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感染白斑综合症病毒(WSSV)对虾相关免疫因子的研究

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摘 要:129尾中国对虾(*Penaeus chinensis*)分别捕自未暴发白斑综合症(WSSV病毒所致)虾池、WSSV暴发虾池以及曾暴发WSSV虾池。用斑点杂交和组织病理学方法确定各尾对虾的染毒(WSSV)程度。用96孔酶标板法测量相应个体血淋巴上清液的抗菌活力(U_a)、溶菌活力(U_L)、酚氧化酶(PO)活性以及过氧化酶(POD)相对活性;用硝酸纤维膜斑点法测定其碱性磷酸酶(ALP)相对活性;用血凝法测定其凝集效价(HAT)。通过对以上免疫指标进行统计分析,结果表明,WSSV感染与对虾血淋巴PO活性以及ALP相对活性变化有紧密联系;不同虾池各免疫因子差异显著,发病虾池虾样各免疫指标平均值均低于其他虾池;曾发病虾池的虾样PO活性较强;WSSV与HPV感染无统计学意义上的相关性;未发病虾池与曾发病虾池实验对虾的U_a与U_L相关性极显著,发病虾池实验对虾U_a与U_L呈负相关;发病虾池对虾PO与ALP活性相关性显著。不同性别中国对虾血淋巴上清液的免疫因子活性没有显著差异。

关键词:中国对虾;白斑综合症病毒;对虾血淋巴;免疫因子
中图分类号:S945.46 **文献标识码**:A

白斑综合症病毒(WSSV)在本实验室早期的报道中暂称为皮下及造血组织坏死杆状病毒(HHNBV)。1993年以来该病毒严重制约着我国对虾养殖业的发展,而提高对虾的自身免疫力,增强其抗病机能是综合防治的根本措施之一。为进一步探讨WSSV在对虾体内的致病机理以及不同WSSV感染状态下中国对虾(*Penaeus chinesis*)血淋巴上清液中免疫指标的活性变化,本研究先用斑点杂交和组织病理法确定单尾中国对虾的带毒状态,然后用96孔酶标板法、硝酸纤维膜斑点法和血凝法测量不同虾池和不同染毒状态下中国对虾血淋巴上清液中各免疫指标的活性,并用生物统计学方法对不同染毒状态下免疫指标的活性进行分析,以期为对虾养殖生产中预防WSSV提供理论依据。

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1 材料与方法

1.1 材料

1.1.1 样品来源 实验中国对虾分别采自胶州市李格氏镇养虾场和胶南市琅岭台养虾场,体长5～8 cm。其中45尾虾样采自李格氏镇未暴发WSSV虾池(GS－ND),29尾虾样采自李格氏镇曾暴发WSSV虾池(GS－PD),32尾采自琅岭台WSSV暴发虾池(YT－D),22尾采自琅岭台未暴发WSSV虾池(YT－ND),后2池相距约500 m,由1人管理。

1.1.2 虾样采集 网捕一定数量的中国对虾,置于大塑料桶内,用1 ml的一次性注射器从对虾的心脏、胸足基部和腹节等处抽取血淋巴,注入洁净无菌

的 1.5 ml 塑料离心管中,置于疫苗冷藏包,回实验室后迅速转移到 -70℃ 超低温冰箱中保存。将相应对虾头胸部一侧的甲壳揭开,取几根鳃丝保存于 SEMP - Tris[1)] 中用于核酸探针斑点杂交,然后用洁净锋利刀片将头胸部纵切开,把没有取鳃的一侧置于 Davidson's AFA 固定液中固定(24 h 后换成 70% 酒精)[1],用于病理制片。

1.1.3　菌株　抗氨卞青霉素的大肠杆菌(*E. coli*)菌株由青岛海洋大学生命学院徐怀恕教授惠赠,活化时用 LB 培养基(含 50 μg/ml 氨卞青霉素)于 37℃ 固体斜面培养;溶壁微球菌由中科院海洋所王雷惠赠,活化时用 LB 培养基于 37℃ 固体斜面培养。实验前用 0.1 mol/L pH6.4 的磷酸钾盐缓冲液把抗氨卞青霉素大肠杆菌和溶壁微球菌洗下,制成菌悬液(大肠杆菌 0.3～0.5 OD_{570},溶壁微球菌 0.1～0.3 OD_{570}),置于 4℃ 冰箱中备用。

1.2　方法

1.2.1　核酸探针斑点杂交分析法检测样品　参照黄海水产研究所病害室研制的"对虾暴发性流行病原核酸探针点杂交检测试剂盒(I 型)"[1)] 提供的操作步骤,用地高辛标记 DNA 探针检测实验样品。在灯光下,根据杂交膜上点样斑点显色的有无或强弱来判断点样斑点上 WSSV 量的多少,从而可以大致推断实验样品的染毒状态和实验样品对 WSSV 的敏感程度。

1.2.2　病理制片　将 Davidson's AFA 固定的样品制备成厚度 5 μm 的石蜡切片,按 Bell 等[1] 方法进行 H - E 染色,中性树胶封片,光镜观察 WSSV 引起实验样品的病变程度,从而初步判断实验样品染毒状态,同时,在光镜下仔细观察虾样的肝胰腺,若发现肝胰腺细小样病毒(HPV)包涵体,虾样相应被定为 HPV 携带者,否则被定为非 HPV 携带者。

1.2.3　确定实验对虾的 WSSV 感染状况　根据斑点杂交显色的强弱定为"+ + + +"、"+ + +"、"+ +"、"+"、"-"5 个等级;光镜观察病理组织切片上 WSSV 引起对虾甲壳下表皮的病变情况,上皮细胞的细胞核肿大数超过同一部位总细胞数的 1/2 者,为"+ + + +",1/4～1/2 者为"+ + +",小于 1/4 且病灶明显者为"+ +",病灶不明显只有很少细胞的细胞核肿大者为"+",没有观察到病变者为"-"。将斑点杂交和对虾甲壳下表皮的病变程度的等级相加,6～8 个"+"者为严重感染;4～6 个"+"者为中度感染;2～4 个"+"者为轻度感染;1～2 个"+"者为潜伏感染;0 个"+"者为没有感染。

