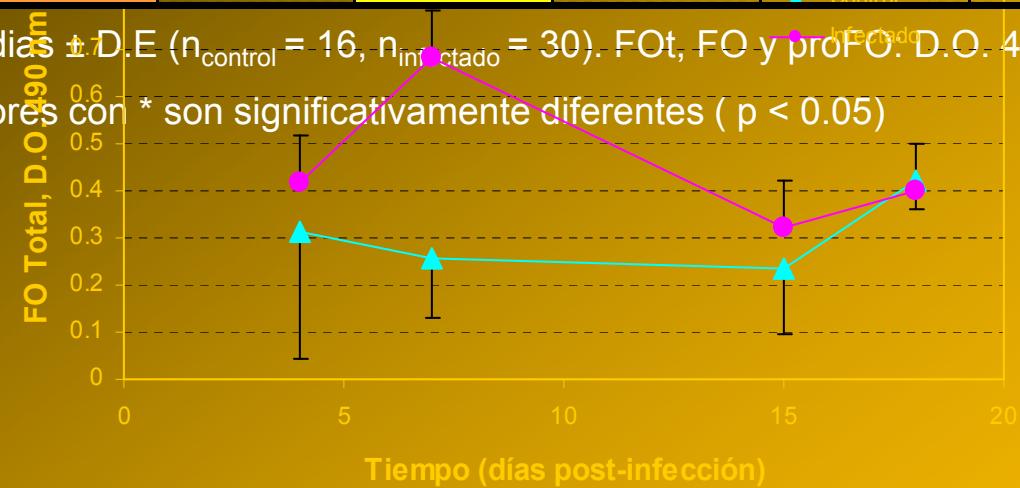
(Pascual y cols., 2003; Sánchez y cols., 2001)

Variables relacionadas con el sistema inmune

Metabolito	Control				Infectados			
	4 d	7 d	15 d	18 d	4 d	7 d	15 d	18 d
Fot	0.31±0.27	0.26±0.12*	0.23±0.14	0.42±0.06	0.42±0.27	0.68±0.34*	0.32±0.24	0.40±0.08
FO	0.047±0.02	0.066±0.02	0.085±0.02	0.052±0.01	0.043±0.01	0.075±0.01	0.10±0.01	0.045±0.01
proFO	0.27±0.02	0.19±0.01*	0.15±0.01	0.37±0.04	0.37±0.02	0.61±0.03*	0.36±0.06	0.36±0.06