1.2.4　抗菌活力(U_a)和溶菌活力(U_L)的测定　采用改进的 Hultmark 等方法[2],在 96 孔酶标板中加入不同对虾血淋巴上清液(10 μl/孔),再向孔内移加 90 μl pH 6.4、0.1 mol/L 磷酸钾缓冲液,读取 490 nm 处的吸光值 A_1,再加入 100 μl 磷酸钾缓冲液,读取 490 nm 处的吸光值 A_2。计算系统误差 K,$K = \Sigma(A_2/A_1)/n$。安排 96 孔板奇数列测血淋巴上清液的 U_a,偶数列测血淋巴上清液的 U_L。分别将待测血淋巴上清液移入酶标板奇数列和偶数列相应的孔(10 μl/孔),再向样品孔内加 90 μl pH 6.4、0.1mol/L 磷酸钾缓冲液,读取 490 nm 处的吸光值 A_K;取出 96 孔板,往相应列的孔内分别移加 100 μl 大肠杆菌悬液和 100 μl 溶壁微球菌悬液,读取 490 nm 处的吸光值 A_0;37℃ 温育 30 min 后,读取 490 nm 处的吸光值 A。按下列公式计算:

$U_a = [[(A_0 - A_K \times K) - (A - A_K \times K)]/(A - A_K \times K)]^{1/2}$;$U_L = [(A_0 - A_K \times K) - (A - A_K \times K)]/(A - A_K \times K)$。如果 $A_0 \leqslant A$,U_a、U_L 均以 0 作为结果。

1.2.5　酚氧化酶(PO)活力(A_{PO})的测定　以 L - 多巴为底物,采用改进的 Ashida 等方法[3] 在 96 孔酶标板中进行。把 10 μl 血淋巴上清液加入 96 孔酶标板中,然后向各孔中加入 200 μl 0.1 mol/L pH 6.0 磷酸钾缓冲液,最后向各样品孔中加入 10 μl 的 L - 多巴(上海伯奥生物科技公司,批号 970901)液(0.01 mol/L),在酶标仪(550, Bio - Rad)中振荡 4 次,每隔 4 min 读取 490 nm 处的吸光值。酶活力以试验条件下,OD_{490} 每 min 增加 0.001 为一个酶活力单位。

1.2.6　碱性磷酸酶(ALP)相对活力(A_{ALP})的测定　借鉴非放射性核酸探针斑点杂交法[4]。双蒸水浸润硝酸纤维膜(NCM),晾干。吸取 1 μl 血淋巴上清液点在 NCM 上。晾干后,装入杂交袋,按膜面积 0.1 ml/cm^2 加入生色底物混合物,置于振荡器上平缓摇动孵育。一俟出现蓝色,把 NCM 膜移到装有 200 μl 0.5 mol/L EDTA (pH8.0) 和 50 ml PBS 的平皿中,5 min 后取出晾干。用扫描仪把显色结果存于计算机中,用软件 Molecular Analyst (V1.5, Bio - Rad) 测定每一样品的显色强度,把所得的平

1) 史成银,黄　倢. 一种新型核酸探针采样液 SEMP - Tris[J]. 1998.

均象素灰度(MC)代入公式 OD=log(255/MC)求得相应的 OD;$OD_{调整}=OD-OD_{空白对照}$;每一样品的酶活 $A_{ALP}=OD_{调整}\times$斑点面积(A_S)。

1.2.7 过氧化酶(POD)相对活力(A_{POD})的测定 采用改进的史成银等方法[5]。在 96 孔酶标板中加入血淋巴上清液(20 μl/孔),然后加入 180 μl 显色缓冲液(7.3 g 一水柠檬酸,11.86 g $Na_2HPO_4\cdot 2H_2O$,双蒸水定容至 1 000ml),置于酶标仪中,读取 490 nm 处的 OD 值(A_3)。向样品所在孔中加 20 μl 显色液(4 mg 邻苯二胺,4 μl 30% H_2O_2,10 ml 显色缓冲液);置酶标仪中摇匀后,避光显色 15 min,读取 490 nm 处的 OD 值(A_4)。血淋巴上清液中 POD 相对活力以 $A_{POD}=A_4-A_3$ 表示。

1.2.8 血淋巴上清液凝集效价(C_{HAT})的测定 取用 Alsevers'液保存的小白鼠血液,转入无菌离心管中,2 000 r/min 离心 10 min,弃上清液,加入 1 ml 0.85 %无菌生理盐水,同上离心;弃上清液,加入 1 ml TBS,2 000 r/min 离心 10 min,重复 1 次,用所得压积细胞配成 1%红细胞悬液,4℃保存备用。在圆底 96 孔酶标板加入 25 μl 血淋巴上清液,用 TBS 作二倍稀释,然后往每孔中加入 25 μl 1%的红细胞悬液。置圆底 96 孔酶标板于振荡器上缓缓振摇 15 min,37℃下放置 2 h,于倒置显微镜下观察。血清凝聚反应的判断标准参考《免疫检测技术》[6]。

1.2.9 对 WSSV 感染的相关免疫进行统计分析 参照杨运清等方法[7],使用计算机分析 WSSV 感染的各相关免疫指标的相关性,比较不同虾池不同 WSSV 感染各免疫指标的差异。

2 结果与分析

2.1 虾样的带毒状况

运用病理学和核酸探针斑点杂交技术对 129 尾中国对虾进行病原检测和病变分析,根据斑点杂交结果和相应虾样的病理变化,将虾样受 WSSV 感染的程度分为潜伏感染、轻度感染、中度感染和严重感染。4 虾池(见 1.1.1)虾样的 WSSV 感染状况和 HPV 的携带情况见图 1(附页)。李格氏镇未暴发 WSSV 虾池(GS-ND)中 91.1%的虾处于潜伏感染,6.1% 中度感染,2.2% 严重感染;35% 携带 HPV。曾暴发 WSSV 虾池(GS-PD)中 96.5%虾处于潜伏感染,3.5%严重感染;3.9% 携带 HPV。琅峙台暴发 WSSV 虾池(YT-D)中,15.6%潜伏感染,3.1%轻度感染,34.4%中度感染,46.5%严重感染;66.7% 携带 HPV。未暴发 WSSV 虾池(YT-ND)中 100%处于潜伏感染;90.9% 携带 HPV。

2.2 WSSV 与 HPV 感染的相关分析

对虾样的 4 种 WSSV 感染状态和是否携带 HPV 给予评分:WSSV 潜伏感染虾样为 0,轻度感染虾样为 1,中度感染虾样为 2,严重感染虾样为 3;携带 HPV 虾样为 1,非携带 HPV 虾样为 0。对 WSSV 和 HPV 感染进行相关分析,YT-D 的 WSSV 与 HPV 感染相关系数为 0.023 7;GS-ND 的 WSSV 与 HPV 感染相关系数为 0.140 8;GS-PD 的 WSSV 与 HPV 感染相关系数为-0.04。对 4 个虾池虾样进行总体分析,WSSV 感染与 HPV 感染的相关系数为 0.178 4。可见,WSSV 感染与 HPV 感染没有生物学意义上的相关性,表明二者不存在干扰现象或协同性。

2.3 不同虾池各免疫指标的比较

4 虾池间的 Ua、U_L、A_{PO}及 C_{HAT}差异显著。Ua 大小顺序为:(GS-ND、GS-PD)>(YT-ND、YT-D);U_L 平均值大小顺序为:(GS-ND、GS-PD)>(YT-D、YT-ND);A_{PO}活性平均值大小顺序为:(GS-PD、GS-ND、YT-ND)>(YT-D);C_{HAT}平均值大小顺序为:(YT-ND、YT-D、GS-PD)>(GS-ND)(图 2,见附页);虾血淋巴 A_{POD} YT-D<YT-ND,但两者差异不显著;虾血淋巴 A_{ALP} YT-D<YT-ND,两者差异显著(图 2,见附页)。

2.4 HPV 感染虾样与非 HPV 感染虾样各免疫指标比较

YT-D 的 HPV 携带者的 A_{PO}的平均值小于非 HPV 携带者,二者差异显著;HPV 携带者与非 HPV 携带者的 Ua、U_L、A_{ALP}、A_{POD}和 C_{HAT}差异不显著(图 3,见附页)。GS-ND 的 HPV 携带者与非 HPV 携带者各免疫指标的差异均不显著(图 4,见附页)。

2.5 不同 WSSV 感染程度虾样的免疫指标比较

对 YT-D 不同 WSSV 感染状态下虾血淋巴各免疫指标中只有 POD 差异显著,其平均值大小顺序为:潜伏感染虾样>中度感染虾样>严重感染虾样,而其 Ua、U_L、A_{PO}、A_{ALP}和 C_{HAT}差异不显著(图 5,见附页)。

2.6 对虾血淋巴上清液各免疫因子的相关性

GS-ND 虾 Ua 与 U_L 的相关系数为0.815(表 1),GS-PD 虾 Ua 与 U_L 相关系数为 0.758(表 2),在 $a=0.01$ 水平上两池 Ua 与 U_L 相关性极显著,

但 YT－D 与 YT－ND 的 Ua 与 U_L 呈负相关(表3)。YT－D 的 A_{PO} 与 A_{ALP} 相关系数为 0.349，二者在 $a=0.05$ 水平上是相关性显著(表3)。A_{PO} 或 A_{ALP}、A_{POD} 和 A_{HAT} 与 Ua 或 U_L 的相关性不显著，不同虾池相关系数不同(表1～3)。

表1　GS－ND 虾样各免疫指标相关系数

Table 1　Correlation of immune factors in Pond GS－ND

$Ua(45)$	1			
$U_L(45)$	0.815**	1		
$A_{PO}(45)$	0.0989	0.018	1	
$C_{HAT}(44)$	0.137	0.143	－0.292	1
免疫指标 Immune index	$Ua(45/44)$	$U_L(45/44)$	$A_{PO}(45/44)$	$A_{HAT}(45/44)$

** —相关性极显著 Extremely significant correlation；
()—计数 Count.

表2　GS－PD 虾样各免疫指标相关系数

Table 2　Correlation of immune factors in Pond GS－PD

$Ua(29)$	1			
$U_L(29)$	0.758**	1		
$A_{PO}(29)$	－0.265	－0.1699	1	
$C_{HAT}(29)$	0.0842	0.0988	－0.0387	1
免疫指标 Immune index	$Ua(29)$	$U_L(29)$	$A_{PO}(29)$	$A_{HAT}(29)$

** —相关性极显著 Extremely significant correlation；
()—计数 Count.

表3　YT－D 虾样各免疫指标相关系数

Table 3　Correlation of immune factors in Pond YT－D

$Ua(32)$	1					
$U_L(32)$	－0.154	1				
$A_{PO}(32)$	－0.06	－0.135	1			
$A_{ALP}(32)$	－0.216	－0.142	0.349*	1		
$A_{POD}(31)$	－0.032	0.015	0.285	－0.027	1	
$C_{HAT}(32)$	－0.065	0.246	－0.054	－0.168	－0.18	1
免疫指标 Immune index	$Ua(32/31)$	$U_L(32/31)$	$A_{PO}(32/31)$	$A_{ALP}(32/31)$	$A_{POD}(32/31)$	$C_{AHAT}(32/31)$

* —相关性显著 Significant correlation；()—计数 Count.