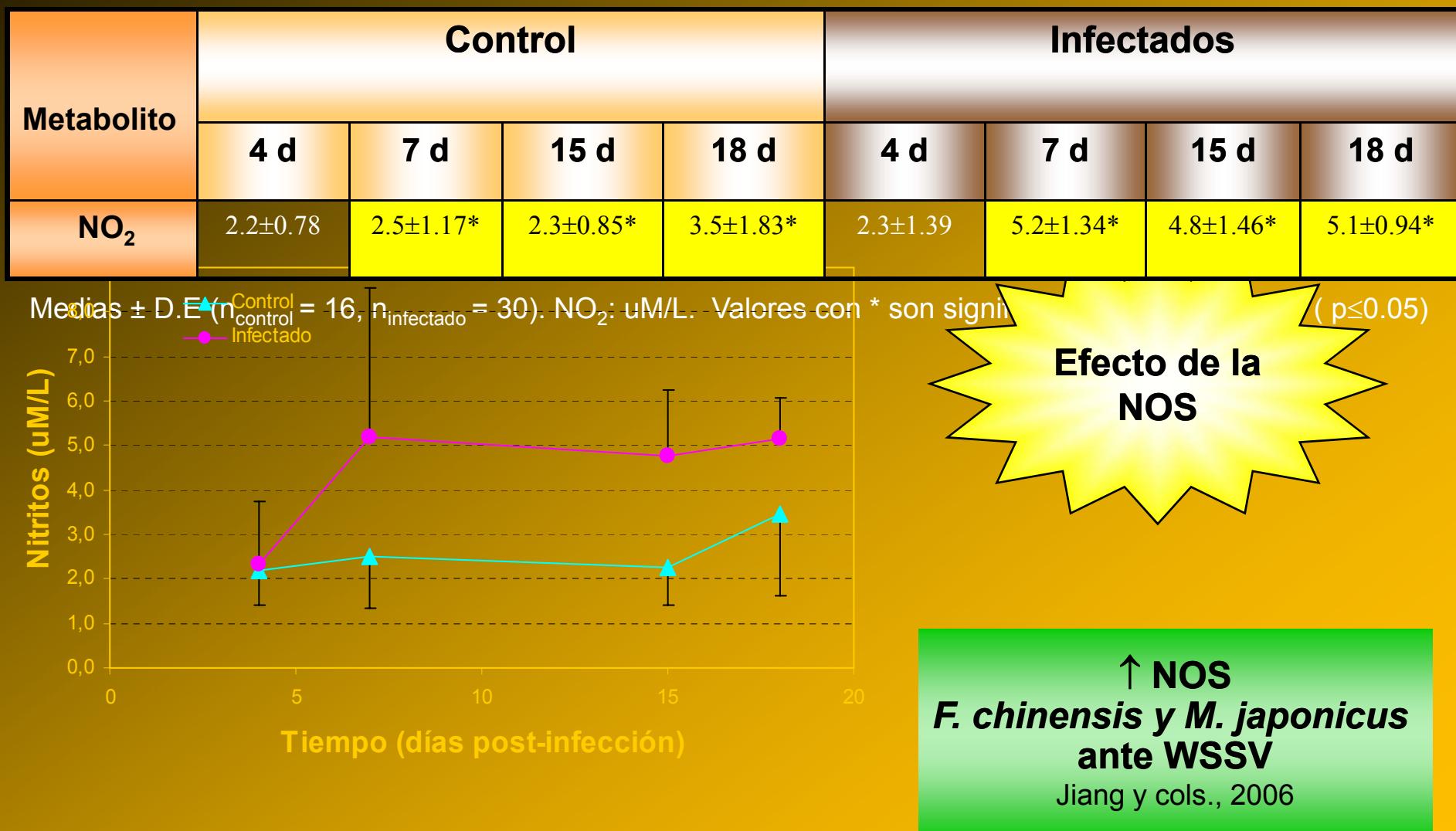
Medias ± D.E (n_{control} = 16, n_{infectado} = 30). FOt, FO y proFO. D.O. 490 nm

Valores con * son significativamente diferentes (p < 0.05)

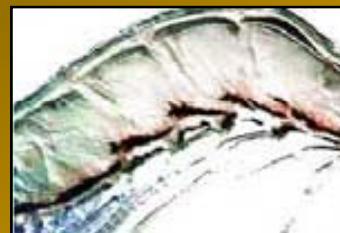


FOT
↑ TSV
(Song y cols., 2003)

Variables relacionadas con el sistema inmune



Determinación de calidad de camarón infectado NHP



Ataca a uno de los principales órganos del camarón (hepatopáncreas), el cuál se encarga de la digestión del alimento, absorción y síntesis de biomoléculas

Análisis de Textura

Flálidez músculo
73 granjas NHP

Textura



CRA

(Cheng y cols., 1979)