2.7　不同性别对虾血淋巴上清液的免疫因子活性比较

2.7.1　抗菌活力比较　从图6(见附页)可以看出，GS－ND、GS－PD、YT－D 和 YT－ND 的对虾，其不同性别血淋巴 Ua 差异均不显著，说明不同性别对虾血淋巴的 Ua 没有显著差异。

2.7.2　酚氧化酶活性比较　从图7(见附页)可以看出，GS－ND、GS－PD、YT－D 和 YT－ND 的对虾，不同性别血淋巴的 A_{PO} 差异均不显著，说明不同性别对虾血淋巴的 A_{PO} 没有显著差异。

2.7.3　碱性磷酸酶相对活性比较　从图8(见附页)可以看出，YT－D 和 YT－ND 的对虾，其不同性别血淋巴的 A_{ALP} 差异均不显著，说明不同性别对虾血淋巴的 A_{ALP} 相对活性没有显著差异。

2.7.4　过氧化酶相对活性比较　从图9(见附页)可以看出，YT－D 和 YT－ND 的对虾，其不同性别血淋巴的 A_{POD} 差异均不显著，说明不同性别对虾血淋巴的 A_{POD} 没有显著差异。

2.7.5　凝聚效价比较　从图10(见附页)可以看出，GS－ND、GS－PD、YT－D 和 YT－ND 的对虾，其不同性别血淋巴的 C_{HAT} 差异均不显著，说明不同性别对虾血淋巴的 C_{HAT} 没有显著差异。

3　讨论

(1)酚氧化酶原(proPO)激活系统是甲壳动物的识别和防御系统，是一个与脊椎动物补体相似的级联系统[8]。其可被 β－葡聚糖[9]、脂多糖[9]和肽聚糖[10]激活，从颗粒细胞释放到血浆中，在丝氨酸蛋白酶的作用下转变为活性的酚氧化酶(PO)，PO 可将酚催化成黑色素，黑色素及其中间产物可将一些病原体杀死[11,12]。因此，PO 的活性强弱在一定程度上反映出对虾抗病力的强弱。

4 虾池虾样 A_{PO} 平均值大小顺序为：GS－PD＞GS－ND＞YT－ND＞YT－D，它们之间差异显著(图2，见附页)，但不同 WSSV 感染状态的虾样，其 A_{PO} 差异不显著，即不同感染状态下的 A_{PO} 平均值并不与 WSSV 感染程度对应(图5，见附页)，说明并非 WSSV 爆发才引起 A_{PO} 活性降低，在对虾体内，除了 WSSV 对 A_{PO} 有一定的影响外还很可能有其他影响因子。引起不同虾池之间 A_{PO} 差异显著(图2，见附页)的原因可能与不同虾池 WSSV 感染程度不同(图1，见附页)有关。

(2)国内外学者借鉴昆虫免疫研究中较为成熟的抗菌肽、溶菌酶活性测定方法用来衡量对虾体液的免疫状态以及药物作用机理。李春猛等[13]和Sung等[14]用细菌的存活指数(SI)来研究菌苗和多糖的作用机理。王雷等[15]以大肠杆菌和溶菌活力为底物,按Hultmark等[2]的方法来测量对虾的抗菌活力(U_a)和溶菌活力(U_L)。以上研究者均把多尾对虾混合后进行测量,这样所得的结果当然是混合血清U_A或U_L,不能体现单独对虾个体的相应指标。本实验对Hultmark等的方法进行改进,用酶标板法来测量单尾对虾的U_a和U_L。

从图3、4(见附页)可以看出,HPV感染与U_a和U_L没有紧密的关联。

YT-ND与YT-D的U_a和U_L没有显著差异,且不同WSSV感染状态下U_a和U_L的差异不显著(图5,见附页),这说明WSSV感染与U_a和U_L之间没有紧密的关联。

在GS-ND和GS-PD中,U_a与U_L之间有密切的关联(表1,2),但在YT-D的U_a与U_L呈不显著的负相关(表3),其原因可能是在暴发WSSV状态下对虾血淋巴的U_a和U_L之间相关联的环节被破坏或被扰乱,并且使得U_a和U_L水平比较低。

(3)ALP是一种磷酸单脂酶,它直接参与磷代谢,并与DNA、RNA、蛋白质、脂质等的代谢有关。它对钙质吸收、骨骼形成、磷酸钙沉积、甲壳质分泌及形成有重要的作用。陈清西等[16]采用Folin-酚试剂法测量ALP活力,吴垠等[17]采用酚试剂法与仪器法研究中国对虾感染WSSV后ALP的相对活性变化。本实验首次用硝酸纤维膜斑点法测量对虾ALP相对活性(A_{ALP})。

YT-ND的A_{ALP}平均值高于YT-D,二者差异显著(图2,见附页),但不同WSSV感染状态的虾样,其A_{ALP}差异不显著,即不同感染状态下的A_{ALP}平均值并不与WSSV感染程度对应(图5,见附页),说明并非WSSV暴发才引起A_{ALP}降低,在对虾体内,除了WSSV对A_{ALP}有一定的影响外还很可能有其他因子影响A_{ALP}。

YT-D的A_{ALP}相对活性平均值低于YT-ND,二者差异显著(图2,见附页),说明WSSV发病对虾血淋巴的A_{ALP}低,对虾体内的磷代谢发生障碍,这可能与甲壳质不正常分泌而在甲壳内侧出现白色花朵样小斑有关。此结果与吴垠等[17]的结果相反,其原因有待进一步研究。

A_{ALP}与A_{PO}的变化趋势相似,即YT-D虾的A_{ALP}和A_{PO}的平均值均小于YT-ND虾(图2,见附页),且A_{ALP}与A_{PO}在$\alpha=0.05$水平上显著相关,说明二者之间有密切关联,另一方面也说明ALP可能是对虾体液免疫因子之一,但从PO和ALP的已知功能来看,二者似乎没有紧密的关联,其原因有待进一步研究。