RC

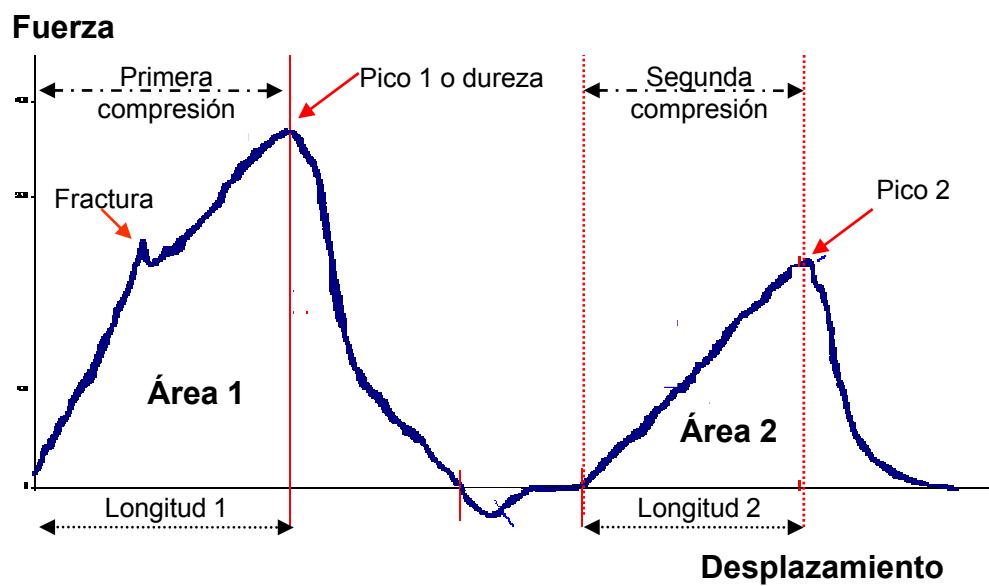
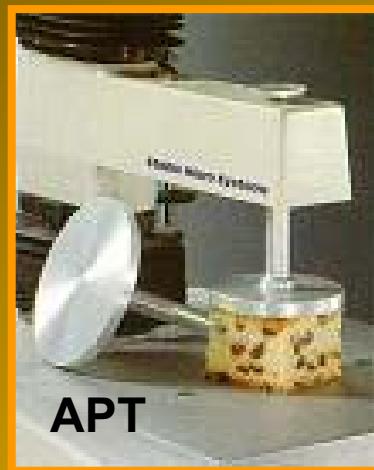
Warner-Bratzler
Zhang y Mittal, 1993

APT

Lamond, 1980



Efecto de la BNHP sobre la calidad del músculo



Pico 1 = dureza
Cohesividad = $(\text{Área 2} / \text{Área 1}) * 100$
Elasticidad = $(\text{Longitud 2} / \text{Longitud 1}) * 100$

Efecto de la BNHP sobre la calidad del músculo cocido

APT Parámetro	Control				Infectados			
	4 d	7 d	15 d	18 d	4 d	7 d	15 d	18 d
Dureza	4982±1477	5783±1458	5870±1055	8733±2175	4719±1422	4127±859	7888±729	6703±1063
Fractura	762±75	1453±1658	727±6	3270±1842	734±3	2440±1230	784±99	1666±1876
Elasticidad	47 ± 2	44 ± 3	51 ± 5	37 ± 2	47 ± 4	51 ± 2	49 ± 3	42 ± 3
Cohesiv.	40 ± 5	37 ± 6	44 ± 8	28 ± 1	41 ± 5	44 ± 8	37 ± 2	32 ± 2

Medias ± D.E ($n_{control} = 6$, $n_{infectado} = 6$). Dureza y fractura: gf. Elasticidad y cohesividad: %. No existen diferencias significativas ($p \geq 0.05$).

Efecto de la BNHP sobre la calidad del músculo crudo

APT Parámetro	Control				Infectados			
	4 d	7 d	15 d	18 d	4 d	7 d	15 d	18 d
Dureza	3098±1275	4399±1771	3210±541	4684±1283	3262±832	3761±1198	4123±929	4008±747
Fractura	1431±457	838±380	939±411	1738±382	850±348	998±613	1412±492	803±167
Elasticidad	31 ± 3	27 ± 6	29 ± 1	26 ± 3	29 ± 3	26 ± 1	31 ± 5	24 ± 3
Cohesiv.	15 ± 4	16 ± 4	16 ± 4	11 ± 1	15 ± 4	15 ± 3	16 ± 3	13 ± 1