(4)过氧化酶广泛存在于真核细胞体内,在清除自由基(O_2^-),防止生物分子损伤方面有十分重要的作用,因此过氧化酶的大小可以用来推断对虾的生理状态。从图2(见附页)可以看出,YT-ND与YT-D A_{POD}平均值的差异不显著,但不同WSSV感染状态之间A_{POD}有显著差异,其平均值大小顺序为:潜伏感染虾样>中度感染虾样>严重感染虾样(图5,见附页)。可见,WSSV潜伏感染状态下,对虾抵抗WSSV的能力较强,其血淋巴A_{POD}较高;WSSV在对虾体内迅速增殖,感染程度加大,对虾血淋巴A_{POD}受到了抑制,也就是说,A_{POD}因WSSV的暴发而降低。不同WSSV感染状态下对虾血淋巴A_{POD}变化趋势与黄灿华等[18]研究SOD活性的结果基本一致,有可能用作对虾病毒检测与诊断的生理生化指标。

(5)血凝法是研究对虾血淋巴凝集效价C_{HAT}的常规方法之一,但对虾血淋巴对小鼠红细胞的凝集能力与血淋巴中的某种与小鼠红细胞有亲和作用的蛋白质有关,这种蛋白质不一定在无脊椎动物的免疫识别系统中起凝集素作用。从本实验看出,HPV感染对HAT没有紧密的关联(图3,4,见附页)。发病虾池(YT-D)与非发病虾池(YT-ND)的血淋巴C_{HAT}差异不显著(图2,见附页),同一发病池(YT-D)中不同感染状态的对虾血淋巴C_{HAT}差异不显著(图5,见附页),说明对虾血淋巴对小鼠红细胞的C_{HAT}可能与对虾对病毒的识别无关。

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Immune factors in haemolymph supernatant of *Penaeus chinensis* infected by WSSV

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Abstract: From four ponds, 129 shrimps *Penaeus chinensis* were captured. Among the four ponds, one was on WSSV breaking out, and the other one once had a WSSV breaking out, and the rest two were never infected with WSSV. The infected degree by WSSV was diagnosed by dot blot hybridization and classical tissue pathology methods. Microplate reader was used to measure antibiotic activity (U_a), bacteriolytic activity (U_L), PO activity (A_{PO}) and POD activity (A_{POD}) of haemolymph supernatant of each shrimp. Dot blotting on NCM was first used to measure ALP activity (A_{ALP}) in the haemolymph supernatant. C_{HAT} was measured by indirect agglutination. All the immune indexes above were analyzed using biological statistics method on computer. The results are as follows: ①the effect of WSSV infection on A_{PO} and A_{ALP} is significant; ②the differences of immune factor activities among the four ponds are significant; the mean activity of each immune factor in WSSV-breaking-out pond is lower than that in any other three ponds; the mean A_{PO} in the once-broke-out pond is the highest in the four ponds; ③statistical correlation of the immune indexes between WSSV infection and HPV infection can not be found; ④the correlation between U_a and U_L is very significant, and the correlation between A_{PO} and A_{ALP} is significant; ⑤there is no significant difference in immune factor activities between sexes.

Key words: *Penaeus chinensis*; WSSV; shrimp haemolymph; immune factor

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Effects of dietary lipid sources on fatty acid composition of rotifer *Brachionus plicatilis*

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Abstract: Rotifer *Brachionus plicatilis* was used as the test species. The diets with different lipid sources were as follows: (1) Baker's yeast, *Saccharomyces cerevisice* (control); (2)algae, *Nannochlorpsis oculata* (diet A); (3) fish oil(5%) + soybean lecithin (5%) + Baker's yeast(90%)(diet B); (4) fish oil (10%) + Baker's yeast(90%)(diet C). The results show that: (1) Considerable difference of fatty acid composition exists in the rotifers fed corresponding diets compared with control, and the highest level of n-3 HUFA fatty acids in the rotifers fed diet C is obtained due to high level of n-3HUFA content in diet. The conclusion is that the HUFA content of diet is a major factor in affecting HUFA levels in the rotifers. (2) The effect of dietary lipid on the fatty acid composition is not only based on dietary level of HUFA, but also the lipid chemical types. (3) The method using dried diet (yeast) mixed with fish oil for the rotifers to enrich n-3HUFA is suitable for large-scale rotifer production mainly for the good effect of HUFA enrichment and avoidance of water contamination.

Key words: *Brachionus plicatilis*; dietary fatty acids source; lipid composition
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Recent studies show that highly unsaturated fatty acids (HUFA, >20C), including eicosapentaenoic acid (EPA, 20: 5_{n-3}) and docosahexaenoic acid (DHA, 22:6_{n-3}) are essential components for marine fish and decapod crustacean[1－4]. These essential fatty acids (EFA) must be supplied in diet to maintain good growth and survival of cultured marine fish and decapod crustacean larvae. Up to now, rotifer *Branchionus plicatilis* has been extensively used as living food due to its appropriate size, rapid production rate and suitability for mass culture under control conditions, but the HUFA in the rotifers fed Baker's yeast usually proved to be nutritional deficiency in lipid composition in terms of fish larval requirement. So, high-HUFA-content source should be provided to improve the dietary quality.

The purpose of this study is to observe the change of lipid composition in the rotifer (*B. plicatilis*) fed different lipid sources.

1 Materials and methods

1.1 Culture of rotifer

At the first experiment, a large number of rotifers were obtained using Baker's yeast as diet in 8 m^3 concrete ponds with sea water (salinity 25), then the rotifers were controlled at the density of 200～

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300 ml^{-1}, totally 12 tanks, each 200 L. The culture was continued with different HUFA-content diets as follows: Baker's yeast, *Saccharomyces cerevisice* (control), algae, *Nannochlorpsis oculata* (diet A, group A), fish oil(5%) + soybean lecithin(5%) + Baker's yeast(90%)(diet B, groupB), and fish oil (10%) + Baker's yeast(90%)(diet C, group C), each with 3 replicates. The fatty acid composition in the diets is presented in Table 1.

Table 1　Lipid compositions in different diets for rotifers feeding experiment

Composition	Group			
	Control	A	B	C
C14:0	0.519	12.862	3.870	7.755
C16:1	13.152	19.460	4.608	8.165
C16:0	32.023	15.467	20.611	19.800
C17:1	7.785	1.002	-	0.352
C18:3	-	1.921	2.011	-
C18:2	-	6.867	20.729	-
C18:1	11.928	8.885	18.222	8.131
C18:0	15.741	0.857	-	4.049
C19:1	7.225	-	-	0.450
C20:5	-	18.044	6.011	13.448
C20:4	-	10.891	0.613	0.866
C22:6	-	-	10.814	20.457
HUFA	-	29.667	16.838	35.453
n-3HUFA	-	18.044	16.825	33.905
n-6HUFA	-	17.758.	21.342	0.866

Note: Control - Baker's yeast; A - algae; B - 5% fish oil + 5% soybean lecithin + 90% Baker's yeast; C - 10% fish oil + 90% Baker's yeast

The rotifers were fed 4 times per day to maintain a constant concentration of 10 $mg \cdot L^{-1}$ diet in water (≈0.2 g/d for 10^6 rotifers). The diets were properly prepared and stored at −20℃ except during feeding time.

1.2 Lipid class analysis

1.2.1 Total lipid The rotifers were sampled after 24 h and concentrated with a 300 mesh scoop, washed with clean sea water, and the surface water was dried with filter paper. For each sample, 1 g (wet weight) was used for lipid analysis and the rest part for water content determination by drying at 60℃ for 24 h. The total lipid was extracted as described by Folch et al[5] and calculated on a dry weight basis. Two thirds of the total lipid was then separated into neutral lipids (NL) and polar lipid (PL) based on Nichol's method[6], and the contents were also determined gravimetrically. One sixth of the total lipid was separated by using thin layer chromatography(TLC) to identify and quantify its NL and PL. NL was separated by using two-step solvent system: (1) the valume ratio is V(isopropyl other): V(aceticacid) = 96 : 4; (2) the volume ratio is V(petroleum ether): V(other): V(acetic) = 90 : 10 : 1[7]. PL was separated as Skispski[8], and the solvent system is V(chloroform) : V(methanol) : V(acetic acid) : V(water) = 25 : 15 : 4 : 2 (volume ratio).

1.2.2 Fatty acid Fatty acid methy ester (FAME) samples were prepared using the total lipids extracted in 1.2.1, i.e. dissolving the lipids with benzene-petroleum(1 ml, volume ratio 1:1)and then esterifying it with KOH-methanol(1 ml, 0.4 ml/L) for 15 min, and then adding into 8 ml distilled water for another 30 min. Two phases were obtained.

The upper phase should be removed and concentrated for GLC(HP5892 Ⅱ) analysis, 0.5～1.0 μl being used for each sample. Column: fused silica, 25 m long, inner diameter 0.25 mm; injection and detector temperature 320℃; column temperature: from initial temperature 60℃ (2 min) to 290℃ (5 min stabilizing) at the rate of 10 ℃/min, then to 60℃. The results recorded on electric computer data processor FAMEs were identified by comparison of retention time of known standard mixture. Individual FAME was quantified refering to the internal standard (C10:0).

1.3 Statistical analysis

Results are presented as means ± sd. The differences among different diets or dietary rotifers were analyzed by one-way ANOVA, followed by a multiple comparison test(Tukey) with the aid of analysis tools of EXCEL, version 7.0.

2 Results and analyses

2.1 Total lipids

Total lipid content (dry weight) in the rotifers after 24 h feeding increased compared with control, but significant increase ($P < 0.05$) only happened in group C (fish oil source) (Table 2). It is noteworthy that the variation of total lipid content in the rotifers

(8.42% ~12.83%) was much less than that in the diets(1.1% ~24.8%) (Table 2).

2.2 Fatty acid composition

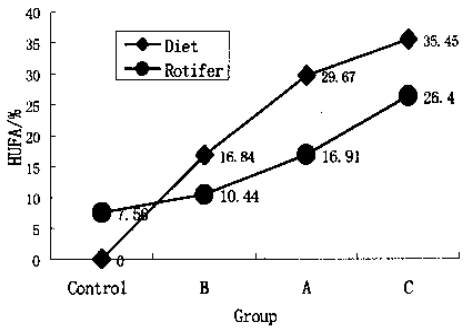
As Table 2 shows, neutral lipid(NL) in the rotifers makes up 50% ~70% of total lipids, and polar lipid (PL) makes up 30% ~50% in different diets. NL was mainly composed of triglyceride (TG), cholesterol, free fatty acids and ester sterol, and PL mainly phosphatidy- lcholine and phosphatidylethanolamine in terms of TLC results. The TLC results also show that fish oil is almost composed of TG and almost all the yeast oil lacks TG; the polar lipid is the dominant lipid (50% ~60%) in algae, and the contents of NL and PL in the rotifers have no proportional relationship with the contents in their correstponding diets.

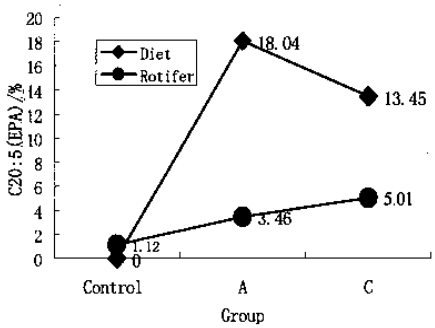
Table 2 Lipid content in rotifers and corresponding diets ($n=5$)

Group	Water content/%	Lipid/% (Dry weight)		PL/TL		Diet TG/TL	Rotifer NL/PL
		Diet	Rotifer	Diet	Rotifer		
Control	88.28±0.84	1.1	8.42 ± 1.53^{a}	–	0.483	0*	0.517
A	89.29±1.00	24.8	9.13 ± 1.32^{ab}	0.50~0.60*	0.327	0.20~0.30*	0.673
B	88.17±1.22	11.1	10.99 ± 1.85^{ab}	0.50	0.416	0.50*	0.584
C	89.34±0.93	10.9	12.83 ± 2.37^{b}	<0.10*	0.475	0.100*	0.535
P	0.157		0.011				

* Values are estimated by TLC result. One way ANOVA. There are significant differences ($P<0.05$) between mean values with different superscript letters in the same column.