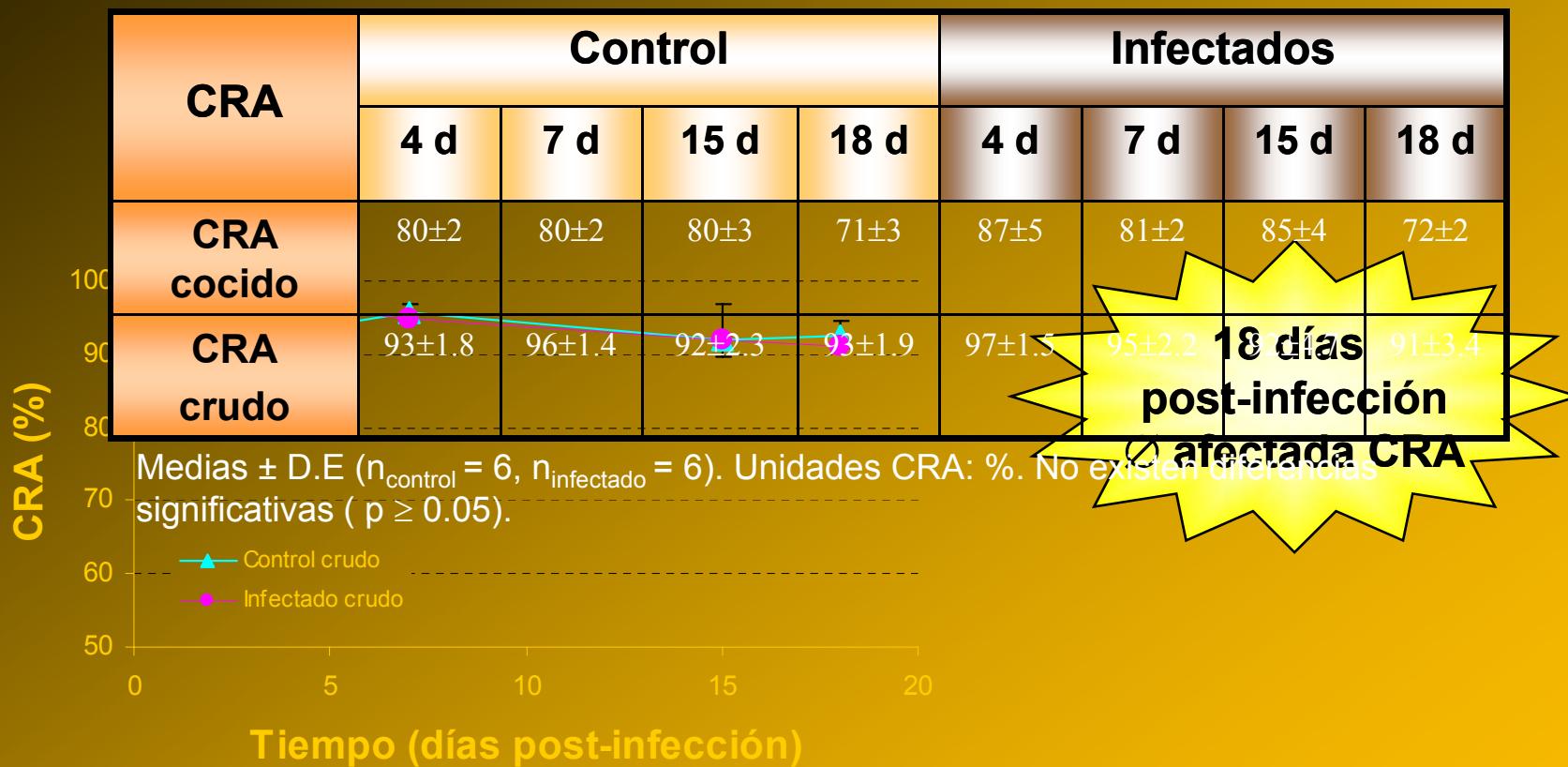
Medias ± D.E ($n_{control} = 6$, $n_{infectado} = 6$). Dureza y fractura: gf. Elasticidad y cohesividad: %. No existen diferencias significativas ($p \geq 0.05$).

Efecto de la BNHP sobre la calidad del músculo RC

RC Parámetro	Control				Infectados			
	4 d	7 d	15 d	18 d	4 d	7 d	15 d	18 d
Dureza cocido	1423±138	1530±118	1648±291	1591±262	1228±98	1605±241	1757±411	1539±116
Dureza crudo	1559±215	1462±215	1773±365	1904±435	1513±387	1701±318	1798±324	1630±257

Medias ± D.E ($n_{control} = 6$, $n_{infectado} = 6$). Dureza y fractura: gf. No existen diferencias significativas ($p \geq 0.05$).

Efecto de la BNHP sobre la calidad del músculo CRA



Variables fisiológicas y texturales en camarón

No se encontraron diferencias
($p \geq 0.05$)



Camarones: grado 1 infección
(Vincent y Lotz 2005)

CONCLUSION

Aun falta mas investigación para el conocimiento y control de NHP.

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¡¡¡Gracias por su atención!!!