The results in Table 3 demonstrate that different lipid sources in diets do affect the fatty acid composition of rotifers, particularly in terms of HUFA (such as C18:2_{n-6}, EPA, DHA), for example, rotifers of diet B which contains high percentage of C18:2_{n-6} (20.7%) has characteristically high level of C18:2_{n-6}, and the lipid in fish-oil-feeding rotifer(diet C, high percentage of EPA and DHA) contains the highest level of EPA and DHA ($P<0.05$). Thus the total HUFA content in diets is a major factor affecting HUFA level in rotifers, and high level of HUFA in diet can result in high level of HUFA in the rotifers (Fig.1).





Control: Baker's yeast; A: algae *N.oculata*; B: fish oil 5%, soybean lecithin 5%, Baker's yeast; C:fish oil 10%, Baker's yeast

Fig.1 HUFA levels in diets and rotifers

Fig.2 EPA enrichment results with different lipid types in diets

Figure 1 also shows that the level of HUFA in the rotifers varys with that in the diets, but there is a little difference. For example, though the levels of HUFA in diet A (29.67%) and diet C (35.45%) have slight difference , the value of HUFA (26.4%) in the rotifers of group C (major lipid is TG) is signif-

icantly higher ($P < 0.05$, Table 3) than that (16.9%) in group A (major lipid phospholipid, 50% ~ 60%). This suggests that TG is a better lipid type for HUFA enrichment in rotifers than phospholipid (PL) type, furthermore this can be obviously seen from Fig. 2 that diet A with major PL type has high level of EPA but low level in diet A-feeding rotifers compared with diet C (TG) and its rotifers.

Although there are very few EPA, DHA and C20:4 in yeast oil, the yeast-feeding rotifers contain low levels of those, indicating that the rotifers have a limited ability to synthesize those fatty acids as demonstrated by Lubzens et al[9] (Table 3).

Whatever contents of C20:4 and EPA were in diets, such as diets B and C, the dietary rotifers contain very small percentage (<1%) of C20:4 and relatively high amount of EPA (>6%) (Table 1), and the percentage of C20:4 in rotifer fatty acids is always higher than that of EPA (Table 3).

Table 3　Fatty acids composition in rotifers fed different diets ($n = 3$)　　%

Fatty acid	Group				P
	Control	A	B	C	
C14:0	3.043±0.865	6.142±3.419	3.177±0.009	5.437±3.001	0.560
C16:1	17.172±1.925	18.498±4.615	9.447±4.546	10.669±1.921	0.220
C16:0	12.925±3.145	22.031±0.875	18.056±4.440	12.400±3.738	0.077
C17:3	2.638±3.732	0.356±0.503	2.821±2.596	5.038±1.010	0.445
C17:0	1.046±0.428	0.763±0.397	1.275±0.345	0.914±0.530	0.861
C18:2	2.044±0.151^a	5.451±0.067^b	8.883±2.408^b	0.847±0.339^c	0.036
C18:1_{n-9}	24.448±2.044	13.101±7.057	20.009±1.335	14.657±1.655	0.122
C18:1_{n-7}	4.336±1.351	4.691±0.744	4.766±1.467	3.443±0.339	0.388
C18:1.	28.784±3.395	17.792±6.313	24.775±0.485	18.099±1.994	0.117
C18:0	7.008±0.124	5.324±0.807	11.276±6.736	5.663±1.814	0.540
C19:1	2.085±3.777	0.218±0.309	1.238±0.245	3.792±1.027	0.666
C20:5	1.119±0.242^a	3.461±1.070^b	2.320±0.332^a	5.007±112^b	0.007
C20:4	2.224±0.664^a	6.688±0.687^b	1.705±0.070^a	6.474(1.254^b	0.033
C20:3	1.230±0.050	1.958±1.112	1.489±0.601	2.602±0.927	0.531
C20:1	3.988±0.779	2.510±0.542	3.226±0.893	2.613±2.639	0.766
C22:6	2.366±1.184^a	3.347±1.137^a	2.930±0.032^a	8.946±3.630^b	0.043
C22:5	0.624±0.882	0.890±1.268	0.492±0.696	4.623±0.111	0.264
Saturated	26.322±5.073	35.479±1.435	32.509±11.167	25.217±7.948	0.713
Mono	56.476±1.614^a	39.676±1.000^b	38.686±5.200^b	36.995±0.880^b	0.041
PUFA(≥18C)	12.300±2.627^a	23.539±2.285^{bc}	19.817±4.186^{ab}	28.138±0.962^c	0.030
n-6	4.267±0.815^a	12.139±1.754^b	12.589±2.672^b	7.673±0.417^a	0.019
n-3	8.032±1.812^a	11.400±0.530^a	5.739±0.907^a	20.464±0.543^b	0.007
EPA+DHA	3.585±1.426^a	6.808±1.067^a	4.250±0.300^a	14.024±3.742^b	0.022
HUFA(≥20C)	7.462±1.258^a	16.910±1.808^{bc}	10.444±1.171^{ac}	26.395±2.567^d	0.002

Note: Values are expressed as mean ± sd from a sample with $n = 3$. Means in the same line with different letters are significantly different ($P < 0.05$). The same below.

Table 4 shows the quantitative difference in the amount of selected fatty acids of rotifers fed different diets. The result is similar to the fatty acid pattern in Table 3. The absolute quantities of fatty acids such as EPA, DHA and C20: 4 get to the highest levels in the rotifers fed diet C (fish oil) in which the HUFA amount is about 9 times as much as those fed yeast, 3 times as much as those fed diet A and diet B.

3　Discussion

3.1　Comparison of techniques for HUFA enrichment in rotifers

There are various enrichment techniques used nowadays to increase rotifer n-3HUFA content, such as feeding rotifers with algae before they are fed as diet of fish larvae or decapod crustacean, or adding oil

emulsions to a Baker's yeast diet[9] or directly into rotifer tanks[10,11]. Direct oil emulsion enrichment technique has a major advantage, i.e. a high level of HUFA enrichment can be achieved in a short time. However, part of HUFA may not be ingested by rotifers, only sticking to the surface of rotifer. This may increase the lipid oxidation and result in water contamination. This disadvantage can be well solved by using microcapsule in which the enriched oil is separated from culture water, but microencapsule process is relatively complex. By using dry enrichment diet containing n-3HUFA lipid source (this study), increasing daily feeding frequency, shortening diet-in-water period to minimize the oxidation, a good result of n-3 HUFA enrichment (fish-oil-source rotifers) is achieved. This dry enrichment technique, for its ease of preparation, may be more suitable for commercial enrichment preparations.

Table 4 Quantities of selected fatty acids in rotifers fed different diets mg·g^{-1}(dry weight)

Fatty acid	Group				
	Control	A	B	C	P
C18:1	7.215±0.336	5.744±1.948	11.017±3.280	12.802±3.006	0.123
C18:2	0.514±0.047	1.765±0.008	4.262±2.145	0.806±0.485	0.08
C20:4	0.549±0.076^{a}	2.161±0.511^{a}	1.675±0.146^{a}	4.644±1.398^{b}	0.022
C20:5	0.430±0.010^{a}	1.123±0.345^{ab}	1.397±0.213^{bc}	3.742±0.287^{d}	0.001
C22:5	0.177±0.250^{a}	0.294±0.415^{a}	0.332±0.470^{a}	1.619±0.127^{b}	0.037
C22:6	0.574±0.202^{a}	1.078±0.673^{a}	1.539±0.068^{a}	6.535±2.216^{b}	0.044
n-3	2.148±0.101^{a}	3.689±0.110^{a}	4.374±2.543^{a}	14.259±1.827^{b}	0.004
EPA+DHA	1.005±0.192^{a}	2.202±0.309^{a}	2.238±0.280^{a}	10.277±3.503^{b}	0.019
HUFA(≥20C)	2.04±0.020^{a}	5.471±0.494^{a}	5.663±1.852^{a}	18.955±3.718^{b}	0.005

Note: Values are expressed as mg/g rotifer (dry weight) with mean ± SD from a sample with $n = 3$.

3.2 Lipid content

Though lipid contents in rotifers are significantly different with different diet sources, the variation is not large compared with the difference in dietary lipid contents. This result is similar to that of Rainuzzo et al.[12], but not to that of Rodriguez et al[11] that there was a rapid increase in total lipid content of rotifer during 24 h enrichment from 13.15% ~ 36.94% at 12% enrichment oil in the medium of rotifers. This difference may result from the different enrichment conditions described above.

3.3 Fatty acids composition

The fatty acids composition, especially HUFA in rotifers, largely depend on that in diet[12-14]. However, rotifers have their own genetic fatty acids pattern because though fatty acids pattern is largely affected by diet fatty acid composition, it is not identical to that of diet, for example, the yeast (control) lacks HUFA but its rotifers still have relatively high amount of HUFA, and whatever the contents of EPA and C20:4 are in diets (this study shows the content of C20:4 in all diets is much less than that of EPA, Table 1), the rotifers always contain higher percentage of C20:4 than that of EPA. This conservative pattern in rotifers may be maintained to some extent by their de novo synthesis and bio-conversion[9].

The enriched HUFA content in rotifers is based not only on fatty acid composition in diet, but also dietary lipid chemical types. This study demonstrates that TG in diet is better than phospholipid type in HUFA enrichment by rotifers, and is more effective for obtaining high level of HUFA in rotifers. The similar results had been described by Rodriguez et al[11].

The result that the C20:4 level is higher than EPA level in the rotifers with different diets is contrast to the report by Ostrowski et al[11], and more differences exist in HUFA composition of the rotifers fed the same algae species, *Nannochlorpsis oculata*. The reason may be that the rotifers, *B. plicatilis*, from different sources may have different genetic strains with different HUFA patterns or abilities to synthesize and bio-converse HUFA. The rotifers used in this study with high content of $C20:4_{n-6}$ implies

they are from freshwater or estuarine origin because organisms from those places tend to have significantly higher n-6HUFA than those from marine origin[15].

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不同脂肪源对褶皱臂尾轮虫脂类和脂肪酸组成的影响

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摘　要: 以褶皱臂尾轮虫(*Brachionus plicatilis*)为实验动物并设计不同脂肪源饵料, 分别为: 面包酵母 *Saccharomyces cerevisice* (对照组), 微绿球藻 *Nannochlorpsis oculata* (A), 5%大豆磷脂＋5%鱼油＋90%面包酵母(B), 10%鱼油＋90%面包酵母(C)。结果表明: (1)接受不同脂肪源的轮虫的脂肪酸组成显著不同, 尤其是高度不饱和脂肪酸(HUFA, 20C)含量存在显著差异, 说明饵料中 HUFA 含量对轮虫体内相应脂肪酸的含量有显著影响。投喂饵料 C 的轮虫具有高含量的 n－3HUFA(26.7%), 而对照组轮虫的 n－3HUFA 只有 7.56%, 所以投喂饵料 C 使轮虫的脂类 HUFA 水平得到了强化, 提高了轮虫的营养价值。(2)轮虫脂类的 HUFA 水平不仅由饵料中脂类的相应脂肪酸组成决定, 而且轮虫脂类 HUFA 的提高和强化效果与饵料中 HUFA 的化学形态密切相关。甘油三酯型饵料 HUFA 的强化轮虫效果高于磷脂型的 HUFA。(3)将富含 HUFA 的鱼油直接添加在干性饵料如面包酵母中, 而不是通过对鱼油乳化后直接强化轮虫, 同样能取得良好的强化效果, 且操作简便, 不易污染水质, 适用于轮虫的规模生产。

关键词: 褶皱臂尾轮虫; 饵料脂肪源; 脂肪酸组成